National Leadership Grants for Museums

Sample Application MG-30-14-0072-14
Project Category: Collections Stewardship

Cincinnati Zoo and Botanical Garden

Amount awarded by IMLS:	$345,777
Amount of cost share:	$421,012

Attached are the following components excerpted from the original application.

- Abstract
- Narrative
- Schedule of Completion

Expanding access and building capacity for African and Asian rhino reproductive care within North American AZA/WAZA facilities: a Rhino Assisted Reproduction Enterprise (RARE)

ABSTRACT:
The Center for Conservation and Research of Endangered Wildlife (CREW), in partnership with SeaWorld & Busch Gardens Reproductive Research Center (SWBGRRC), requests funding of $427,952 over three years for a project focused on expanding access and building capacity for African and Asian rhino reproductive care within North American Association of Zoos and Aquariums (AZA)/World Association of Zoos and Aquariums (WAZA) facilities. Current rates of reproduction in captive African and Asian rhinos remain insufficient for long-term sustainability. This demonstration and investigative research based project will benefit collections charged with managing African and Asian rhino populations for genetic health and viability and help ensure these charismatic animals are around in zoological settings for many years to come.

Previous IMLS funded research conducted by CREW demonstrated the value and feasibility of utilizing genome resource banks (GRBs) and assisted reproductive technology (ART) in Asian rhinos. As a result, requests for Indian rhino ART assistance have increased, and there has been a call for expansion of this work to African southern white rhinos. Due to the rapidly developing interest in using ART to facilitate rhino breeding/management programs among zoos, a critical next step involves training additional scientists/veterinarians to conduct these procedures so that there are enough qualified individuals to handle the demand. Eight such facilities have committed to building a **Rhino Assisted Reproduction Enterprise (RARE)** in collaboration with CREW. In addition, there are specific next steps worth pursuing to improve the efficiency of ART for assisting the management of rhino populations. During this grant, we aim to assist multiple North American AZA/WAZA facilities in producing rhino pregnancies through staff training and technology transfer of proven and novel ART. Specific objectives of the grant are to: 1) contribute to the genetic management and propagation of captive Indian rhinos through artificial insemination (AI); 2) enhance southern white rhino fertility through exogenous hormone administration prior to natural breeding or AI; 3) build upon national rhino gamete rescue centers at CREW and SWBGRRC; and 4) provide collaborating facilities with individualized training and/or support in rhino ART (exogenous hormone protocols, ultrasonography, endocrine analysis, AI, and sperm collection, sorting and cryopreservation).

The project will be directed by Monica Stoops, CREW Reproductive Physiologist. Dr. Justine K. O'Brien, SWBGRRC Scientific Director will serve as project co-director. The project team of ten CREW/SWBGRRC scientists and research technicians will be supported by an advisory group of veterinarians and rhino keepers that have been involved in previous rhino ART procedures in collaboration with CREW. IMLS funds will support the endocrine research associate and technician required to perform the hormone analysis critical for timing natural breeding or AI. In addition, it will provide the purchase of equipment and supplies needed for the execution of the work, travel to and from facilities for the purpose of performing ART procedures and travel for project team members to disseminate results at conferences aimed at our target audience.

This project will provide much-needed access and capacity for rhino ART within our nations' facilities that will be utilized now and in the future to help meet the needs of captive rhino species. The offspring produced as a result of this work will be living proof of the value of rhino ART and GRBs. Through this project, we will expand and develop novel rhino ART procedures for those rhinos that are not breeding naturally or are behaviorally incompatible with their mates. Many zoos will welcome the use of ART procedures as an alternative to the risky and largely unsuccessful efforts to introduce certain animals for natural breeding. As a result, pregnancies/offspring will be produced between pairs that would otherwise not have reproduced. Furthermore, the genetic lifespan of many individual rhinos will be expanded as a result of sperm banking, which can be used to bolster populations now or decades from now. AI, exogenous hormones and sperm sexing technology will be integrated into the available set of ART tools that rhino managers will have at their disposal to meet the genetic and demographic needs of captive breeding programs for African and Asian rhinos.

