Sample Application

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Research

Memphis Zoological Society
Memphis, TN

Development of Assisted Reproductive Technologies for Endangered North American Amphibians: Phase II Salamanders

Development of Assisted Reproductive Technologies for the Conservation of Endangered North American Amphibians: Phase II Salamanders

Abstract

It is estimated that nearly one-third (32%) of known amphibian species are threatened with extinction world-wide. Captive assurance colonies are rapidly being established by zoos and governmental organizations for many of the nearly extinct amphibian species in North America as a response to the amphibian crisis. Endangered, threatened or state-listed salamander species that have established captive colonies and will be the focus of our proposal include the Alabama warrior waterdog (*Necturus alabamensis*), hellbender (*Cryptobranchus bishopi*), blue-spotted salamander (*Ambystoma laterale*), and flatwoods salamander (*Ambystoma bishopi*). Species of least concern that will serve as models include the mudpuppy (*Necturus maculosus*), tiger salamander (*Ambystoma tigrinium*), and lesser siren (*Siren intermedia*).

Parties responsible for the population recovery of these endangered salamanders have all been in contact with the principal investigator's team in order to establish partnerships to address a similar and recurring problem: namely, genetically valuable animals are dying without being represented in the captive collections and the animals have never bred in captivity making the captive collections unsustainable. Therefore, this research proposal aims to develop assisted reproductive technologies and biomaterial banking of reproductive cells for these endangered amphibian species. The specific objectives of this study are to: 1) develop novel hormone regimens for sperm and egg collection in our model species, the tiger salamander, mudpuppy, and lesser siren; 2) test artificial fertilization techniques in our model species for their application to endangered salamanders as evaluated by fertilized embryo development; 3) generate sperm freezing protocols in the model species for their application to gene banking of sperm from endangered species as evaluated by post-thaw survivability; and 4) increase the reproductive output of our targeted endangered amphibian species by applying what we learn in Objectives 1-3 to developing innovative hormone regimens and artificial fertilization techniques that can easily be used by zoo, aquarium, and government curators.

This is a multi-institutional inter-disciplinary three year project with many supporting institutions including our official partners Mississippi State University and the Fort Worth Zoo along with our collaborators at the Omaha Zoo, Jacksonville Zoo, Cincinnati Zoo, U.S. Fish and Wildlife Service, Amphibian Ark, IUCN/CBSG, St. Louis Zoo, and Missouri Department of Conservation. Results from our project will be broadly disseminated through publications, internet resources for rapid sharing, conferences, book chapters, training symposiums, proceedings and zoo signage sharing the study's results with our visitors. Furthermore, the project team has devised a research plan that is easily replicated and exportable to other zoos, aquariums or government organizations for the conservation of endangered amphibians, thus optimizing increases in reproductive output as well as the preservation of existing genetic diversity from founder salamander populations. Lastly, this project will create new leaders in the field of applied zoological research by supporting post-doctoral, graduate and undergraduate students early in their professional career.

Development of Assisted Reproductive Technologies for Endangered North American Amphibians: Phase II Salamanders.

1. Assessment of Need: ***The Amphibian Extinction Crisis:*** Amphibian conservation has received a great deal of attention in the last decade, owing to the large number of species that are presumed to have gone extinct or are experiencing rapid population declines. It is estimated that nearly one-third (32%) of known amphibian species are threatened with extinction world-wide [6]. A comprehensive world-wide amphibian survey estimates that 38 species have likely gone extinct; however, it is projected the number more closely approaches 158 because many of the animals in the critically endangered column have not been seen for years, and in some cases a decade [6]. Of greatest concern is the fact that nearly half of all amphibians are continuing to decline in population, suggesting the number of threatened species can be expected to rise in the near future. In North America, 21% of its 262 species are threatened, representing 55 species [19]. Amphibians are often viewed as an 'indicator species' for environmental health because of their dependency on a mix of aquatic and terrestrial habitats, as well as the highly porous nature of their skin which causes them to be particularly susceptible to environmental contaminants and changing global climates. Confounding the crisis, these species appear to be more susceptible to a rapidly spreading fungal disease known as chytrid fungus (*Batrachochytrium dendrobatidis*) [14]. The catastrophic epidemics caused by the spread of this fungus have caused mass extinctions of amphibian populations worldwide [1,2,15,18]. The current rate of amphibian extinctions exceeds the total extinction rate for all vertebrate species over the last 350 million years [16] and has been compared to the loss of the dinosaurs. In an effort to bring awareness to this extinction crisis, the American Association of Zoos and Aquariums (AZA) and the World Association of Zoos and Aquariums (WAZA) declared 2008 as the *Year of the Frog*.

The Problem: Captive assurance colonies are rapidly being established by zoos and governmental organizations for many of the nearly extinct amphibian species in North America as a response to the amphibian crisis. While the amphibian assisted reproductive technologies thus far have focused mainly on anurans (frogs and toads), very little has been done for salamanders, which are facing a far greater threat of extinction (49.8% of salamanders are threatened compared to 31.6% of anurans) [6]. Endangered, threatened or state-listed salamander species that have established captive colonies will be the focus of our proposal and include the Alabama black warrior waterdog (*Necturus alabamensis*), hellbender (*Cryptobranchus bishopi*), blue-spotted salamander (*Ambystoma laterale*), and flatwoods salamander (*Ambystoma bishopi*). Species of least concern that will serve as models include the mudpuppy (*Necturus maculosus*), lesser siren (*Siren intermedia*), and tiger salamander (*Ambystoma tigrinium*). The captive breeding programs for these four endangered species have three main goals: 1) to maintain the genetic diversity of the original founders; 2) to produce offspring for established reintroduction programs; and 3) to provide animals for research in reproduction, nutrition, disease control and genetics aimed at improving long term conservation of the species. Parties responsible for the population recovery of these four endangered species targeted in this proposal have all been in contact with the principal investigator concerning a similar and recurring problem: namely, these species are not reproducing in captivity. Because of this, genetically valuable animals are dying without being represented in the captive collections making the programs unsustainable. *Loss of reproductive output limits the number of offspring from captive collections that can be reintroduced to the wild and assist in the overall species recovery.* As genetic diversity decreases for these assurance colonies, poor reproductive output will continue to be a problem. Therefore, it is prudent to develop hormone protocols for exerting external control over these individuals' reproductive patterns, design artificial fertilization (AF) and assisted reproductive technologies (ART), and conserve as much of the genetic diversity as possible in biomaterial resource banks for reproductive cells. In 2007, the Association of Zoos and Aquariums (AZA) Amphibian Taxonomic Advisory Group (ATAG) created a Regional Response Plan (RRP) for addressing declining North American Amphibians (see supplement). Three salamander species given high priority in this RRP were the hellbender, Alabama waterdog and flatwoods

