Wade Znosko

Assistant Professor, Biology

Home: 391 Timbercreek Drive, Farmville, VA 23901

Office: 303 Chichester Science Hall

Phone: 724-882-5720 (h); 434-395-2668 (o)

Grant sought: Faculty Development

Grant Period: 3-4 years

Amount Requested: $11,000

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Applicant:

(Wade Znosko)

Department Chair:

(Mark Fink)

Dean:

(Chuck Ross)

II. Project Abstract:

Zebrafish have been used as a model to study many different biological areas, including neurobiology, development, and behavior. Zebrafish form a functional, beating heart just two days after inception. During this time, in addition to a heart, a brain, eyes, kidneys, and most other organs are also formed. This rapid organ formation allows zebrafish to be examined to determine the critical events that take place early during development. I previously identified a specific family of proteins that are responsible for proper heart formation. Normally, all zebrafish hearts loop in a certain direction. Without these proteins present, the heart is looped in completely the opposite direction, or is not looped at all. Interestingly, these proteins do not function directly upon heart cells during development, but rather alter cilia movement in a completely different region of the developing embryo. My future work will involve looking at other novel, uncharacterized proteins within zebrafish to analyze a possible role in heart development.

A second, more immediate goal is to examine specific agricultural contaminants found within local Virginia waterways and determine what types of developmental effects these contaminants are having on aquatic animals. In addition, this study would fill a void currently noticed within water quaility programs. A main goal of this study is to determine the short-term and long-term effects that specific agricultural contaminants found in our waterways have on aquatic life.

III. Narrative Description:

Critical signaling during development for proper heart development

A. Rational: The zebrafish is an established model system to address questions in areas of cell biology, developmental biology, neurobiology, and animal behavior. The advantages of rapid generation time, high fecundity, and the ability to manipulate the transparent embryo have allowed this model system to be used in many biological studies. Both classical loss-of-function and gain-of-function assays can be performed with ease to characterize gene function during zebrafish development.

Due to my graduate research projects, I have gained expertise in the area of gene expression during zebrafish development (16, 17, 18). As a mentor to undergraduates in the Summer Undergraduate Research Program (SURP), I have taught undergraduates about gene expression patterns, how proteins function within a signaling pathway, and proper organ formation in development. During my graduate study, I had assistance from several undergraduate students in many aspects of my research, indicating this type of work is interesting to undergraduates and can be driven by their efforts. Importantly, the questions my research will ask are fundamental questions in developmental biology. Techniques such as *in situ* hybridizations to determine expression patterns, micro-injections of RNA into zebrafish embryos for gain-of-function studies, and the use of MOs for loss-of-function studies, are all heavily used experimental techniques that allow students to follow the scientific method as well as prepare students who would like to continue research in zebrafish biology, developmental biology, or cell/molecular biology.

Importantly, this type of research, specifically my second project, can bridge the two halves of our department, as it can generate great interest to both environmental scientists as well as cell/developmental biologists. This could perhaps be one the largest contributions of this research. In addition, I fell this second project can also be used as a tool to educate local public

school students. The methods and results of this type of study rely heavily on basic biological concepts that even younger scientists can understand fully. By both allowing high school students to get involved in this project by collecting water samples, and going to local schools to give a lecture about the findings of this study and the impact of pollution, I feel local public school students could be positively impacted and play a large role in this study.

Finally, this proposal fits well in an institution with the size and breadth of Longwood University. The research I propose in my first study involves examining uncharacterized genes, allowing for novel connections to be made between the expression pattern and functions of these genes with zebrafish development, but it is also suitable for an undergraduate-driven lab to address these questions. Due to the nature of characterizing novel genes, the outcomes of these studies have the ability to be published in peer-reviewed journals and have the potential to be externally funded. Furthermore, the zebrafish model system is a great teaching tool to study developmental biology and physiology, with the hopes of gaining great collaborations within the biology department and among other departments at Longwood.