PROJECT JUSTIFICATION

The Center for Conservation and Research of Endangered Wildlife (CREW) at the Cincinnati Zoo & Botanical Garden (CZBG), in partnership with the SeaWorld & Busch Gardens Reproductive Research Center (SWBGRRC), seeks a National Leadership Grant of $427,952 over three years in the Collections Stewardship category. This would be a research grant focused on expanding access and building capacity for African and Asian rhino assisted reproductive care within Association of Zoos and Aquariums (AZA)/World Association of Zoos and Aquariums (WAZA) facilities across North America. Previous IMLS funding enabled CREW to demonstrate the value and feasibility of utilizing genome resource banks (GRBs) and assisted reproductive technology (ART) in Asian rhinos. As a result of this work, requests for Asian rhino ART assistance have increased, and there has been a call for expansion of this work to African rhinos. Due to the rapidly developing interest in using ART to facilitate rhino breeding/management programs among zoos, a critical next step involves training additional scientists/veterinarians to conduct these procedures so that there are enough qualified individuals to handle the demand. In addition, there are specific next steps worth pursuing to improve the efficiency of ART for assisting the management of rhino populations. During this grant, we aim to assist multiple North American AZA/WAZA facilities in producing rhino pregnancies through staff training and technology transfer of proven and novel ART. Specific objectives of the grant are to: 1) contribute to the genetic management and propagation of captive Indian rhinos through artificial insemination (AI); 2) enhance southern white rhino fertility through exogenous hormone administration prior to natural breeding or AI; 3) build upon national rhino gamete rescue centers at CREW and SWBGRRC; and 4) provide collaborating facilities with individualized training and/or support in rhino ART (exogenous hormone protocols, ultrasonography, endocrine analysis, AI, and sperm collection, sorting and cryopreservation).

This collaborative project will significantly advance the application of ART to African and Asian rhinos and directly benefit collections charged with managing these populations for genetic health and viability. Most importantly, it will follow up on earlier work conducted by CREW[1] that demonstrated AI can be performed on rhinos at remote facilities using a standing sedation approach. As a result, AI pregnancies were established in Indian rhinos at three facilities (born January 2008, October 2010, June 2013 and one due June 2014) using cryopreserved sperm from three different males. The value of this type of endeavor was discussed at the AZA Rhino Taxon Advisory Group (TAG) and Research Council meetings in August 2013, and the effort has since been endorsed by the Species Survival Plan (SSP) coordinators for Indian and African southern white rhinos (*Supporting Document Letters of Support*). It was evident from the meetings that there is a pressing need to continue the use of ART and further develop these capabilities in rhino species experiencing difficulties with reproduction and/or limited genetic diversity in captivity.

It is anticipated that, through this project, CREW – a leader in reproductive breakthroughs with rhinos will collaborate with AZA/WAZA facilities to develop their institutional capacity for rhino ART. Eight such facilities have committed to building a **Rhino Assisted Reproduction Enterprise (RARE)** in collaboration with CREW (see Project Work Plan; *Supporting Document Letters of Support*). These facilities will contribute the veterinary and rhino keeper staff time needed to learn and implement rhino ART, with the necessary training, tools and laboratory support provided by CREW. The successful transfer of rhino ART to other facilities will help fully integrate these options into the managed breeding programs for African and Asian rhinos. In addition, it will foster closer working relationships and ensure continued commitment by AZA/WAZA rhino facilities to the application of this acquired skill set.

In addition, CREW will partner with SWBGRRC- a leader in research on sperm sorting and AI for pre-sexed births of marine and terrestrial wildlife species (*Supporting Document Partner Letter*). Since 2006, CREW and SWBGRRC have worked in partnership to develop sperm sorting and preservation technologies for sex ratio modification in Asian and African rhinos[2]. The ability to preferentially produce female or male offspring using sperm sorting/preservation and AI technologies could significantly enhance the management and sustainability

of zoological populations of species such as the rhino. SWBGRRC will contribute staff time, equipment and expertise in this area. Together, we will demonstrate how coordination of GRBs at different zoological institutions can facilitate best assisted rhino pairings. Finally, an advisory team (*Supporting Document Advisory Group Letters*) of experts in the areas of rhino management/training and veterinary care will support the project team throughout and provide essential feedback, input and ideas.

Background: One of the primary goals for keeping rhinos in zoological collections is to maintain populations that are genetically viable and self-sustaining to guard against any unforeseen extinction in the wild. Another is to have animals for exhibition to educate and inspire the public about the significant role these species play in their ecosystem. Unfortunately, the reproductive rate of captive African and Asian rhinos remains insufficient to sustain independent populations, relying on importation of individual animals from facilities overseas or the wild. Captive rhino populations are not alone in facing a serious loss of critical genetic diversity. Wild rhino populations are experiencing a poaching crisis of unprecedented scale that is endangering their very existence. Currently, in South Africa alone, one rhino is killed every 10 hours for the purpose of poaching for horn. Because the pressure for rhino horn results from ancient and deeply rooted beliefs, it is not anticipated to decline any time in the near future. This insatiable demand has exponentially increased poaching to a rate that, if left unabated, will render wild rhino populations no longer sustainable. It is possible that zoos may be the last places these animals exist. Therefore, it is imperative to address and remedy those difficulties associated with reproduction that have limited gene diversity in zoological populations of rhino species to ensure they are around for many years to come.