salamander and the ATAG identified that assisted reproduction and genetic resource banking of reproductive cells will be crucial to these species survival. This research proposal has been designed to address the needs of several audiences (Table 1), each with a key role in the curation and management of salamander collections or wild populations and to provide a practical means to facilitate each in meeting those needs:

Table 1: Research Project Audience Needs & Benefits

Audience 1: ***National & international zoo & aquarium curators/professionals attempting to save critically endangered salamander species***	
Needs: Reliable methods are needed to reproduce critically endangered salamanders and curatorial standards are necessary for biomaterial banking what remains of the captive founder populations.	**Benefits:** New protocols for assisted breeding will be developed for the endangered Alabama water dog, hellbender, and flatwoods salamander; viable gametes will be gene banked which can be used for species recovery programs and transfer of gametes between institutions.
Audience 2: ***Governmental organizations in the U.S. actively engaged in recovery programs for federally listed endangered species***	
Needs: Reintroduction efforts by the U.S. Fish and Wildlife Service (USFWS) for North American endangered salamanders are hampered due to poor reproduction and loss of genetic diversity due to a dwindling stock of captive animals.	**Benefits:** An increase in the number of salamander larvae that can be translocated for reintroduction efforts by the USFWS; ability to preserve the existing genetic diversity of the captive population long-term and the ability to reintroduce genes from deceased individuals.
Audience 3: ***Academic researchers engaged in ecological and/or disease monitoring surveys for declining amphibian species***	
Needs: By the simple nature of their declining status, academic researchers are struggling to answer questions related to amphibian disease transmission, nutritional ecology, genetics and behavior.	**Benefits:** Methodologies for the continued propagation of endangered species in captivity and long-term gene banking will continue to provide a resource for academic studies on salamander biology and the pathology of disease.
Audience 4: ***Visitors to the Memphis Zoological Society and other collaborating institutions involved with salamander conservation***	
Needs: It is critical to connect our visitors to wildlife and their habitats so that when they leave our institution they have a greater appreciation for nature and the animals they observed. To this end, we are trying to instill a conservation ethic that includes all living things including some of the smallest and least appreciated, such as amphibians and especially salamanders. The Memphis Zoo intends to increase awareness of the amphibian extinction crisis through more interactive signage and highlight how zoos are involved with conservation research.	**Benefits:** One of the most frequent comments from visitors leaving the zoo is "we had no idea that the zoo was involved with so much conservation work for the species and habitats they are exhibiting". We firmly believe that the visitors will benefit from our increased informal education about the conservation research we are engaged in and how our activities are making a difference for the recovery of species in the wild. Visitors who make this connection are often inspired to make a difference themselves either through financial contributions or wildlife activism.

2. National Impact and Intended Results:

The premise of our proposal is that the development of ART and biomaterial banking of reproductive cells for endangered salamanders will increase reproductive output and help maintain the existing genetic diversity from the founder populations. To meet these goals we will hire one post-doctoral fellow, two graduate students and several undergraduate interns, providing professional training and early career development opportunities. This highly collaborative project is comprised of key project staff and supporting institutions representing zoos, academia, and government agencies. The Memphis Zoo has created an official **partnership** with Mississippi State University (MSU) and Fort Worth Zoo (FWZ) to address conservation issues related to declining salamander populations. In addition to our partnership, we have also established as **collaborators** the Omaha Zoo, Cincinnati Zoo, Jacksonville Zoo, St. Louis Zoo, Missouri Department of Conservation (MDC), U.S. Fish and Wildlife Service (USFWS), ATAG, AZA, Amphibian Ark, and IUCN (letters of collaboration can be found in supporting documents). Currently, we are funded by a 2009 IMLS Phase I NLG grant (#LG-25-09-0064-09) to develop ART and a frozen genetic bank repository for several endangered frog and toad species that will benefit their conservation and management. Species in the 2009 Phase I grant include the Mississippi gopher frog, leopard frog, boreal toad, Wyoming toad, Puerto-Rican crested toad, and Fowler's toad. This Phase II proposal is a continuation of our amphibian conservation work but expanded to salamanders and overlaps with the last year of the Phase I project in order to take advantage of the expertise and knowledge obtained by the

previous post-doc and graduate students. Their experience will create a peer relationship program that is complemented by the key senior personnel and leadership. Advanced professional training of the new students, by overlapping them with the Phase I candidates, will ensure multiple teaching moments and foster a network of collaborators that will have gone though team-building research exercises and help maintain long term career cooperation in the field of amphibian conservation. The partnership between the Memphis Zoo and MSU was originally created in the Phase I study to specifically address the pressing conservation challenges faced by our collaborators and friends in the amphibian community.