B. Goals: The main focus that I intend to pursue would have strong implications within developmental biology and toxicology, in addition to environmental science. As importantly, research will be conducted in Farmville's local waterways, having large implications for the Farmville community.

a). Identification and function of uncharacterized genes in zebrafish heart development. Through a screen for developmentally regulated genes in zebrafish, clones from a normalized cDNA library were tested using high-throughput *in situ* hybridization. Of the 2765 clones screened, 347 genes had patterns restricted to a portion of the developing zebrafish embryo (1). Of these, only half represent previously characterized zebrafish genes. Of particular interest are the uncharacterized factors involved in zebrafish heart development. Initial studies indicate several genes are localized in the anterior lateral plate mesoderm (ALPM), an area giving rise to heart tissue. During my graduate research, I determined that Etv5, another uncharacterized gene

from this initial screen, was needed for proper heart formation. Further analysis of results from this screen is critical to understand the importance of specific genes during zebrafish heart development. Primarily, determining the expression pattern of these genes throughout all of heart development is essential. Of particular interest will be genes expressed only in specific regions of the heart, such as the ventricle or atrium. Next, MOs will be generated to knock-down these genes. Following micro-injection of these MOs into embryos, phenotypic analysis as well as *in situ* hybridizations will be performed to further reveal the developmental impact of these specific proteins. Finally, utilizing antibodies specific for the heart, immunostaining will be executed on MO-injected embryos to determine the role of these specific proteins in later heart development. In addition, several transgenic lines, such as *Tg(CMLC2:dsRed^{nuc})*(15) can be used as an effective tool for analysis of heart formation. This transgenic line drives nuclear dsRed only in cardiac cells. Injecting MOs generated from uncharacterized genes expressed in the heart into this transgenic line will further reveal if noted heart phenotypes are a result of a decrease or increase in number of cardiac cells.

b). Examination of pollutants in local Virginia waterways and determine their impact on zebrafish heart development. Another area of interest includes a project that will bridge the biological and environmental science areas of our department. For this project, I will be collaborating with another member of the Biological and Environmental Sciences Department, Dr. David Buckalew. Dr. Buckalew has been involved in monitoring Virginia waterways and conducting research on them for the past 10 years. This student-driven research has lead Dr. Buckalew and his student researchers to 25 presentations at local, national, and international conferences in addition to four peer-reviewed publications. The current project will first examine the pollutants in our local Virginia waterways, an area that is already a strong interest to several researchers within our department. To increase the relevance of this study, I will examine the effects of these pollutants on aquatic life, again utilizing zebrafish as a model system. A recent study has found that environmental pollutants can have a large impact on zebrafish development,

specifically in heart development (19). After determining which pollutants (both bacterial and agricultural) are most common in local waterways, I will then perform assays with zebrafish examining developmental processes in the presence of these pollutants, looking particularly at heart development, but also examining other critical processes, including brain formation.

C. Subjects: Animal subjects to be used during this research are the typical, wildtype zebrafish, *Danio rerio*, that are being used in my research lab to study developmental biology. Many published protocols are available regarding proper care of these animals. These protocols will be strictly followed to ensure the health of our zebrafish subjects. The proper Animal Research Consent Forms can be found as an addendum to the end of this proposal.

D. Procedures: To achieve the goals of this proposal and examine the effects of local agricultural contaminants on zebrafish development, it is necessary to use the expertise of an outside source. Using a reputable external company (GCMS-Express), we will identify through Gas Chromatography-Mass Spectrometry specific agricultural chemicals found within these waters based on an exhaustive library of known agricultural chemicals. To have a full evaluation of several of the most contaminated creeks in the area, The total cost of this analysis will be $3,000. When comparing this same service between several companies, GCMS-Express provided the most cost-efficient options in addition to having a very reputable service. It is also extremely advantageous that this is a local company found within our area. In addition to this service expense, lab fees such as 96-well plates to monitor zebrafish development, microscope use, and fish husbandry will be $1,000 of this grant.

In addition, a second cost expense is a micro-manipulator that is critical to conduct zebrafish experiments. This would allow me to conduct not only the agricultural contaminants studies, but also allow me to continue looking for genes that are required for proper zebrafish heart development. Importantly, this micro-manipulator is a common tool used in many aspects of biology experimentation, including cell biology, genetics, and neurobiology. Due to this, several other faculty within the Biology Department at Longwood have expressed interest in

using this piece of equipment in their research with students. Therefore, the money received from this grant will be used to finish purchasing the entire micro-manipulation system to analyze my specific project goals, in the hopes to be completed within 3 years, although the equipment will be utilized for experimentation well into the future. I have slowly been obtaining pieces of the apparatus, but have never received enough funding (through ETF funds or other Longwood University internal funding) to purchase all components to this system. The remaining components cost $7,000. Also just as important, the techniques on how to use this equipment can be easily learned and practiced by students. This is a piece of equipment that can be utilized by both students and faculty.