White Rhinos- The African southern white rhino remains the most popular rhino species held in US zoos. Presently, a total of 156 southern white rhinos are maintained in 46 AZA facilities[3]. Its popularity can be attributed to its highly tractable nature in comparison to other rhino species, and the fact that females can be kept in large groups for display. Although captive breeding has been successful and a sufficient number of calves are being produced to consistently maintain the population, the proportion of breeding recommendations resulting in offspring is quite low. If reproduction were more reliable, the best strategy would be managed breeding targeted at recruiting remaining potential founders and equalizing founder representation. Current founder representation in the southern white rhino population is highly skewed. A single animal out of 65 founders is responsible for >10% of the genetic representation. With 14 potential founders still remaining, it is essential that they contribute to the population.

It is estimated that <30% of all wild caught and <20% of captive born southern white rhinos have reproduced in captivity[3]. These numbers reflect a major impediment to achieving a sustainable ex situ breeding program for this species. A primary reason for the low reproductive rate is that a vast majority of females display long periods of acyclicity. In addition, many captive males do not respond to females exhibiting silent heat, or for that matter, females displaying regular estrous cycles. The SSP has resorted to moving individuals and pairing animals with mis-matched mean kinships in an attempt to stimulate breeding in rhinos that have not yet reproduced. This approach has been met with some success. Because many of these pairings do not have well-matched low mean kinships, the goal of equal founder representation remains unmet. However, it provides a means that previously did not exist to achieve successful reproduction in select individuals. Because there still remains a large subset of rhinos that have failed to reproductively respond to new pairings, it became necessary to develop novel ART to overcome this management challenge.

One part of the proposed research will be to continue and expand upon a preliminary CREW investigation conducted in collaboration with several AZA/WAZA facilities to develop an exogenous hormone protocol to initiate reproductive activity in previously acyclic southern white rhinos. During this initial study (*Supporting Document Preliminary Data figures 1&2*), administration (n=4 females) of a single injection of sustained release progesterone (P4)[4]: 1) achieved serum P4 concentrations >1ng/mL; 2) ensured release of P4 over a 10-

14 day time period; 3) inhibited follicle growth during P4 release and 4) promoted growth of a preovulatory follicle following exogenous P4 decline. Follicular growth rate following P4 withdrawal was normal for the species (~.2cm/day; 9 day follicular phase). We also determined that GnRH (Cystorelin®) administration induces ovulation in the white rhino and results in a luteal phase the duration of which is consistent for this species (*Supporting Document Preliminary Data figure 3*). While the current protocol stimulates growth of preovulatory follicles of a normal size, females fail to show signs of behavioral estrus and rebuff advances by a male to breed; preliminary evidence suggests a failure to elicit an endogenous GnRH (and hence LH) surge. However, because females are responsive to exogenous GnRH, our protocol appears adequate if AI were to be performed. It still remains imperative that an exogenous hormone regime be developed that facilitates natural breeding so that more individual rhinos and institutions will benefit.

This preliminary study generated several new avenues of question and is the basis for the novel research outlined in this proposal (*Project Work Plan, Activity 2*). The research questions we aim to answer are: 1) What is the fertility rate of follicles that grow and ovulate as a result the current hormone protocol? ie, how many pregnancies are achieved in these animals following AI using frozen-thawed sperm? 2) Will addition of sustained release estradiol (E2) over 1-2 days during late follicular phase elicit estrus, induce an endogenous GnRH and LH surge and facilitate natural breeding? 3) Will the new hormone regime inititate regular, spontaneous reproductive activity in treated females?

Indian Rhinos- The captive Indian rhino population in North America has grown since its inception as a result of successful captive reproduction and importation, reaching the current size of 55 animals distributed throughout 23 zoos (including 19 AZA facilities)[5]. Indian rhinos are primarily solitary and territorial. Males and females are exhibited separately except during introductions for breeding. Natural breeding attempts can frequently result in severe aggression between Indian rhino pairs, and this behavioral incompatibility has made genetic management of this species a challenge. Four founders are responsible for over 50% of all genes found in the present population. Therefore, the genetic foundation and diversity of this population is limited and inadequate for long-term viability. Unlike the 14 potential founders (11 females; 3 males) that still remain in the southern white rhino population, none of the 23 founder Indian rhinos remain capable of contributing via natural breeding. Within the last five years, two captive-born male Indian rhinos were imported from India and now serve as new founders for North America. Sperm has been cryopreserved from one original and one new founder[6]. These samples currently reside in GRBs at CREW and SWBGRRC.

Behavioral incompatibility is not the only issue impacting the reproductive management of captive Indian rhinos. Fertility declines when females undergo extended lengths of time without carrying a pregnancy. Female Indian rhinos are prone to the development of leiomyomas within their reproductive tract. As this pathology appears under the influence of cyclic changes in reproductive hormones, continuous estrous cycles without intervening pregnancies spell a recipe for disaster. The earlier first pregnancies occur and the quicker subsequent pregnancies can be established, the longer the reproductive lifespan will be for female Indian rhinos. The sex ratio of an ex situ population is also a factor to consider when managing a sustainable breeding program, particularly for species like the Indian rhino which reproduce slowly and have considerable housing requirements to minimize aggressive interactions. Currently, slightly more females (n=30) than males (n=25) are held in the North American zoological population[5]. It will be desirable to maintain this sex ratio skew favoring females in the long-term to maximize the population's growth rate and ensuing sustainability, whilst still ensuring that there exist an adequate number of genetically diverse males for natural breeding.