The objectives listed herein (Section 3), and the specific questions posed throughout the proposal, directly result from our collaborators' expertise in the field as their input was integrated early on and will continually be sought throughout implementation. We have an extremely active and productive collaboration with all the organizations listed in this proposal, some going back over ten years, and the authors visit several of the respective institutions annually. The intended results from this project will produce technologies and protocols that are affordable, easily replicated and *fully exportable* to other institutions. We have already seen the national impact of our studies from the Phase I grant, replicated and implemented by our collaborators without our presence, highlighting the project's transferability. For example, in 2007 Colorado's Native Aquatic Species Restoration Facility (NASRF) produced fewer than 10,000 tadpoles for repatriation, using ineffective hormone regimens and without employing AF. After consulting and working with the authors, NASRF produced nearly 30,000 endangered tadpoles, 5,000 of them by AF, which were subsequently released into the Rocky Mountain National Park. To the research team's knowledge, there is no other example in the world where ART has had such a significant impact on captive breeding and reintroduction programs for endangered species as the work conducted for declining amphibians under our multi-institutional collaborations. To date, nearly 10,000 endangered tadpoles from three different species have been produced by *in-vitro* fertilization (IVF) and repatriated to the wild, primarily due to *exportation and implementation by our collaborators* of the technologies developed by a prior IMLS CPS grant (#IC-01-03-0199-03) and our current IMLS NLG Phase I amphibian grant (#LG-25-09-0064-09). Furthermore, our previous IMLS grants have assisted with the creation of the first-ever genetic resource bank (GRB) for amphibians, and led to the world's first endangered toad species (Wyoming) produced by IVF with the subsequent release of over 2,000 tadpoles into the wild [4], as well as the production of Fowler's toad tadpoles from IVF using frozen-thawed spermatozoa held in cryostorage. In 2010, the world's first transfer of non-invasively collected chilled frog sperm was shipped successfully across country from the Memphis Zoo to the Omaha Zoo and used for IVF, producing more than one hundred critically endangered Mississippi Gopher Frogs. This news story made headlines all over the world and was part of a larger IVF experiment that led to more than 1,400 endangered gopher frog tadpoles produced (the most ever in captivity). At the writing of this proposal USFWS is reviewing options for releasing or head-starting these animals into the wild.

Our research team and collaborators have seen the potential benefits of our earlier studies and feel that even greater impacts on conservation can be achieved if we could improve our captive breeding success and produce more amphibians for increasing wild populations. Currently, most federal and state programs tasked with species recovery have secured additional new discharge sites, however too few animals are available for release due to absent or low reproductive output. Our proposed studies will have a significant impact on increasing salamander reproductive output, and a critical research component for this goal will be to explore ways to improve the use of exogenous hormones for manipulating the salamander reproductive cycle. Advantages to manipulating salamander reproductive cycles include: 1) bypassing unknown environmental cues that stimulate reproduction; 2) induction of reproductive events outside of their annual breeding cycle; 3) circumventing hibernation requirements (known to induce bacterial and fungal infections) for inducing reproductive behavior; and 4) synchronization of egg and sperm release to optimize fertilization [10,11]. The success of such conservation technologies is even greater when considering most salamanders have a high fecundity rate (tens to hundreds of eggs per female), which has the potential for producing large populations that can be reintroduced into the wild. Due to the limited space and resources available to zoos and aquariums,

these institutions will be unable to preserve the growing number of endangered salamander species worldwide in need of assistance. It is therefore prudent to secure populations representing salamander evolutionary diversity in both captive facilities and GRBs until more definitive solutions to the crisis are available. Although GRBs have been established for various charismatic mega-vertebrates [17] amphibians have been virtually ignored, placing them in greater danger of permanent widespread extinction than mammals. To the authors' knowledge, there are no salamander reproductive live cells (sperm, eggs, or embryos) banked down anywhere in the entire world. In 2008 an IMLS NLG was awarded to the San Diego Zoo (SDZ) for studying amphibian diseases and establishing a GRB for non-reproductive skin cell cultures. The authors of this current proposal are working closely with SDZ investigators by providing them tissues; however, the SDZ group's research is not focused on gene banking of reproductive cells as our study proposes. Thus, the GRB we have been developing for many years is complementary to SDZ's IMLS project but different in scope and application. Frozen reproductive cells stored in the Memphis Zoo's amphibian gene bank can contribute directly to species captive breeding and salamander reintroductions to the wild for *on-the-ground restoration* of threatened species. This proposal fills that urgently needed assistance to the captive breeding programs. The potential impact of a GRB is dependent not simply upon successful methods for cryostorage of sperm or (eventually) embryos, but also on related ART for manipulating reproductive processes such as ovarian cycles to facilitate the utilization of banked spermatozoa [5,9,11]. Our earlier studies indicate that the gametes we have cryobanked can be successfully used to reproduce endangered amphibians. These conservation milestones were the first steps to a comprehensive plan for applying this technology to other endangered amphibians including salamanders.

We see the additional following national impacts to this program: 1) leadership training of professional fellows and undergraduate students for a career in zoological research and curation; 2) a model for other amphibian based partnerships that is fully *exportable and easily replicated*; 3) building of an interdisciplinary and collaborative network of curators, scientists, zoos, and state/federal agencies for the conservation of salamanders; and 4) creation of a sustainable program for amphibian research that will increase reproductive output for endangered species while maintaining existing genetic diversity for captive assurance colonies at risk. Ultimately, these studies will increase the number of larvae or tadpoles that can be repatriated to the wild.

Long-term considerations: All the endangered species described in this proposal are part of carefully managed breeding programs to monitor and maintain genetic diversity. The animals chosen for our studies, which may generate offspring (larvae), will be pre-determined by the recovery program coordinators to insure appropriate pairings to maximize genetic diversity. A small proportion of random eggs/larvae will be sent to the SDZ disease laboratory and sampled for presence of disease prior to release. Once animals are deemed clear for release, eggs or larvae will be provided to the USFWS or respective state agencies for repatriation. Due to challenges associated with metamorphosis and size, there is currently no technology to do mark-recapture studies on larvae and follow their impact on wild populations. However, a post-doc with the Memphis Zoo and Rhodes College is currently testing the migration of micro-chips and elastomer dyes to see how these affect the physiology of larvae and metamorphs in order to track survivorship of reintroduced animals produced by ART.