E. Expected Outcomes: As mentioned previously, these proposed projects could help to build a bridge between the two sections of our department, Biology and Environmental Science, a feat that has yet to be accomplished within our current areas of research. There is already great interest by students to become involved in this study, as I already have 3 student researchers this semester. The equipment purchased from this grant would benefit students and faculty in several subject areas, including cell biology, developmental biology, genetics, and neurobiology. The equipment can be used as part of a teaching tool in laboratory components of introductory and upper level courses, in addition to aided current faculty and research students in their studies. On my specific goals for this study, since we would be working with novel genes, I believe publication is extremely favorable in a time period of about 3 years. This data would also be conducive to present at local, regional, and national meetings, particularly by students.

F. Current Status of Project: The focus of my graduate research involved a transcription factor, *etv5* (ETS-variant 5) that was identified in a random *in situ* hybridization screen to exhibit a similar expression pattern during zebrafish development as *fgf8* and *fgf3* (*1*). Etv5, a member of a larger family of Pea3 ETS transcription factors, contains the highly conserved ETS DNA binding domain, which allows the transcription factor to bind to DNA targets and activate transcription. Since this factor displayed similar expression during

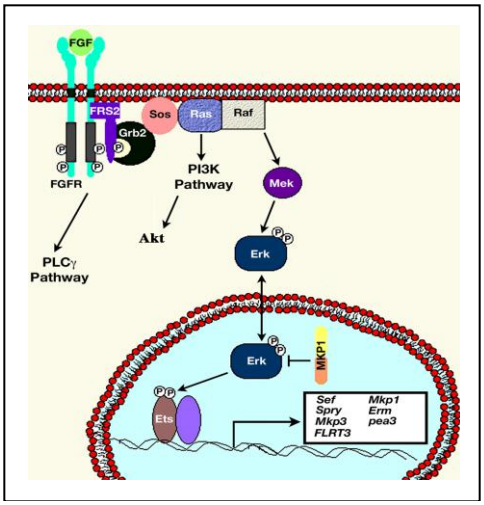

Figure 1. The FGF pathway during development.

development as Fgf ligands, we hypothesized that ETS transcription factors were important in the FGF signaling cascade (Figure 1). My research interests focused on the role for ETS transcription factors in FGF-mediated developmental processes. To further examine the role of ETS factors, anti-sense morpholino oligonucleotides (MOs) were utilized to knock-down Pea3 ETS genes. I focused on the role of these factors in heart and brain development; two processes know to require FGF signaling (2-8). In *3EtsMO*-injected embryos, knocking-down ETS factors had an effect on heart looping, where hearts were looped in the opposite direction (Figure 2). Altered looping arises when there is incorrect left-right patterning within the embryo. Since cilia within Kupffer's vesicle (KV), a structure analogous to the mouse node (9-13), has been shown to play a role in left-right patterning, KV cilia within MO-injected embryos was analyzed. I was able to conclude that there was a significant decrease in the amount of cilia, thus resulting in improper left-right patterning and altered heart looping (Figure 2).

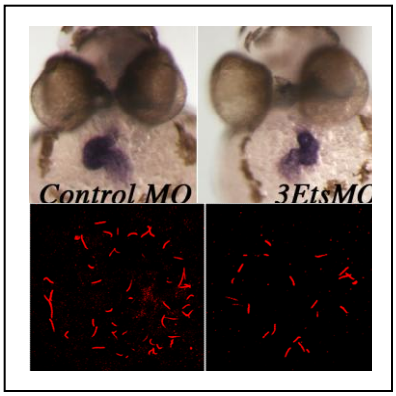

Figure 2. When knocking-down ETS factors, left-right patterning defects occur in the heart due to a decreased number of cilia within Kupffer's vesicle.