As a result of previous CREW work[1], AI has proven successful in Indian rhinos with a conception rate of 50% achieved under optimal AI conditions. We propose through this project to apply ART to more facilities and individual Indian rhinos to overcome some of challenges faced in the genetic and reproductive management of this population (*Project Work Plan, Activity 1*). Concurrently, we will provide to appropriate personnel at other

AZA/WAZA facilities (*Project Work Plan, Activity 4*) the knowledge and skill set required to successfully perform AI procedures.

CREW and SWBGRRC Research to Date- CREW scientists are committed to research to enhance breeding success and develop ART to help ensure genetically fit populations of captive rhinos. The heart of CREW is the GRB, better known as the CryoBioBank. It was established through an IMLS exceptional project awarded in 1990. If not for the CryoBioBank, CREW would not have had the foundation for the rhino sperm bank currently utilized in ART procedures (*Supporting Document Rhino GRB*). Since 2002, we have performed a total of 49 electroejaculation (EEJ) procedures on male Indian, white and black rhinos in collaboration with six AZA facilities[6,7]. Procedures resulted in ejaculates containing high quality sperm fractions that were cryopreserved and are presently maintained in GRBs at CREW and SWBGRRC (n= 13 males). CREW has also established a successful gamete rescue protocol for post-mortem male rhinos[8,9]. Of the 16 gamete rescue attempts that have been conducted for male Indian, white, black and Sumatran rhinos, successful sperm cyropreservation was achieved in half of these attempts. In the other half, either the male was too young to be producing sperm or the harvested sperm exhibited too low motility or numbers to meet the criteria for cryopreservation. Since 2009, seven post-mortem gamete rescue attempts on white and black rhinos have been performed at SWBGRRC. Sperm from two of these males were of adequate quality and successfully cryopreserved. The concerted efforts by CREW and SWBGRRC have ensured that the non-sorted sperm required to conduct the AIs proposed in this grant are secured (*Project Work Plan, Activities 1&2*).

Previous IMLS funding (2009-2012) helped CREW develop the methodologies for successful AI in Indian rhinos at remote facilities and demonstrate that our protocol for freezing and thawing sperm can be used to produce ART calves (three term pregnancies 2008, 2010, 2013 and one ongoing pregnancy due June 2014). As a result, GRBs and AI are now valuable tools that can be integrated into the management strategy for the captive Indian rhino population. Currently, CREW is the only facility performing AI in this species. A logical next step is to train additional colleagues in this procedure at facilities across North America.

In 2006, CREW teamed up with SWBGRRC to develop sperm sorting and preservation technologies for sex ratio modification in Asian and African rhinos. Since that time, there have been 14 EEJ attempts in white and Indian rhinos. As a result of this work, optimum methods for sorting African rhino sperm were established[2] and X-enriched samples have been frozen from one southern white rhino for use in future AI trials. Concurrent efforts with Indian rhinos were delayed as a species-specific interaction between their seminal plasma and sperm transport extenders prevented resolution of X and Y sperm populations. In 2012, different extenders and processing methods were trialed. Results were promising and serve as the basis for the research proposed in this grant (*Project Work Plan, Activity 3*).

With an exogenous hormone protocol in place that appears sufficient for white rhino ART procedures, we are set to commence AI trials during this project using non-sorted cryopreserved sperm. Even though optimum methods for sorting and cryopreservation of white rhino sperm have been established, banking of adequate numbers of X-enriched samples and validation of the proposed AI methodology (*Project Work Plan, Activity 2*) are required for sorted sperm AI trials in the future. Inconsistent ejaculate quality is the greatest challenge to the application of sorting technology and its integration into an AI program for white rhinos. Across all EEJs conducted on this species, semen quality was high enough for sorting trials in 44% of collections. In those ejaculates that did not meet criteria for sorting trials, semen quality was diminished as a result of urine contamination.

In an effort to improve ejaculate quality and increase the numbers of X-enriched samples for white rhinos (*Project Work Plan, Activity 3*), we will examine if 1) urethral catheterization of anesthetized male white rhinos prior to EEJ will result in non-urine contaminated sperm samples; 2) the same drugs used to chemically induce

a concentrated ejaculate from domestic stallions can provide an alternative and/or superior method than EEJ to obtain sperm.