3. Project Design and Evaluation Plan:

***Hypothesis*:** Development of assisted reproductive technologies and biomaterial banking of reproductive cells for endangered salamanders will increase reproductive output and help maintain the existing genetic diversity from the founder populations. This collaborative project will expand partnerships between academia, government and non-profit zoological institutions to address a critically important conservation issue.

Specific Objectives:

1) Develop novel hormone regimens for sperm and egg collection in our model species, the tiger salamander, mudpuppy, and lesser siren.

2) Test artificial fertilization (AF) techniques in our model species for their application to endangered salamanders as evaluated by fertilized embryo development.

3) Generate sperm freezing protocols in the model species for their application to gene banking of sperm from endangered species as evaluated by post-thaw survivability.
4) Increase the reproductive output of our targeted endangered salamander species by applying what we learn in Objectives 1-3 to develop species-specific hormone regimens and AF techniques that can easily be used by zoo, aquarium, and government curators.

***Species to be studied*:** The four endangered species that will be the focus of our experiments include the Alabama black warrior waterdog (*Necturus alabamensis*), hellbender (*Cryptobranchus bishopi*), flatwoods salamander (*Ambystoma bishopi*), and blue-spotted salamander (*Ambystoma laterale*). None of these salamanders have ever bred in captivity, despite having been in some collections for several decades (e.g. hellbenders). The first three species above are all listed as endangered by the IUCN red-list and are candidates for federal listing under the Endangered Species Act. The blue-spotted salamander is listed as common but rapidly declining in population and has been listed as endangered in some states (e.g. Iowa). Currently, the USFWS and state agencies (e.g. Missouri and Iowa Departments of Conservation) have translocation sites and monitoring programs in place for these threatened species, but due to their lack of reproduction in captivity no reintroductions have occurred and their situation is becoming critical. The hellbender displays external fertilization and although efforts by the St. Louis Zoo have led to the collection of sperm and eggs for possible IVF attempts, these collections were never synchronized to afford fertilizations. The other three species all have internal fertilization and no one in North America has ever attempted AF in salamanders. St. Louis Zoo, Omaha Zoo and Fort Worth Zoo have large numbers of hellbenders for us to work with (in excess of 500 animals), while the other species have very small numbers in captivity (10-20 animals/species). The low number of animals in captivity makes these assurance colonies particularly susceptible to failure and loss of the founders if some kind of intervention is not initiated soon. Species of least concern that will serve as models and display external and internal fertilization modes include the mudpuppy (*Necturus maculosus*), lesser siren (*Siren intermedia*), and tiger salamander (*Ambystoma tigrinum*). What we learn in our model species will also prove valuable to other endangered species such as the California tiger salamander, dwarf siren, and Chinese giant salamanders. Mudpuppies (*N*=50) will be purchased from several commercial dealers and the Omaha Zoo will provide us with 30-50 tiger salamanders from their collection as part of the partnership. The lesser sirens have already been amassed by the USFWS for the Memphis Zoo and are currently in our collection, although we will be supplementing the current number to reach a sample size of 20 or more. For additional information on the species listed in this proposal see the IUCN species accounts found in the supplemental material.

***Project Design*:**
Objective 1 - Develop novel hormone regimens for sperm and egg collection in our model species, the tiger salamander, mudpuppy, and lesser siren. We propose to test two different hormones, Luteinizing Hormone Releasing Hormone (LHRH) at 0.1, 0.25, 0.5 and 1.0 μg/g body weight and human Chorionic Gonadotropin (hCG) at 50, 100, 250, and 500 IU and a combination of the two optimal hormone doses on gamete production in male and female salamanders. Hormone injections will be given intra-peritoneal in 100 μl of sterile saline. For the males, we will evaluate sperm production, percent motility, forward progressive motility, concentration, and sperm viability over time. Viability will be assessed using the fluorescent live/dead stain SYBR-14. Sperm will be collected by either manually stripping sperm while gently massaging the abdomen, collection of a spermatophore, or inserting a small plastic catheter a few mm into the cloaca. Samples will be collected hourly over 24 hours. Similarly, various hormone concentrations will be tested on the females and percent responders, time to egg laying, and number of eggs laid will be determined. If single ovulatory injections are not generating a response, we will try priming hormones to induce egg maturation (low doses of the ovulatory hormone given twice over several weeks) prior to an ovulatory hormone dose. These studies will allow us to optimize assisted breeding for these three species using exogenous hormones. The two hormones, LHRH and hCG, at these concentrations have been used extensively by our lab for ART in frogs and toads [3,4,7-13]. In a separate

experiment, we will also place males and females together following various hormone stimulations described above and record breeding behaviors through time-lapse videography.

Objective 2 - Test artificial fertilization techniques in our model species for their application to endangered salamanders as evaluated by fertilized embryo development. In vitro fertilization (IVF) is becoming common practice in frogs and toads, but virtually nothing is known regarding how to accomplish this for salamander species. We propose to use our optimal hormone protocols from Objective 1 for the collection of sperm and eggs in our model species. In brief, we will conduct IVF by mixing sperm and eggs together in a Petri dish, additionally we will conduct artificial fertilization (AF) by inseminating a male's spermatophore directly into the cloaca of the female. For IVF, spawned eggs will be removed immediately from the female's enclosure and clumps/strings of eggs from each female will be placed in Petri dishes and fertilized. Fertility will be assessed at 4-8 hours post-fertilization (4-32 cell stage) using a stereo-microscope. Parameters to be tested include short-term storage of the sperm, sperm concentration and volume for optimal fertilization, time for gamete mixing during IVF, and timing of AF. Measured responses include egg numbers, fertilization rate, neurulation rate, numbers of larvae, and/or metamorphs produced.