G. Personnel: I have recently published a research article in 2010 that provided the necessary experimentation that will be built upon during this study (18). Many of the techniques have already been implemented previously, so once equipment is purchased, it will not be difficult to start generating data. As evidenced by 5 students currently doing research in my laboratory, there is great excitement within the department and by the students for this type of research to grow and continue with the department.

IV. Proposed Budget:

To achieve the goals of this proposal and examine the effects of local agricultural contaminants on zebrafish development, it is necessary to use the expertise of an outside source (GCMS-Express). We will identify through Gas Chromatography-Mass Spectrometry specific agricultural chemicals found within these waters based on an exhaustive library of known agricultural chemicals. To have a full evaluation of several of the most contaminated creeks in the area, The total cost of this analysis will be $3,000. When comparing this same service between several companies, GCMS-Express provided the most cost-efficient options in addition to having a very reputable service. It is also extremely advantageous that this is a local company found within our area. In addition to this service expense, lab fees such as 96-well plates to monitor zebrafish development, microscope use, and fish husbandry will be $1,000 of this grant. Since research involving zebrafish is a novel model system being utilized at Longwood, the final portion of the funding from this grant will go toward critical zebrafish-specific equipment, mainly a micro-manipulation setup. This is currently equipment that is not present within the department, but once acquired could be used by several faculty in the BES department based on their research interests in addition to being utilized for research students. I also envision this equipment being easily transitioned into the classroom to facilitate laboratory and lecture exercises. Based on discussions with GCMS-Express and World Precision Instrument representatives, the breakdown of expenses includes:

GCMS-Express analysis	$3,000
Completion of micro-manipulator (stand, needle puller, injector)	$7,000
Disposables for setup	$1,000
	$11,000

This equipment, including disposables for setup, would total $11,000.

V. Previous Grants:

Cook-Cole Research Grant - December 2010 - $8,000 awarded

College of Graduate and Professional Studies Summer Research Grant – July 2011 - $2,000 awarded

Addendum:

Consent form for animal research

Title: The Role of Fibroblast Growth Factor (FGF) Signaling for Proper Heart Development

Purpose of Research: The goal of this research is to analyze uncharacterized genes during zebrafish development to determine the importance of specific genes during heart development. The research is being conducted as a student/faculty collaborative effort, under the supervision of Dr. Wade A. Znosko.

Methods and Procedures:

Participants - *Danio rerio* (zebrafish) are the animals used in this proposal. We will use several strains of zebrafish, including: AB*, EK, TL, *ace* and *Tg(mkp3:d2eGFP)*. Both female and male zebrafish ranging in ages of three months to two years will be used. We will utilize about 20 tanks of zebrafish for this study, to use for embryo collection and propagate the strains. Zebrafish are widely recognized as model systems in which to study vertebrate development. Some advantages zebrafish have over other model vertebrate systems are: 1) the embryos are spawned outside of the mother in large numbers, 2) the zebrafish embryos are transparent allowing visualization of all cells throughout early development; 3) zebrafish developmental processes, including organogenesis, are very similar to mammalian; 4) a large and expanding number of zebrafish researchers provide valuable resources from many fields of study; 5) zebrafish sexual maturity occurs around three months resulting in rapid generational turnaround; and 6) zebrafish are easy to maintain making them an affordable animal model for experiments requiring large population sizes.

Procedures - Zebrafish breed when the water quality conditions, photoperiod and feedings are optimal as per published protocols. Zebrafish exhibit a carnivorous nature that compels them to eat their own eggs. Therefore, zebrafish male/female pairs or groups are kept in breeding tanks with a false bottom (or other devices may be used) allowing fertilized eggs to be collected. Adult

zebrafish may be chemically immobilized using 0.168 g/L tricaine. This treatment is required for DNA sampling from fins, a necessary procedure when analyzing transgenic lines. The immobilization is typically less than three minutes, and the fish are removed from system water for less than thirty seconds. Adult zebrafish are first anesthetized by emersion in tricaine (MS-222; 0.168 g/L; as per IACUC approval) or clove oil (25-100 mg/L; FDA approval received) until gill movements cease then euthanized by transferring to an ice bath.

Possible risks – If following the appropriate, published procedures, as mentioned above, there are no risks for causing unnecessary harm to the animal models. All techniques have been published to be the most humane ways to house, immobilize, and euthanize zebrafish as per IACUC standards.

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