Intended Project Results- The proposed project sets out to address two critical next steps towards ensuring the long-term survival of the North American captive breeding programs for African and Asian rhinos. First, from a biological perspective, more founder rhinos will contribute to the tiny gene pool that currently exists within these populations. Second, from a collaborative perspective, the tools, training, laboratory support and analysis needed for successful rhino ART will be transferred to and implemented at other AZA/WAZA facilities. In so doing, institutional and individual capacity in the use of ART to manage rhino collections will increase.

The most tangible results of this project will be the products produced. First and foremost are the offspring that will be born from genetically valuable pairs. Substantial numbers of sperm will be banked and numbers of individual male rhinos represented in GRBs will increase. These samples may be used tomorrow or 50^{+} years from now to produce offspring that will bolster Indian and white rhino populations. Higher numbers of sorted sperm will be cryopreserved from white rhinos which will facilitate AI trials using X-enriched sperm samples in the future. Sperm sexing technology will be established in Indian rhinos so that frozen-thawed X- or Y-enriched sperm sample AI trials can similarly occur sometime in the future. Another intended result will be enhanced fertility among female Indian and white rhinos. The establishment of pregnancies from either species will help ensure reproductive viability for many years to come. Instead of the irregular, prolonged and anovulatory estrous cycles currently exhibited by a large number of female white rhinos, there will be established exogenous hormone protocols in place to: 1) promote estrus and natural breeding; 2) induce repeated estrous activity and; 3) ensure ovulation following ART procedures.

Project Benefits- A significant number of zoos, individuals and organizations will benefit from the project's activities and accomplishments. Because of its direct impact on the reproductive success of individual rhinos within managed breeding programs (SSPs) and the fact that there are numerous zoos participating in each SSP, our project should have far-reaching effects. Zoo visitors will be rewarded and inspired by the opportunity to see baby rhinos produced from enhanced natural breeding or ART. The SSP will benefit by the production of calves from rhino pairings that are genetically desirable for the population. One of the most tangible achievements of this project will be the ability to maintain each species' genetic diversity and ensuing sustainability in the long-term through enhanced reproduction and founder representation. Benefit will be also be gained from the collaborative, institutional and individual development that occurs between and within AZA/WAZA facilities as a result of this work. Access to the tools, resources and knowledge base required for successful rhino ART will be expanded. In turn, North American rhino collections will benefit from the increased capacity for African and Asian rhino reproductive care.

PROJECT WORK PLAN:

RARE is a three-year project with four distinct activities that support the overarching goal to apply and enhance ART for ex situ African and Asian rhinos to ensure their optimal genetic management and long-term sustainability. These activities involve demonstration and investigative based research.

Activity 1: Facilitate the genetic management and propagation of captive Indian rhinos through AI

This activity will largely be demonstration-based and focused on the development of additional personnel at AZA/WAZA facilities (*Supporting Document Letters of Support; African Lion Safari, Buffalo Zoo, Denver Zoo, Cincinnati Zoo & Botanical Garden*) to conduct AI procedures. Urine samples will be collected on a frequent basis from female Indian rhinos and shipped to CREW for enzyme immunoassay (EIA) analysis of progesterone metabolite (PdG) and estrogen conjugate (EC) concentrations[10,11]. Rhino keeper staff at each facility will condition females to enter a chute for the purpose of performing AI. The standing sedation protocol already established for successful intrauterine AI in this species will be implemented one day prior to expected

ovulation date. Each facility will observe at least one AI before conducting the next AI under CREW supervision. In order to ensure long-term success and efficient use of valuable GRB samples, personnel from each facility will be required to visit CREW or SWBGRRC for ongoing accreditation in semen thawing and assessment/observation of AIs. The Project Director will work in conjunction with the Indian rhino SSP coordinator to determine the best assisted rhino pairings based on the available GRB sires. CREW and SWBGRRC GRBs currently contain sperm from 11 Indian rhinos.

Activity 2: Enhance southern white rhino fertility through exogenous hormone administration for natural breeding and AI

A key component of this project is to test and evaluate exogenous hormone protocols to promote ART and natural breeding. Collaborating facilities (*Supporting Document Letters of Support; African Lion Safari, Indianapolis Zoo*) have four founder females that have not yet contributed to population. Two of these institutions have already participated in our preliminary research to establish an appropriate exogenous hormone protocol for ART procedures in white rhinos. Therefore, keeper and veterinary staff are already familiar with the frequent blood collection and rectal ultrasound examinations that will be required for the next phase of the study. We will determine if addition of sustained release (1-2 day) estradiol to our current protocol (P4+GnRH) will result in estrus and breeding. Estradiol will be administered one day prior to GnRH. A second trial will be conducted in which females will receive P4+estradiol, but will not receive GnRH. This will establish if estradiol induces endogenous GnRH and LH surges (necessary for ovulation). Serum samples will be sent to CREW for EIA analysis of P4 concentrations. Collaborators are familiar with introducing male and female white rhinos for the purposes of breeding, so they are prepared to conduct pairings during late follicular phase and watch for signs of behavioral estrus. Females that do not breed naturally will have standing sedation AI procedures performed utilizing the most effective hormone protocol as identified from our research. The intrauterine AI technique will be based on that developed for the Indian rhino. The Project Director will work in conjunction with the white rhino SSP coordinator to determine the best assisted rhino pairings based on the available GRB sires. CREW and SWBGRRC GRBs currently maintain sperm from eight white rhinos.