Objective 3 - Generate sperm freezing protocols in the model species for their application to gene banking of sperm from endangered species as evaluated by post-thaw survivability. The purpose of this study is to evaluate the application of frog and toad sperm cryopreservation protocols, currently being used by our laboratory, for application to salamanders. In brief, sperm will be collected following hormone administration and evaluated as described above for Objective 1. Sperm samples will be diluted 1:1 in one of two non-permeating buffer systems (10% Trehalose or TES-Tris egg yolk buffer). Extended sperm samples will be cooled to 4°C at which point two permeating cryoprotectants will be added (glycerol and dimethylsulfoxide) to sub-samples at three different concentrations (2.5, 5, and 10%). Spermatozoa will then be loaded into commercial freezing straws and frozen in liquid nitrogen using a programmable freezer. After 1 week of storage in liquid nitrogen, sperm samples will be thawed quickly in room temp water and reactivated by dilution of spermatozoa into distilled water containing 0.2% BSA. Spermatozoa will be evaluated pre- and post-thaw as described above. This experimental strategy will allow the investigators to determine species-specific protocols for salamanders by varying extenders, cryoprotectant concentrations, and cooling rates.

Objective 4 - Increase the reproductive output of our targeted endangered amphibians. Lessons learned from Objectives 1-3 on our model species will be used to develop an experimental approach and appropriate hormones for delivery to our targeted endangered species, the black warrior water dog, hellbender, blue-spotted salamander and flatwoods salamander. We will work with our partners at various zoos to see if we can collect gametes and conduct IVF, AF, or sperm cryopreservation for these species that will have immediate impact on their conservation. Sperm and egg collection along with parameters measured and responses recorded will be similar as above.

Statistics: Data from the female studies, number of responders, egg numbers, cleavage, neurulation, larvae and metamorphosis rates, will be expressed as the means ± SEM and differences will be considered significant at $p<0.05$. Power analysis was performed using over 8 years of standard deviation data to determine that a sample size of 10 or more animals is needed for each treatment group above. Data collected from the males (sperm motility, forward progression, concentration, viability and number of responders) will be analyzed similarly. All percentage data will be arcsine transformed prior to analyses and tested for normality and homogeneity of variance using the Shapiro-Wilk W and Barlett tests. Non-parametric analyses may be necessary when data are not normally distributed. Chi-square tests will look at the differences in percentages of animals responding to treatments. Tukey-Kramer least significant difference will be used for multiple comparisons of means and Student *t*-tests for pairs of means. Analysis of variance (ANOVA), repeated measures ANOVA or linear mixed models for evaluations over time will be used where appropriate. One component of our evaluation plan will require examining the output measures we collected statistically to make sure we are providing accurate statements between the partners, among our collaborators and to the general community regarding our research findings.

Evaluation Plan: The **first phase** of our evaluation plan will be to determine the outcome of our studies on expanding the use of reproductive technologies by the collaborating institutions (Omaha Zoo, Jacksonville Zoo, Fort Worth Zoo, Cincinnati Zoo, St. Louis Zoo and MDC). The institutional contact for each collaborating facility will complete detailed pre- and post-surveys designed to obtain metrics that will review changes in assisted breeding protocols from the start of the project to the end of the project (e.g. number of larvae produced by AF, number larvae repatriated to the wild) as well as level of interest, continued involvement and perceived benefits to their program. In addition, breeding report records will be obtained to determine how many assisted breeding trials were conducted on their own and quantify the results for comparison to when researchers were assisting in order to understand whether the technology transfer is easily exportable and sustainable. These breeding record reviews will be conducted at the end of year two and three of the project; an increase in breeding attempts with successful offspring produced will assess the quality of the program compared to the previous two years before the start of the project. A long-term **second phase** evaluation of this proposal is related to the *Leadership* component of the IMLS grant submission. It is our intent to follow the professional career and placement of the post-doctoral fellow, Ph.D. and M.S. student and nine undergraduate students involved with this three year program to understand the impact the mentoring and leadership opportunity had on shaping early-career development for young scientists. We will follow each participant in the program for 5 years following their completion of the program using permanent email communications established with Yahoo or Gmail. Metrics to be measured will fall into several categories including demographics, field of occupation, number of majors supported, impacts on professional development, publications, and impact on the institution (zoo).

4. Project Resources: Budget, Personnel and Management:

Budget: The budget was developed by Drs. Kouba, Vance and Willard from our previous experience in conducting research on endangered amphibians, developing these technologies under the first Phase I IMLS-sponsored grant, and in consultation with the department of sponsored research at Mississippi State University where the Ph.D. and M.S. student will be acquiring their degree. Our post-doctoral fellowship salary was determined after consultation with Mississippi State University to make sure we were offering a competitive stipend and health care for the region. These are the two largest expenses being requested from this proposal. Funding for the graduate students would begin in January 2012 to coincide with the beginning of the spring semester and would support research on this project for three years, whereas the post-doctoral fellow could be hired immediately upon announcement of the award. We are also requesting financial support for travel to our collaborating institutions and for some necessary equipment to conduct the gene banking at MSU and Memphis Zoo. The Memphis Zoo and MSU will be providing matching costs toward this program as presented in the budget justification section.

Facilities: Much of the equipment needed for the success of this project (liquid nitrogen storage tanks, reserve tanks, dry shippers, programmable freezers, microscopes, small laboratory supplies and some animal holding cages) have already been purchased for both the Memphis Zoo and university location. Moreover, the Zoo's conservation department maintains an 800 square foot state-of-the-art facility for conducting the amphibian research described herein, and all animals will be maintained in the herpetarium or aquarium. All of the institutions we are collaborating with have laboratory space for the graduate students and post-doctoral fellow including microscopes for analyses. Any additional equipment needed will travel with the research associates. A brand new completely remodeled 400 square foot laboratory at MSU has been set-aside for the amphibian conservation partnership and was fully furnished with standard laboratory equipment under the Phase I IMLS NLG grant to conduct the research (less some specific cryopreservation equipment we are requesting in this proposal).