Activity 3: Build upon national rhino gamete rescue centers at CREW and SWBGRRC

For this activity, EEJ procedures will be conducted on Indian and white rhinos (*Supporting Document Letters of Support; African Lion Safari, Buffalo Zoo, Lowry Park Zoo, the Wilds*) to obtain sperm for demonstration, investigative research and long-term gamete banking purposes.

<u>White Rhinos</u>- With a sperm sorting technology already established in this species[2], the primary focus will be on banking additional samples. Because inconsistent ejaculate quality has challenged the application of sorting technology for white rhinos, we will concurrently investigate alternative methods for collecting sperm in the hopes of obtaining higher quality samples. White rhino ejaculates containing elevated creatinine concentration (due to urine contamination) display reduced *in vitro* sperm quality after sorting. Urine contamination likely occurs as a result of bladder stimulation during EEJ. A potential side effect of the alpha-2-adrenergic agonists (commonly used in white rhino sedation) is semen release into the urethra[12]. Therefore, anesthetized males (n= 8 males) will undergo urethral catheterization prior to EEJ in order to determine if non-urine contaminated sperm samples can be collected. In addition, we will investigate whether chemically induced ejaculates can be obtained from bull white rhinos using a combination of oral imipramine and IV xylazine[13]. The method we are proposing is considerably less invasive than EEJ and because the amount of xylazine utilized will be low, it will not result in full sedation of the animal. Therefore it could be performed more frequently or used in males that cannot be anesthetized due to health concerns. We routinely use oral imipramine prior to Indian and white rhino EEJ procedures and are comfortable with administration and dosage of this drug. However, because white rhinos are more sensitive to anesthetics, we will begin with a low dose from the recommended dose range for IV xylazine, gauge response and increase the dose as seen fit by the individual animal. Only male rhinos that are already conditioned for blood collection will be used for this study.

Indian rhino- Our initial attempts to transfer the sperm sexing technology developed for African rhinos to Indian rhinos were met with unexpected results. We discovered that the protein source (egg yolk) used in the traditional rhino semen extender utilized for shipment to the sorting laboratory was forming a precipitate with Indian rhino seminal plasma, thereby preventing uptake of the fluorescent dye required to resolve X and Y sperm populations. Therefore, alternative extenders with different protein sources were examined using sperm collected from two male Indian rhinos via EEJ. A skim milk-based extender showed promising results in achieving resolution between X- and Y- sperm from the Indian rhino. The investigative research we propose to undertake during this project will involve conducting sorting trials using this new extender, and comparing two freezing methods (straw versus directional solidification). We will target five different male Indian rhinos and collect sperm via EEJ. The numbers of sperm needed to perform these trials may be less than the total number of sperm collected. Any remaining sperm will be cryopreserved using established methods for non-sorted sperm and stored in CREW or SWBGRRC GRBs.

Activity 4: Provide partner facilities with individualized training and support in rhino ART
This demonstration-based activity will focus on facilitating the application of established rhino ART to AZA/WAZA facilities in an effort to expand the capacity for rhino reproductive care in North America. *Endocrine analysis* will be employed to track the estrous cycles and time ART procedures in female Indian and white rhinos. *Ultrasonography* will be utilized to monitor follicular growth, ovulation and diagnosis of pregnancy in both rhino species. In addition, it will document semen deposition following AI procedures. Ultrasound images (jpg or video) will be transmitted via email to the Project Director for input and feedback. Both metrics will provide critical data for exogenous hormone trials and AI. *Exogenous hormones* will be shipped directly to partner facilities. They will be provided with detailed instructions/timelines for administration and use of P4, GnRH and estradiol. The Project Director will be onsite for *semen collection* via EEJ and process samples for transport to SWBGRRC for *sorting*. If spermic ejaculates are obtained as a result of chemical induction trials, subsequent trips will be planned by the Project Director to examine sample quality and determine use for sorting/cryopreservation. Collaborating facilities will be outfitted with the equipment needed to successfully thaw rhino sperm and perform *AI* procedures. They will be instructed on the proper use of these materials by the Project Director and be accredited yearly. Endocrine, ultrasound or sperm data will be entered into excel worksheets for each rhino. Quality control for EIA (endocrine) and flow cytometric (sperm sorting) data will be ensured CREW and SWBGRRC, respectively. Completed worksheets and accompanying graphs will be shared with partner facilities for interpretation, discussion and agreement on overall conclusions.