Personnel: *Dr. Andy Kouba*: Dr. Kouba is director of conservation and research for the Memphis Zoo and will contribute 10% of his time to the project. Dr. Kouba has extensive experience in cryopreservation techniques and over eleven years of experience with assisted reproductive technologies for wildlife including amphibians

[2, 3, 6-11]. He is currently the AZA reproductive advisor for the Amphibian Taxon Advisory Group, Puerto Rican Crested toad SSP, Wyoming toad SSP, and steering committee member for AZA's Reproductive Scientific Advisory Group and the Biomaterials Banking Advisory Group. Dr. Kouba will primarily be responsible for the mentoring and supervision of the post-doctoral fellow.

Dr. Carrie Vance: Dr. Vance is a research associate with Mississippi State University and has worked with Dr. Kouba on amphibian reproductive technologies for eleven years and will contribute 10% of her time to the co-supervision of the Ph.D. and M.S. graduate student and will serve on the individuals' committees. In her role she will assist with design, implementation and analysis of experiments that the Ph.D. student will be conducting. She has extensive experience with cryopreservation, gene banking, biochemistry and cell systems.

Dr. Scott Willard: Dr. Willard is professor and chair for the department of Biochemistry and Molecular Biology at Mississippi State University and will provide 3% of his time to the project. As chairman of the department, Dr. Willard is able to bring significant resources to the partnership (see attached partnership statement). He will act as the major advisor for the Ph.D. and M.S. students' training and assist with development of curriculum, mentoring and analysis of experiments. He is a reproductive physiologist by training and is uniquely qualified to assist with development of the technologies in this proposal.

Fields Falcone, M.S.: Fields is a wildlife ecologist and has been with the Memphis Zoo for 1 year - she will contribute 10% of her time to the project. Fields will assist with lab experiments, supervision of interns, reporting, lab management and ordering of supplies, and animal care.

Dr. Jen Germano: Dr. Germano is a herpetologist specializing in reproductive physiology and coordinates the amphibian genome project at the Memphis Zoo. Dr. Germano will contribute 10% of her time to the project and will assist Dr. Kouba with supervision and training of the graduate students along with design and analysis. Dr. Germano will assist the M.S. and Ph.D. student with assisted breeding studies and gene banking trips at other sites. We will not show her time as a match in the budget as she is contractually paid for by another grant.

Post-doctoral fellow, M.S. and Ph.D. Student: The majority of activities detailed within this proposal will be accomplished by the post-doctoral fellow, M.S. and Ph.D. student. Thus, we do not expect this proposal to interfere with on-going museum/zoo activities either at the Memphis Zoo or MSU. The post-doc will be primarily responsible for conducting assisted reproductive research and biomaterial banking on the lesser siren, mudpuppy and hellbenders while the Ph.D. and M.S. student will be responsible for developing similar technologies for the tiger salamander, blue-spotted salamander, flatwoods salamander and black warrior waterdog. The post-doc, Ph.D. and M.S. student will contribute 100% of their time to the project (less the M.S. & Ph.D. students' classes).

Management: The Memphis Zoo will be hosting the post-doctoral fellow and one undergraduate per year to be supervised by Drs. Kouba, Vance and Germano. The Ph.D. and M.S. student along with two undergraduate students per year will be hosted at MSU and will be supervised by Drs. Willard and Vance. Weekly lab meetings between mentors and trainees will occur at each institution and a monthly lab meeting between both facilities will be held so that the entire research team is communicating directly. *In addition to these regularly established meetings, a list-serve will be established by the zoo for communicating between the two partner organizations as well as with our collaborators (institutions holding the animals).* Input from our collaborators is extremely important since they work with these amphibians daily and we feel that a ***list-serve*** is an excellent way to communicate our findings in near real-time. Weekly results from studies can be posted on the list-serve by senior and junior investigators as well as the undergraduate students and allows everyone to be more engaged with the project. In addition to the list-serve, the key personnel will also organize a quarterly conference call with our collaborators using the Qwest FTS audio conference center assistance lines. These live discussion conference calls have been useful in reinforcing our internet communications and create another forum for adaptive management that is useful for periodic evaluations to make sure progress is proceeding according to the project plan. Dr. Andy Kouba will serve as the principal investigator and is responsible for reporting to IMLS. Dr. Carrie Vance will serve as moderator for the list-serve.

5. Dissemination:

Rapid dissemination to amphibian community: The urgent and critical need for sharing our findings with other biologists and the scientific community cannot wait for normal publication processes. We propose to utilize several internet portals to reach the broadest audience quickly. First, the Memphis Zoo will create and host a website specifically for the amphibian assisted breeding program. It will be linked under the conservation section at www.memphiszoo.org. The website will be used to highlight the objectives of the program, the leadership students involved, the results, FAQ and protocols developed. This website could also be linked to IMLS. The website and its content will be advertised through 30 different list-serves that we have identified which will reach the majority of the amphibian global scientific community and professional societies. Each threatened and endangered amphibian species in the U.S. has a recovery team (whether State, Federal or a combination of both) assigned to its conservation. Each of these recovery teams utilizes internal list-serves to communicate within the group and we will communicate within these list-serves to share protocols and information on our website. This form of dissemination also has the advantage of reaching policy makers (State and Federal) directly associated with policies related to a species recovery. However, our finding should also be disseminated on other portals. We will therefore provide our protocols, as they are developed and modified, to the internationally accessed companion animal and non-domestic species (CANDES) assisted reproduction website hosted by our collaborators at the Omaha Zoo [http://www.omahazoo.com/iets/candeshomepage.htm].

Training*:* AZA hosts a seven day amphibian biology and management professional training course that is attended by keepers, curators, academic professionals and students. We are in discussions for participating in this course and including in its curriculum how to use assisted breeding for species difficult to reproduce and how to gene bank reproductive cells. Furthermore, we will offer to host wet-labs at each annual recovery group meeting for all threatened and endangered species in the U.S. that are interested in such technologies due to low reproductive output and declining genetic diversity. We will be visiting our collaborating institutions yearly and the techniques developed from our studies will be incorporated into their standard best management practices.

Publications, Conferences and Husbandry Manuals: The research team will publish its findings in peer-reviewed journals throughout the course of the program as well as a book chapter at the termination of the grant. It is also critical to attend conferences such as the AZA annual ATAG meeting and the Society for the Study of Amphibians and Reptiles (SSAR). Our findings have already been incorporated into the Wyoming toad husbandry manual and PRC toad husbandry manual, highlighting how the information from our study filters down to standard breeding protocols for our targeted species.

Zoo visitors: The Memphis Zoo has an in-house communications team that will develop educational messages associated with the amphibian crisis and our research activities for each species listed in the study. These educational messages will be displayed in the herpetarium and using rotating electronic picture albums that will allow us to provide more adaptive information to our visitors. This displayed material on the amphibian crisis and our research has the ability to reach over 1 million visitors per year. Moreover, our conservation research studies will be incorporated into the educational classrooms and be regularly distributed through the zoos monthly publication, *Exzoobearance*, which reaches over 20,000 members annually.

6. Sustainability:

New Leadership: The principal and co-investigators will recruit and train twelve new professional scientists (one post-doctoral fellow, one Ph.D. and one M.S. student & nine undergraduates) in the field of zoological research and curatorial responsibilities. It is anticipated that upon completing their programs the post-doc or graduate students will enter the zoological or aquarium field and continue to contribute towards the amphibian conservation studies they began in Memphis or MSU. As they pursue their professional careers, it is likely they will pursue additional partnerships, work with other imperiled species and seek funding for their conservation projects. Moreover, undergraduate students from MSU, University of Memphis, Christian Brothers University, and Rhodes College will participate in the Memphis Zoo's summer biodiversity fellowship program that creates opportunities for young biologists to pursue research experience that will help them secure graduate or

veterinary school positions in higher education. Each year the Memphis Zoo will place three undergraduates on this proposal to assist the post-doctoral fellow and graduate students. The key senior personnel feel mentoring twelve additional staff over three years is reasonable, and any more and would impact normal operations. The post-doctoral fellow and graduate students would mentor the undergraduates as part of their training.

Capacity Building: Biologists at our collaborating organizations Omaha Zoo, Fort Worth Zoo, Jacksonville Zoo, Cincinnati Zoo, St. Louis Zoo and Missouri Department of Conservation will be trained on the protocols developed as a result of this research proposal. This training will allow these facilities to use assisted breeding technology and gene banking for increasing their reproductive output beyond the termination of the proposed project. An example of this type of capacity building has already taken place at the NASRF facility and at Omaha's Henry Doorly Zoo under our Phase I IMLS grant. Both organizations have greatly improved the reproductive output of the toads and frogs that they work with, with NASRF already releasing 5,000 artificially fertilized tadpoles into the wild. These facilities are working side by side with the investigators learning our techniques and incorporating them into their standard breeding protocols. *The example above demonstrates how our major collaborators are using these technologies to ensure the project is sustainable; a conservation milestone output has already been demonstrated by our collaborators highlighting the incorporation of our study outcomes to applied on-the-ground conservation.* We have found that the best way to capitalize on knowledge gained by our studies is to travel to our collaborators facilities, conduct the research with them side-by-side, and perform training exercises with their staff. Furthermore, information we learn from our studies will be incorporated into the husbandry manuals for these species and provide a guidepost for other agencies working with endangered species. These technologies will be rapidly disseminated as described in the previous section.

Long-term Commitment*:* The conservation and biomaterial banking of reproductive cells for endangered amphibians was established as one of our institution's signature projects in 2001. Since that time, our zoo has invested a significant amount of resources towards the long-term management and use of the material placed into our GRB for amphibians. The resources we have placed into this repository will be used for research and breeding purposes long after the project is completed and new support from IMLS will assist in the expansion of assistance that we can provide to zoos, aquariums and government facilities involved with amphibian conservation. Our continued sustainability toward this program is documented in our recent five year strategic plan (2008-2013) for the conservation and research department (see supporting documents) illustrating the zoo's commitment toward sustaining this program past the termination of the proposal. Long-term commitments to our proposed conservation activities have been incorporated into the 2005 'Amphibian Conservation Action Plan' drafted by the IUCN and CBSG. Ownership of the material (reproductive cells) placed into the GRB is under specific control by the state or government agencies responsible for the species recovery and they are on loan to the Memphis Zoo (due to ESA regulations related to endangered species). We are able to provide reproductive cells for captive breeding, recovery and research studies to outside organizations once permission is received from federal or state agencies.

***Partnership with Mississippi State University*:** The partnership between the Memphis Zoo and MSU has grown substantially over the last five years and our institutions have been involved with many projects together. By creating a specific laboratory at MSU for amphibian conservation, the proposal will help build a long-term program between our facilities that will be sustained beyond the termination of the proposal and provide numerous more opportunities for professional leadership development of young scientists early in their career. A five-year memorandum of understanding (MOU) for the sustainable partnership is in review that will formalize our continued partnership and can be provided to IMLS once the University's legal department has finished its evaluation.

BUDGET FORM: Section B, Summary Budget

	$ IMLS	$ Cost Share	$ TOTAL COSTS
1. Salaries and Wages	$137,000.00	$69,812.00	$206,812.00
2. Fringe Benefits	$17,936.00	$10,280.00	$28,216.00
3. Consultant Fees	$0.00	$0.00	$0.00
4. Travel	$26,400.00	$26,680.00	$53,080.00
5. Supplies and Materials	$39,224.00	$24,000.00	$63,224.00
6. Services	$0.00	$0.00	$0.00
7. Student Support	$0.00	$0.00	$0.00
8. Other Costs	$43,161.00	$11,500.00	$54,661.00
TOTAL DIRECT COSTS (1-8)	$263,721.00	$142,272.00	$405,993.00
9. Indirect Costs	$36,279.00	$21,341.00	$57,620.00
TOTAL COSTS (Direct and Indirect)	$300,000.00	$163,613.00	$463,613.00