Project Resources: Personnel, Time, Budget- CREW's collaborators in this project are AZA/WAZA facilities, with SWBGRRC as a partner on specific aspects of the project. The direction of this project will be shared between CREW and SWBGRRC, with CREW's Dr. Monica Stoops serving as Project Director. CREW will bring a total cost-share of $619,611 to this three-year project. CREW will use a combination of institutional resources and private donor support to meet cost-share requirements. IMLS funds will be directed towards portions of salaries and fringe for CREW. Full IMLS support is allocated towards disseminating findings at conferences, purchasing supplies, equipment, shipment costs and travel to/from **RARE** collaborating facilities to perform rhino ART procedures. We have verified the timeliness of the proposed activities along with those of the management, labor, resources, facilities and equipment of CREW, AZA/WAZA facilities and SWBGRRC.

CREW: **Dr. Monica Stoops**, Reproductive Physiologist, CREW (80% time commitment) will serve as **Project Director**. Dr. Stoops will ensure proper oversight concerning all administrative activities. She will manage relationships between CREW, AZA/WAZA facilities, SWBGRRC and the Rhino Advisory Group, ensuring clear and close lines of communication and well-defined roles. She will ensure timelines are effective and planning activities are relevant and achievable. She will provide overall project supervision. **Katherine MacKinnon**, Endocrine Research Associate, CREW (60% time commitment) will carry out the proposed EIA

analysis. **Patricia Hermes**, Part-Time Endocrine Research Technician, CREW (15hr/week time commitment) will assist with endocrine activities. **Dr. Terri Roth**, VP Conservation and Science/CREW Director, CREW (5% time commitment) will assist with semen collection/AI procedures as needed.

SWBGRRC: **Dr. Justine O'Brien**, Scientific Director, SWBGRRC (40% time commitment) will serve as **Project Co-Director** and provide oversight of the sperm sorting research. Dr. O'Brien has led sperm sorting research for numerous marine and terrestrial wildlife species since 2000. **Dr. Todd Robeck**, VP Theriogenology, SWBGRRC/SeaWorld Parks and Entertainment Inc. (5% time commitment) will assist with semen collection procedures as needed. **Jacqueline Posy**, Research Technician, SWBGRRC (5% time commitment) will operate flow cytometers for the sperm sorting component of the proposed research. **Karen Steinman**, Laboratory Manager, SWBGRRC (5% time commitment) will assist with sperm sorting, cryopreservation and GRB activities. **Amanda McDonnell**, Research Technician, SWBGRRC (5% time commitment) will assist with sperm sorting and cryopreservation activities. **Dr. Gisele Montano**, Research Associate, SWBGRRC (2% time commitment) will assist with sperm quality analysis activities as needed.

AZA/WAZA Facilities: The majority of AZA/WAZA facilities outlined in the grant have previously collaborated with CREW on rhino reproductive research. They will contribute valuable keeper and veterinary staff time to the project by collecting biological samples, training rhinos for ART and conducting ART procedures. Points of contact have been established at each institution and the same will hold true for any new facilities that come board with the project. With the support of the Project Director, facilities will test protocols, document progress, and provide feedback and input.

Rhino Advisory Group: A group of experts in the fields of rhino management/operant conditioning and veterinary care will support the project team throughout and provide essential feedback, input and ideas. They will serve as external resources for the collaborating facilities' rhino keepers and veterinarians to consult. Regular updates through email and Skype will occur so they can monitor the overall direction of the project, review the testing conducted thus far, and provide input concerning aspects of rhino behavior, reproduction, management and health. The following members have committed to this group: Dr. Jack Kottwitz, Dr. Scott Citino, Joe Hauser and Randal Pairan (*Supporting Document Advisory Group Letters*)

Dissemination of Results- The information gained from this project will be shared with the zoological and rhino community through direct communication and scientific/veterinary/keeper conferences/workshops. The Project Director will disseminate information directly to the SSP coordinators, and relevant findings will be sent to the AZA rhino TAG listserve. CREW and the Project Director have shown a high level of commitment to disseminating results from IMLS-funded projects through publications in peer-reviewed journals and oral presentations[14-20]. Therefore, relevant data from this project will also be dispersed via journal manuscripts and oral/poster presentations presented at conferences/workshops targeted at our project audience. Project staff, collaborators and partners will submit abstracts for inclusion in four conferences: International Rhino Keeper Workshop, International Society of Wildlife Endocrinologists (ISWE), American Association of Zoo Veterinarians (AAZV), and International Elephant and Rhino Conservation and Research (IERCR) Symposium. Interactive training sessions will occur at collaborating facilities. It is anticipated that this will also provide a forum for questions and answers. Media releases by CREW, SWBGRRC and collaborating facilities will help disseminate **RARE** results to the public. They will be posted on partner and collaborator websites and linked to their organizational Facebook and Twitter accounts. CZBGs website has an interactive blog with subcategories specifically for 'CREW' and 'Indian Rhino' that will be updated throughout the project.

PROJECT RESULTS: Given the primary goal of this project to enhance the genetic vigor of captive African and Asian rhino populations in North America by building capacity for rhino ART within AZA/WAZA facilities, the results will certainly have substantial life beyond the grant. The project's far-reaching impact will

be felt across the nation's zoos and SSPs and beyond to global breeding programs. Any pregnancies that result in live calves will increase both animal numbers and genetic diversity of captive Indian and southern white rhino populations for decades to come. Similarly, the training, knowledge and experience gained in rhino ART by collaborating facilities will have long-term beneficial impact on captive breeding programs for African and Asian rhinos that we hope will lead to additional ART procedures, natural breedings, pregnancies and calves over the years. Furthermore, the close working relationships that develop as a result of this project will establish a formalized network of **RARE** researchers, veterinarians, keepers and managers focused on captive African and Asian rhino reproductive care across North America. Consequently, the knowledge base of rhino reproductive physiology will increase.

This project will provide much-needed access and capacity for rhino ART within our nations' facilities that will be utilized now and in the future to help meet the needs of captive rhino species. If SSPs are going to achieve their goal of building self-sustaining populations of African and Asian rhinos for continued exhibition in zoos, they will be forced to increasingly rely on GRBs and ART at some level or they will not be able to sustain genetically viable populations of either species far into the future. The offspring produced from this project will be living proof of the value of rhino ART and GRBs. Through this project, we will expand and develop novel rhino ART procedures for those rhinos that are not breeding naturally or are behaviorally incompatible with their mates. Many zoos will welcome the use of ART procedures as an alternative to the risky and largely unsuccessful efforts to introduce certain animals for natural breeding. As a result, pregnancies/offspring will be produced between pairs that would otherwise not have reproduced. Furthermore, the genetic lifespan of many individual rhinos will be expanded as a result of sperm banking, which can be used to bolster populations now or decades from now. AI, exogenous hormones and sperm sexing technology will be integrated into the available set of ART tools that rhino managers will have at their disposal to meet the genetic and demographic needs of captive breeding programs for African and Asian rhinos.

We will know we are meeting our goals, objectives and outcomes by the following: 1) complete endocrine and in some instances ultrasound characterization of ovarian activity and reproductive cycles of the female Indian and white rhinos at collaborating facilities; 2) accurate decision making for timing AI or natural breeding introductions based on biomarkers (behavior, endocrine, ultrasound); 3) successful mating between female and male white rhinos following exogenous hormone administration; 4) enhanced technical skills, data interpretation and decision making demonstrated by collaborating rhino ART facilities; 5) high quality semen collected, sorted, cryopreserved and accessioned into GRBs from genetically valuable male Indian and white rhinos; 6) pregnancies established in female rhinos at collaborating facilities; 7) calves produced; and 8) research data presented or published to the scientific and zoo community.

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Expanding Access and Buildling Capacity for Rhino Reproductive Care in North American AZA/WAZA Facilities: a Rhino Assisted Reproduction Enterprise

Phase of Project

Dec-14 | Jan-15 | Feb-15 | Mar-15 | Apr-15 | May-15 | Jun-15 | Jul-15 | Aug-15 | Sep-15 | Oct-15 | Nov-15 | Dec-15 | Jan-16 | Feb-16 | Mar-16 | Apr-16 | May-16 | Jun-16 | Jul-16 | Aug-16 | Sep-16 | Oct-16 | Nov-16 | Dec-16 | Jan-17 | Feb-17 | Mar-17 | Apr-17 | May-17 | Jun-17 | Jul-17 | Aug-17 | Sep-17 | Oct-17 | Nov-17 | Dec-17 | Jan-18

ADMINISTRATION

IMLS Proposed Project Period

IMLS Reporting

Rhino Advisory Group Skype Conference

STAFFING

Project Director (80% time)

Endocrine Research Associate (60% time)

Endocrine Research Technician (15hr/wk)

GENERAL PROJECT ACTIVITIES

Urine and Serum Hormone Analysis

Ultrasonography

Artificial Insemination (AI)

Rhino ART Training

Indian Rhino Electroejaculation/Sorting Trials

White Rhino Electroejaculation/Catheterization Procedures

White Rhino Sperm Sorting, Cryopreservation & Banking

White Rhino Chemical Induction Trials

White Rhino Exogenous Hormone Trials

EVALUATION & INTERPRETATION OF RESULTS

Manuscript Writing

DISSEMINATION

AAZV Conference

ISWE Conference

Rhino Keeper Workshop

IERCR Symposium

Online - CREW 'Rhino' Website Updates

Online- Web Blogging