Project Funding for the Entire Grant Period

1. Grant Funds Requested from IMLS	**$300,000.00**
2. Cost Sharing:	
a. Applicant's Contribution	**$62,180.00**
b. Kind Contribution	**$101,433.00**
c. Other Federal Agencies*	**$0.00**
d. TOTAL COST SHARING	**$163,613.00**
3. TOTAL PROJECT FUNDING (1+2d)	$463,613.00
Percentage of total project costs requested from IMLS	**64.71 %**

*If funding has been requested from another federal agency, indicate the agency's name:

SCHEDULE OF COMPLETION – shaded areas are when the experiments will occur

Objective	Species	2011-2012											
		Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct
Objective 1: Develop novel hormone regimens for sperm and egg collection in our model species, the tiger salamander, mudpuppy, and lesser siren.	**Mudpuppy**												
	Tiger Salamander												
	Lesser Siren												
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												
Objective 2: Test artificial fertilization (AF) techniques in our model species for their application to endangered salamanders as evaluated by fertilized embryo development.	**Mudpuppy**												
	Tiger Salamander												
	Lesser Siren												
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												
Objective 3: Generate sperm freezing protocols in the model species for their application to gene banking of sperm from endangered species as evaluated by post-thaw survivability.	**Mudpuppy**												
	Tiger Salamander												
	Lesser Siren												
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												
Objective 4: Increase the reproductive output of our targeted endangered salamander species by applying what we learn in Objectives 1-3 to develop species-specific hormone regimens and AF techniques that can easily be used by zoo, aquarium, and government curators.	**Mudpuppy**												
	Tiger Salamander												
	Lesser Siren												
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												

SCHEDULE OF COMPLETION – shaded areas are when the experiments will occur

Objective	Species	2012-2013											
		Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct
Objective 1: Develop novel hormone regimens for sperm and egg collection in our model species, the tiger salamander, mudpuppy, and lesser siren.	**Mudpuppy**	■	■	■	■	■	■	■	■	■	■	■	■
	Tiger Salamander	■	■	■	■	■	■	■	■	■	■	■	■
	Lesser Siren	■	■	■	■	■	■	■	■	■	■	■	■
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												
Objective 2: Test artificial fertilization (AF) techniques in our model species for their application to endangered salamanders as evaluated by fertilized embryo development.	**Mudpuppy**	■	■	■	■	■	■	■	■	■	■	■	■
	Tiger Salamander	■	■	■	■	■	■	■	■	■	■	■	■
	Lesser Siren	■	■	■	■	■	■	■	■	■	■	■	■
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												
Objective 3: Generate sperm freezing protocols in the model species for their application to gene banking of sperm from endangered species as evaluated by post-thaw survivability.	**Mudpuppy**	■	■	■	■	■	■	■	■	■	■	■	■
	Tiger Salamander	■	■	■	■	■	■	■	■	■	■	■	■
	Lesser Siren	■	■	■	■	■	■	■	■	■	■	■	■
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												
Objective 4: Increase the reproductive output of our targeted endangered salamander species by applying what we learn in Objectives 1-3 to develop species-specific hormone regimens and AF techniques that can easily be used by zoo, aquarium, and government curators.	**Mudpuppy**												
	Tiger Salamander												
	Lesser Siren												
	Blue Spotted Salamander					■	■	■					
	Alabama Waterdog							■	■	■	■		
	Flatwoods Salamander	■									■	■	■
	Hellbender	■	■	■								■	■

SCHEDULE OF COMPLETION – shaded areas are when the experiments will occur

Objective	Species	2013-2014											
		Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct
Objective 1: Develop novel hormone regimens for sperm and egg collection in our model species, the tiger salamander, mudpuppy, and lesser siren.	**Mudpuppy**	■	■	■	■	■	■						
	Tiger Salamander	■	■	■	■	■	■						
	Lesser Siren	■	■	■	■	■	■						
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												
Objective 2: Test artificial fertilization (AF) techniques in our model species for their application to endangered salamanders as evaluated by fertilized embryo development.	**Mudpuppy**	■	■	■	■	■	■	■	■	■	■	■	■
	Tiger Salamander	■	■	■	■	■	■	■	■	■	■	■	■
	Lesser Siren	■	■	■	■	■	■	■	■	■	■	■	■
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												
Objective 3: Generate sperm freezing protocols in the model species for their application to gene banking of sperm from endangered species as evaluated by post-thaw survivability.	**Mudpuppy**	■	■	■	■	■	■						
	Tiger Salamander	■	■	■	■	■	■						
	Lesser Siren	■	■	■	■	■	■						
	Blue Spotted Salamander												
	Alabama Waterdog												
	Flatwoods Salamander												
	Hellbender												
Objective 4: Increase the reproductive output of our targeted endangered salamander species by applying what we learn in Objectives 1-3 to develop species-specific hormone regimens and AF techniques that can easily be used by zoo, aquarium, and government curators.	**Mudpuppy**												
	Tiger Salamander												
	Lesser Siren												
	Blue Spotted Salamander					■	■	■	■				
	Alabama Waterdog							■	■	■	■		
	Flatwoods Salamander	■									■	■	■
	Hellbender	■	■	■								■	■

SCHEDULE OF COMPLETION – important notes.

Hormonal sperm induction and cryopreservation, and artificial fertilization techniques for the mudpuppy, tiger salamanders and lesser sirens can occur throughout the year as we will have research colonies present at both the Memphis Zoo and Mississippi State University. Experiments testing sperm induction and cryopreservation for endangered and threatened salamanders will occur at various times of the year, based on the holding institution's captive management reproductive program and the natural reproductive season.

Blue spotted salamander (Nebraska/Iowa): March-June
Alabama Waterdog (Ohio): May- August
Flatwoods Salamander (Florida): Autumn
Hellbender (Texas and Missouri): September-January

Experiments that will possibly result in the production of larvae (Sperm and ovulation induction and short-term storage of sperm followed by IVF) will be set-up to coincide with the species' normal breeding cycles in the wild. This is because any larvae produced as a result of our experiments will be given to the USFWS for their reintroduction programs. Cyclical breeding for each species is shown above: