

# Abstracts from the Amphibian Ark Biobanking Advisory Committee Workshop *Towards a Biobanking Strategy for Amphibian Conservation*

September 6–8, 2010  
London and Portsmouth, UK

*The abstracts that follow demonstrate the broad range of topics covered in the oral and poster contributions presented at the workshop*

## Introduction

Approximately one-third of the 6260 amphibian species assessed to date are described as critically endangered or vulnerable. Extinctions not only result in the loss of whole species but also the scientifically (e.g., DNA, RNA, and protein) and even therapeutically (e.g., biomedicines) valuable information contained within their cells together with the possibility to revitalize or save animal populations that have become genetically depauperate and require an injection of genetic diversity (e.g., from sperm, eggs, or embryos) to survive. Only a small proportion of endangered species are expected to be saved by amphibian conservation breeding programs, such as the global Amphibian Ark (AArk) initiative, whose vision is to keep "... the world's amphibians safe in nature" and whose mission is to ensure "... the global survival of amphibians, focusing on those that cannot currently be safeguarded in nature." The AArk recognizes biobanking (also known as genome resource banking) as a vital back-up strategy.

A small group of interested parties took advantage of a general meeting on biobanking held in Trier, Germany in June 2008 to discuss amphibian biobanking. Key findings of this meeting were: (1) few individuals world-wide seemed to be actively engaged in amphibian biobanking and (2) communication between stewards of amphibian populations, both in zoos and in the wild, and biobanking practitioners appeared to be lacking. This small group of people became the AArk Biobanking Advisory Committee (ABAC).

In response to these key findings, the nascent ABAC organized a three-day workshop on amphibian biobanking where experts on endangered amphibian, *Xenopus* (the laboratory frog) and fish biobanking and stewards of amphibian populations were encouraged to come together for a series of presentations, discussions, and practical demonstrations.

Approximately 30 people, from as far afield as Japan, the United States, Australia, and Canada, attended the presentations and discussions held at the Zoological Society of London (ZSL) on days one and two of the workshop. A variety of topics were covered including: species and sample prioritization, data recording, biosecurity, and existing and emerging amphibian biobanking methodologies. Twenty people then attended the practical demonstrations on day three of the workshop, held at the state-of-the-art European *Xenopus* Resource Centre (EXRC) in Portsmouth. This was an opportunity for attendees to gain hands-on experience in biobanking and related methodologies including: sperm cryopreservation, *in vitro* fertilization (including sperm nuclear injection), embryo vitrification, and tissue cell culture cryopreservation.

The main outcome of this workshop will be an amphibian biobanking strategy document for presentation to the wider conservation community, within which will be guidelines on which species and samples should be biobanked, which biobanking methodologies are most appropriate in each case, and which places can process and/or store samples.

This document should significantly aid those engaged or looking to engage in amphibian biobanking, boosting the number of amphibian biobanks worldwide. This in turn should help abate the ongoing catastrophic loss of amphibian biodiversity.

Address correspondence to:  
R. E. Lloyd  
Amphibian Ark Biobanking Officer  
Institute of Zoology  
Regent's Park  
London, NW1 4RY  
United Kingdom

E-mail: Cryobanking@amphibianark.org

## Oral Presentation Abstracts

### 1 History of Biobanking in Zoos and Applications in Conservation

Gordon McGregor Reid

North of England Zoological Society, UK

Natural living organisms are the original source of biomaterials. Many scientists argue that life originated on earth abiotically, via inanimate precursor molecules, some 3.5 Ga (billion years ago). Other authors have advanced proofs that life is cosmic and ubiquitous, most recently via the analogy of a microbe *Deinococcus radiodurans*, which can survive in simulated extraterrestrial conditions. Contemporary humans are arguably known from *ca* 2.4 Mya and their original interest in biomaterials and their preservation relates to survival needs: shelter, food, drink, and clothing; and, later human and animal preservation and burial in cultural contexts. Today we would, in the context of sustainability, rank these biomaterial benefits among “ecosystem services.” “Prescientific” preservation was mainly through drying. Aristotle (384–322 BC), the “father of zoology,” preserved animals for his “Mouseion” but did not establish a permanent collection as material evidence for his hypotheses. The first permanent, dry zoological collections arose in the sixteenth and early seventeenth centuries—and wet preservation, with alcohol, followed in the mid to late seventeenth and eighteenth centuries. Permanent frozen or freeze-dried museum collections date from the late nineteenth century, and this initiative extended into zoological gardens through the twentieth century, to complement research work on the living collections. In the twenty-first century, the crucial idea of systematically biobanking animal specimens, their constituents, and products extends into the realm of applied conservation science. Today, zoos and allied institutions use biobanks or “frozen zoos” to help conserve threatened species *in situ* and *ex situ*.

### 2 Prioritizing Amphibians for Biobanking

Richard Gibson

Amphibian Ark, UK

There are currently more than 6600 described species of amphibian in the world. Six thousand two hundred and eighty-four of these have been assessed by the IUCN and of these almost two-thirds (3913) are considered threatened or insufficiently well known to assess. More than 500 species are already Extinct, Extinct in the Wild, or Critically Endangered—meaning they are at risk of imminent extinction.

While every effort must be made to save as many as possible in the wild where they belong, it is increasingly apparent that numerous species cannot or will not be saved in time. For these species it is essential we implement alternative strategies to traditional *in situ* measures in order to maximize future options for their preservation and eventual return to the wild. *Ex situ* conservation is one such strategy and is identified as an essential

component of the Amphibian Conservation Action Plan (ACAP). The Amphibian Ark (AArk) was conceived as an umbrella organization to facilitate and coordinate *ex situ* conservation efforts for amphibians, and it has been highly successful in raising both awareness of amphibian conservation needs and funds for implementation of conservation programs. However AArk’s core responsibility and focus is on the identification of priority species for *ex situ* programs and the development of local capacity to facilitate these programs. To date AArk has run 19 Amphibian Conservation Needs Assessment (ACNA) workshops and assessed more than 37% of the world’s amphibian species. With its many collaborators it has also been instrumental in the delivery of more than 42 *ex situ* conservation workshops to more than 1300 students from at least 26 countries.

*Ex situ* conservation need not apply solely to living populations. A second, and complimentary, strategy for buying time in the face of imminent species extinction is biobanking—the cryopreservation of gametes, embryos, and somatic and stem cells. Safely frozen genetic material from endangered and even extinct species may be of immense value in their long-term conservation and represents a cost-effective means of adding to the *ex situ* gene pool. Frozen amphibian sperm for example, may be stored in a tiny space and at minimal cost for later introduction to the living population through modern assisted reproductive technologies.

Building upon the foundation of the IUCN Red List and ACNA workshops by AArk and its collaborators, it should be possible to construct a relatively simple system of establishing amphibian biobanking priorities. Those species Extinct in the Wild or Critically Endangered and identified for *ex situ* Ark and Rescue programs will likely form the bulk of our priorities, and especially those which are difficult to maintain and/or problematic to reproduce *ex situ*. However it may also be prudent to consider other criteria such as evolutionary distinctiveness and life history. Agreed priorities can be communicated to the relevant communities through global databases like the IUCN Red List and AArk Community Portal (<https://aark.portal.isis.org/Lists/Prioritization%20workshop%20results/ConservationActions.aspx>).

A coordinated network of biobanking specialists must collaborate closely with both the *ex situ* and *in situ* conservation communities to keep them informed as to which tissues/cells are needed, to train them how to collect and store them, and establish protocols to transport them to regional biobanks. Where possible, biobankers should visit zoos and aquariums and perhaps even join field programs to collect relevant samples for themselves.

### 3 Fundamental Aspects of Cryobiology: Sample, Individual, and Species Differences

Bill Holt

Zoological Society of London, UK

Cryobiology as a discipline can be separated into two distinct strands. While one branch deals with the investigation of

naturally occurring physiological adaptations and responses to cold temperatures, the other is largely concerned with preserving viable cells and tissues. The cryopreservation of cells and tissues differs from other forms of cold storage because the ultimate aim is that the samples are as viable upon thawing as they were before freezing.

The cells and tissues in question are usually poorly adapted to cooling and storage, especially if they come from animals that regulate their body temperature, and finding out how to prevent tissue and cell damage during cooling and freezing has been a technological challenge that has faced scientists for decades. Cooling and ice formation in cells and tissues are both unnaturally rapid processes that initiate damaging changes to both the plasma membrane (e.g., lipid phase transitions enforce the lateral redistribution of membrane proteins) and cytoplasm (formation of extracellular ice leads to the formation of pockets containing hyper-concentrated salt solutions and the osmotic efflux of water). The extent of these changes is influenced by complex interactions between the cell membrane composition (e.g., whether the plasma membrane contains high levels of polyunsaturated fatty acids and sterols) and the rate of cooling applied (if cooling is too rapid, there is insufficient time for cells to lose water by osmosis, with the result that lethal intracellular ice is formed). Cells derived from different species, and even different subspecies, breeds and individuals, are known to differ in plasma membrane lipid content with the result that species differences in the cryopreservation techniques needed are almost inevitable. This situation applies especially to spermatozoa, which seem to be more sensitive to cryoinjury than other cell types. Membrane lipid composition can only be a minor factor in determining the optimal cooling rate for spermatozoa; however, because there is considerable evidence that susceptibility to cryoinjury even differs between individuals of the same species.

Solving the problem of species differences and individual variation with respect to cellular cryoinjury has resisted the best empirical approaches over at least the last six decades, and the time has come for improved theoretical understanding of the cellular basis of cryoinjury. Measuring membrane permeability to water and cryoprotectants has proved extremely useful for the cryopreservation of mammalian oocytes and embryos, but not for spermatozoa. These approaches have also been of little use for fish and amphibian oocytes and embryos, where nature has provided these objects with highly impermeable outer coatings that prevent the efflux of water during freezing and the influx of cryoprotectants prior to freezing. There is some hope that vitrification, a process that involves cooling without ice formation, may overcome some of these problems.

#### 4 Databank Systems for Biobanks

*Haiko Wick*

*Fraunhofer-Institut für biomedizinische Technik, Germany*

Cryobanking, storage below  $-130^{\circ}\text{C}$ , makes it possible to keep somatic cells, stem cells, sperm including high quality DNA, etc. almost without physical or chemical change over centuries. Protecting genetically diverse populations by cryogenic conservation is important for the preservation of endangered or rare species. A broad collection of genetic material keeps open the option of reviving a species' population in the future. It can also be used to track mutations and environmental effects and assemble advanced knowledge of biological systems.

With new technologies, storage concepts and procedures for safe cryogenic conservation the complexity of related data and sample information grows significantly. Primary data includes

specimen details (e.g., phylum and class), sample details (e.g., identifiers, freezing curves, source and type) and storage information. Secondary data can also be added. This highly sample-related but diverse data can range from form based textual data collections, microscopic images or blood analysis results to genetic sequences. Thus, the growing data amounts and complexities lead to large databases or semi-structured data-spaces in which individual records are only loosely coupled and mostly not easily accessible or findable.

Cross-sample or cross-population analysis as well as the need for efficient sample and collection organization raise the demand for standardized and harmonized management of data. The secure deposition in a shared management system that respects the individual requirements of collections is of great importance for purpose- and goal-oriented collection management. However, the focus of future data-management is on data relations as, for example, the backtracking of data to an individual sample and related samples is of high interest. Adding tertiary data as study results or analysis of secondary information complicates the tracking of data-flow as this data is typically more unstructured and no longer in a strict sample-data-relation.

Especially for heterogeneous networks of sample and data sources as well as locally separated depots that collect, distribute and share samples and related information, a new kind of data management system has to be developed that allows a detailed linkage of data. Hence, here we present an information system architecture based on the concept of a subject integrating "Data-Sample-Data"-loop. Designed as an overlay for distributed data spaces, the system enables the user, even after multi-level analysis, to trace results back to the primary sample, its aliquots and related processes and data. Additional tools for search and research are provided, e.g., for collection and data compilation, but they can also be easily developed and integrated by third parties. This hub for data management and exchange is supported by automated data aggregation and linkage (e.g., data push on related aliquots) processes, an overall workflow, integrating local and specific workflows, as well as a strong policy concept. Thus, different requirements and scenarios can be met. A finely granulated abstraction allows the short-term usage of the system in new projects and scenarios or enables existing users to expand their variety of stored data and samples without the need for a new and customized data management system.

#### 5 Biobanks: Genetic Resources for Informing Conservation Strategy

*Jim Groombridge*

*Durrell Institute of Conservation & Ecology, University of Kent, UK*

The science of conservation genetics for restoring endangered species has advanced sufficiently to be able to offer a number of important benefits to conservation managers, including the amphibian research and conservation community working to save species from extinction amid the current amphibian crisis. Biobanks offer a valuable tool for long-term genetic management as well as for population restoration. Information that is valuable to know when genetically managing endangered populations includes the extent and geographic distribution of existing levels of genetic diversity, information on the evolutionary distinctiveness of populations, evidence for localized and regional adaptation.

While some of this information is ideally required prior to and during collection of material to initiate a biobank, biobanks can fulfill important roles once they have been established. First, they can be integrated into captive-breeding and reintroduction programs set up to restore threatened amphibian populations, either

by acting as an insurance policy against catastrophic events that trigger a population decline, such as disease outbreaks, or being used to provide sources of genetically diverse material for re-introduction programs. In many instances, levels of genetic diversity in captive, wild, and/or reintroduced populations may require “topping-up” due to natural erosion of genetic diversity over time by random genetic drift, and here, biobanks have the potential to play a pivotal and potentially long-term role in maintaining adequate levels of genetic diversity during the period of population recovery.

An example of a group of isolated, threatened amphibian populations for which biobanks might have a role are the Sooglossid frogs endemic to the Seychelles archipelago, including a recently discovered population on Praslin island, for which field surveys and molecular genetic studies are currently underway to assess the viability of this population and its evolutionary distinctiveness from populations on other Seychelles islands. In their most applied role, biobanks could be used for purposes of initiating “genetic rescue” of threatened amphibian populations, whereby problems associated with loss of genetic diversity and inbreeding depression in captive/wild and/or reintroduced populations can be redressed by the introgression of novel genetic diversity from biobanked sources. In non-amphibian taxa, ‘genetic rescue’ has been demonstrated to have clear beneficial effects, for example in inbred adder populations, prairie chickens and Florida panthers. While the evolutionary processes and ethical considerations that underpin genetic rescue are complex and non-trivial, this technique provides a potent tool for amphibian conservation and is well-suited to the role that biobanks can provide. Encouragingly, “genetic rescue” is already being applied to some degree to conserve some amphibians, and biobanks will undoubtedly provide a means to optimize these types of conservation interventions.

## 6 Disease Risk Analysis for Biobanking

*Tony Sainsbury*

*Zoological Society of London, UK*

There is a potential for all stored biological samples to include infectious agents, and these agents could remain viable for prolonged periods in storage. The risk of significant infectious disease outbreaks as a result of the use of biobanked samples associated with infectious agents will be greater if, (i) many years have elapsed since the samples were stored, (ii) the samples are introduced to a geographical area not previously exposed to this material (which have crossed an ecological boundary), and (iii) the samples are in contact with species that have not previously been exposed to similar biological material. In order to evaluate the hazard posed by biobanked samples, a disease risk analysis would, if possible, be carried out before the samples are collected, and this analysis would include consideration of practical mitigation measures to reduce the disease risks of collection, storage and future use.

## 7 Initiating the Biobanking of Caribbean Amphibians

*Blair Hedge*

*Pennsylvania State University, USA*

Biobanking (or cell banking) and captive breeding are often considered last resorts in the many options for protecting species from extinction, but they are better viewed as insurance policies on species, and considered as mainstream options. Caribbean

amphibians are ideal candidates for these insurance policies, because the forest habitat on Caribbean islands is much more degraded than in Central and South America, threatening all biodiversity, and amphibians in general are more sensitive to human-caused environmental change. About 90% of original forests have been destroyed on the Caribbean Islands and more than 99% in Haiti. Yet Haiti has a large frog fauna, with many endemic and critically endangered species that occur only in that country and are not shared with the neighboring Dominican Republic. National parks exist but they are not protecting the species, and there is no promising future ahead for the biodiversity of Haiti. The immediate challenge will be to successfully find, biobank, and establish breeding colonies for these Haitian frogs that are literally at the brink of extinction. Locating the species often involves logistical challenges, and exporting biological materials from any country usually involves political challenges. In a country like Haiti, recently ravaged by an earthquake, these challenges are significant.

## 8 Progress Towards Amphibian Biobanking in Australia and New Zealand: Roadblocks, Strategies, Opportunities, and Target Taxa

*John Clulow*

*University of Newcastle, Australia and Helen Robertson,  
Perth Zoo, Australia*

The frog faunas of Australia and New Zealand (ANZ) are highly endemic and/or diverse. Evolution in isolation has proven no protection against recent extensive species’ declines and extinctions. Four Australian species are listed nationally as extinct since 1980; many more are endangered. Only four of the seven indigenous New Zealand species are extant. Complicating responses to amphibian declines (with implications for biobanking sampling strategies), the taxonomy of the amphibian fauna is not fully resolved with new species regularly being described. Biobanking may play an important role in preserving and managing the biological and genetic diversity of ANZ species (managing small threatened populations and their genetic diversity, and extinction insurance). To date, it has not been actively implemented as a management tool in ANZ, nor has the concept gained a prominent role in planning and implementation of recovery and management plans (although it is mentioned peripherally in some).

What could be done to change this situation? One approach is to present a mechanism for the establishment of genome resource banks that is feasible, affordable, and practical. A small but active research base exists in ANZ that could supply the capacity to develop procedures and protocols. We contend the essential infrastructure is already in place for an amphibian genome resource banking network. In Australia, at least, the major museums hold extensive collections of frozen material. One example, the South Australian Museum, holds over 9000 frozen amphibian specimens at  $-80^{\circ}\text{C}$ , including highly threatened and extinct species (raising the possibility of species recovery through nuclear transfer). This infrastructure is capable of retrievably storing live cell lines, reproductive cells and tissues that could be used to regenerate live organisms. New Zealand also has infrastructure in place that could function in the same role (eg. New Zealand Centre for Conservation Medicine, Auckland). A paradigm shift through advocacy is needed to convince institutions holding frozen collections of the benefits of accepting a biobanking role, and conservation agencies that biobanking is affordable and has substantial immediate and future benefits.

### 9 Assisted Reproductive Technologies and Cryobanking Genetic Resources at the Toronto Zoo: Past, Present, and (Amphibian-Friendly) Future

Stacey Hayden

Toronto Zoo, Canada

The Toronto Zoo is the largest zoo in Canada with at least 29 species of amphibians on site. With leadership from a Curator of Reptiles and Amphibians and a Curator of Reproductive Programs, the zoo as a whole is committed to participating in many strategies for amphibian conservation, captive breeding and re-introduction. We are the only zoo in Canada with a reproductive physiology department that is actively investigating and applying assisted reproductive technologies, cryopreservation and genome resource banking strategies for mammals from our own zoo and occasionally from other institutions. We will offer an overview of the species and types of samples that have been and are currently being collected and stored at the Toronto Zoo, including the challenges that are faced within our zoo and within Canada. With growing interest in the amphibian extinction crisis, we are moving towards including non-mammalian species in our technical repertoire. Our plans include the initiation of amphibian research meetings within our zoo and other Canadian zoological and academic institutions to evaluate techniques and strategies that can be implemented in the short- and long-term. A summary of species of immediate interest and potential will be outlined.

### 10 Establishing a Genome Resource Bank for the Panamanian Golden Frog (*Atelopus zeteki*)

Gina Della Togna

Center for Species Survival, Smithsonian Conservation Biology Institute, USA

The Panamanian Golden Frog (*Atelopus zeteki*) is an iconic amphibian extinct from the wild that only exists in captive breeding facilities. To preserve the genetic integrity over the long term, the genetic diversity of captive populations needs to be maintained. Assurance colonies of golden frogs therefore could benefit from assisted reproduction in association with Genome Resource Banking. This approach will allow us to conserve genetic material from founding populations that could be lost due to diseases or genetic drift. However, there is a lack of knowledge in golden frog's gonad and gamete physiology which prevents us from developing appropriate techniques for gamete preservation and assisted reproduction. Our first objectives therefore are to (1) better understand the gametogenesis, (2) develop safe stimulation methods to induce spermiation and ovulation to recover viable gametes, and (3) and study the mechanisms of sperm motility activation and fertilization. Specifically, structural and functional properties of sperm cells will be investigated using flow cytometry and near infrared spectroscopy. These techniques will allow us to characterize cellular and molecular mechanisms related to motility activation and fertilization. Results of these fundamental studies will be used to explore optimal long-term preservation methods for viable gametes and gonadal tissues. We hope to use these insights to assist with the genetic management and reproduction of other threatened *Atelopus* species, including newly established captive assurance colonies of 3 closely related *Atelopus* species collected as part of the Panama Amphibian Rescue and Conservation Project.

### 11 Establishing and Cryopreserving Cell Cultures: Procedures Developed for the Frozen Zoo®

Marlys Houck

San Diego Zoo's Institute for Conservation Research, USA

Since 1975 the San Diego Zoo has supported the establishment and maintenance of a frozen cell repository of viable fibroblast cell lines from endangered and other threatened species. Improvements in primary culture initiation techniques and media development have made it possible to optimize growth conditions of cell lines from a wide variety of taxa. The "Frozen Zoo®" bioresource collection at the Institute for Conservation and Research (<http://www.sandiegozoo.org/>) contains somatic cell lines from more than 8000 individuals providing access to the genomes of approximately 800 species and subspecies, including many that are listed in the IUCN Red List of Threatened Animals. The largest and most diverse of its kind, this collection currently contains cell lines from 45 vertebrate orders including 20 mammalian, 19 avian, three reptilian, two fish, and one amphibian order.

Efforts to establish, propagate, and cryopreserve viable amphibian cell lines at the San Diego Zoo began in 2006 and intensified in 2009 with grant support provided by the Institute of Museum and Library Services. Although amphibian tissues have historically proven more challenging to culture than mammals, birds, and reptiles, cell lines from several species have been successfully accessioned into the Frozen Zoo®, including fibroblasts from a White's tree frog (*Litoria caerulea*) individual that died of chytridiomycosis. Biobanking amphibian cell lines provides a unique resource for studies that have direct application to the conservation and management of this group and is a crucial component for combating the amphibian extinction crisis. It is especially critical to establish and cryopreserve cell lines from amphibian species that might not be available in the future. The living cells can be utilized to obtain chromosome preparations, expanded to generate large quantities of DNA and RNA, and in the future could potentially be used for somatic cell nuclear transfer or generating induced pluripotent embryonic stem cells.

### 12 Spermic Urine (Collection, Short-Term Storage, Cryopreservation and Use in *In Vitro* Fertilization)

Andy Kouba

Memphis Zoo, USA

Amphibian captive assurance colonies are being established worldwide for populations threatened with extinction. These founder animals represent a portion of the genetic diversity that could potentially be reintroduced back to the wild should original threats to the population be alleviated. However, this back up plan of maintaining a reservoir of captive genes can easily fail if steps are not taken early in a program to secure biobanked material and develop protocols for successful reproduction. By necessity, gamete collection protocols must be non-invasive and minimally stressful for critically endangered animals. Our lab has been successful at determining the exogenous hormone concentrations for stimulating spermiation or ovulation in numerous amphibian species including *Rana sevosia*, *Rana pipiens*, *Bufo boreas*, *Bufo fowleri*, *Bufo americanus*, and *Peltophrynes lemur*. Moreover, we can store spermic urine for long periods of time, transport sperm between institutions, and conduct *in vitro* fertilization (IVF) using gametes held in short-term storage. Collectively, these technologies have produced more than 20,000 endangered amphibians released to the wild; about 7,000

produced by IVF. In addition, our lab has produced *Bufo fowleri* tadpoles using frozen-thawed spermatozoa collected from live animals; however, to date the technique is minimally effective with fewer than 10 tadpoles produced in each trial. Future challenges in developing amphibian reproductive technologies focus on improving hormone selection and dosage to secure good quality eggs for IVF and improving cryopreservation of amphibian sperm collected from live animals. Our successes, failures and current direction will be shared with workshop participants.

### 13 The European *Xenopus* Resource Centre

Matt Guille

European *Xenopus* Resource Centre, UK

The aim of the European *Xenopus* Resource Centre (EXRC) is to provide cell and developmental biologists with transgenic, mutant and wild-type *Xenopus*, together with the molecular reagents used to study them. In addition to this core activity, the EXRC acts as a point of contact for scientists using the *Xenopus* model. This has allowed staff to test for disease (e.g. chytrid fungus) in many of Europe's lab colonies and to disseminate the very well established procedures used for *Xenopus* propagation to those involved in research with other frogs.

I will describe how our *Xenopus* are used and how the Centre is operated in the hope of stimulating discussion about how the knowledge gained from this model organism might be used to underpin research into amphibian biobanking.

### 14 Transgenesis Procedures in *Xenopus* Now and in the Future

Shoko Ishibashi

University of Manchester, UK

*Xenopus laevis* is an excellent system for elucidating the molecular mechanisms of early development. Embryos develop externally and are large and robust providing advantages such as the availability of molecular and cellular methods including microinjection, dissection, and transplantation. Generally *in vitro* transcribed RNAs are injected into developing embryos to express the gene of interest. However, since RNAs are translated soon after injection, this method has been used mainly for studying early stages of development. To manipulate gene expression in temporal and spatial manner during later stages of development, transgenic technology based on restriction enzyme mediated integration (REMI) was developed. The REMI transgenesis protocol can be divided into three parts: 1) preparation of high-speed egg extracts, 2) sperm nuclei preparation, and 3) sperm nuclear transplantation. Since the transgene is incubated with sperm DNA directly, oocytes subjected to sperm nuclear transplantation develop as transgenic animals. This approach enables stable, non-mosaic expression of cloned gene products in *Xenopus* embryos, allowing a broader range of feasible experimentation than that previously possible by transient expression methods.

*Xenopus tropicalis*, a closely related frog, has emerged as a new vertebrate model system. Retaining *X. laevis*'s embryological and cell biological advantages, *X. tropicalis* can be used for genetic studies due to its diploidy and shorter generation time. The genome sequence of *X. tropicalis* was recently published. While the REMI transgenesis has been adapted to *X. tropicalis*, the technique does not work as efficiently as it does in *X. laevis*. It has also

been shown that, with the REMI procedure, the transgene generally integrates as a concatemer, which potentially causes genomic instability. For these reasons, the REMI transgenesis is not suitable in *X. tropicalis*. Therefore we have been generating transgenic *X. tropicalis* using I-SceI meganuclease method, which was originally developed in medaka fish. I-SceI is a commercially available nuclease isolated from *Saccharomyces cerevisiae*. The method achieves low copy integration by co-injection of I-SceI and a DNA construct containing the 18 base pairs I-SceI recognition site. Since the transgene integrates in early cleavage stages, the transgenic animals generated by this method are mosaic. However because it is a very simple and easy procedure, many laboratories have started using this method with some success to establish transgenic *X. tropicalis* lines. Several mobile DNA, transposons have also been tested as a tool for transgenesis in many species. Tol2 transposon has been shown to be highly active in *X. tropicalis*. In this method, transposase RNA and a DNA construct containing the transposon sites are co-injected into the embryos. This method also produces mosaic animals. To overcome mosaicism, we have generated the transgenic frogs expressing Tol2 transposase ubiquitously. Since the transgenic female frogs are expected to lay eggs containing the Tol2 transposase protein, injecting these eggs with transposon DNA would be expected to give rise to transgenic animals more efficiently and with less mosaicism. In addition, methods for performing large-scale insertional mutagenesis screens in *X. tropicalis* will also be discussed.

### 15 Gynogenesis and Genome Manipulations: Strategies and Applications for Amphibian Conservation

Lyle Zimmerman

MRC National Institute for Medical Research, UK

Whole genome manipulations of many amphibians can be readily accomplished using a variety of simple methods. Haploid genetics and gynogenesis (maternally-derived asexual reproduction) have been developed primarily for genetic analysis of *Xenopus* embryogenesis, but related techniques may be useful for conservation of threatened species. Haploid embryos may be created by ultraviolet (UV)-irradiating sperm or eggs prior to *in vitro* fertilization. Haploid embryos are typically viable for several days of development, often displaying specific posterior defects. Gynogenesis by polar body suppression takes advantage of the fact that externally-fertilized amphibian embryos are deposited before the completion of Meiosis II. Formation of the second polar body usually occurs shortly after fertilization, but can be efficiently disrupted in many species by pressure or temperature shock, resulting in triploid embryos or rescue of haploid embryos produced by UV-irradiated sperm to viable diploidy. Rescue of haploid embryos can also be accomplished by suppressing first cytokinesis following S-phase, again using temperature shock or pressure. Rescue efficiencies are much lower, but the resulting embryos are completely homozygous ("isogenic"). Animals grown from experimentally diploidized haploids can be viable and fertile, and a breeding population could be recovered from a single female. Success depends critically on sex determination mechanisms, which vary widely even among closely-related amphibian species. Our group routinely uses a simple "early cold shock" (ECS) procedure to accomplish polar body suppression gynogenesis in *Xenopus tropicalis* for genetic screens. Hundreds of viable embryos can be recovered from a single *in vitro* fertilization with UV-irradiated sperm. We have also rescued haploid embryos obtained from UV-irradiated sperm to viable diploidy by suppression of first cleavage.

Embryos rescued by suppression of both polar body formation and first cytokinesis have been raised to adulthood and are being evaluated for fertility. Androgenesis, in which the genomes of offspring are completely paternally derived, has been experimentally accomplished in fish and urodeles by UV-irradiation of eggs and *in vitro* fertilization followed by suppression of first cleavage. Androgenesis is theoretically compatible with recovery of diploid genomes from cryopreserved sperm, using eggs of related species.

## 16 Fish Biobanking: Current Activities, Lessons, and Opportunities

David Rawson

LIRANS Research Institute, University of Bedfordshire, UK

It is estimated that over 30% of amphibian and fish species are endangered. The percentage of species that have been carefully assessed is still small, with the status of only 14% of fish species known. The nature of the habitats of both groups make regular review of species numbers difficult, as is in the case of marine fishes of the ocean depths. Closer to home, the United Kingdom's freshwater and marine environments are known to be home to some 300 species of fish, and of these 41 species are already considered to be threatened, most critically the cod and common skate.

While it is understandable that the immediate concerns of many *ex situ* efforts are those species already recognized as being critically endangered, it is important to recognize that global changes in climate and habitat quality can result in the loss of a much wider range of species, including many that are today not considered to be endangered. Clearly, it is going to be very difficult to have material from all species cryo-banked, especially as ideally there should be a multiple specimens for each species. But with global species diversity in a state of flux, we should be ensuring that as far as possible we have a permanent record of the life on Earth in the first decades of the millennium.

The fish cryobank at LIRANS has set out to bank material from both critically endangered species, as and when they become available, and also for species found in UK waters, in effect an environmental specimen bank for UK fish species. Collecting critically endangered species for the cryobank is opportunistic in nature, usually from other *ex situ* collections (zoos and aquaria), while environmental specimen banking involves field based collections in the main. Material is being cryobanked in two ways: (1) viable tissue explants and cell lines and (2) tissue samples preserved and cryobanked to simply ensure retention of long-chain DNA integrity. Fish from which such material has been taken are then held as voucher specimens for future classification confirmation. Most of the LIRANS fish collection is now held at the Natural History Museum, London. To date, 112 fish species are represented in the LIRANS cryobank as both DNA preserved and viable material. In addition, cell lines have been established from 20 of these species, and these are also cryobanked. The real benefits of any bank rest on the ability to make both "deposits" and "withdrawals." While non-viable tissue samples are the easiest to bank, they do present a real problem in terms of access to a finite amount of such material. The establishment of cell-line cultures helps overcome this limitation, and also ensures that the full molecular moieties of the species are preserved. It is also possible to speculate that in the longer term such viable material may provide opportunities for revitalization of small populations through transgenics, and even re-establishment of species with nuclear and mitochondrial transplantation and cloning.

## 17 Embryo Vitrification and Primordial Germ Cell Transfer in Fish

Shogo Higaki

Tottori University, Japan

An increasing number of wild fish species are in danger of extinction. Cryopreservation of gametes and embryos is of great importance for preserving biodiversity. Although successful cryopreservation of spermatozoa has been achieved for many fish species, reliable cryopreservation methods for fish oocytes and embryos have not been developed. In zebrafish (*Danio rerio*), surrogate production using cryopreserved primordial germ cells (PGCs) is thought to be an alternative method for cryopreservation of gametes and embryos.

Surrogate production in fish is a technique used to obtain the gametes of a certain genotype through the gonad of another genotype. This technique in teleost fish is achieved by inducing the germ-line chimeras. In small teleost fishes (such as goldfish, medaka and zebrafish), the following three methods for inducing germ-line chimera have been reported: (1) blastomere transplantation, (2) blastoderm-graft transplantation, and (3) transplantation of green fluorescent protein (GFP)-labeled PGC.

In blastomere transplantation, donor blastomeres are randomly sucked from blastula-stage embryos and transplanted into recipient blastulae. In this method, germ-line chimeras are induced occasionally, because donor cells do not always contain PGCs. In the transplantation of a blastoderm graft, lower-part blastoderm of blastula, in which PGCs are contained, is transplanted into a recipient embryo. In this method, germ-line chimeras could be always induced, but donor cells tend to aggregate in the host embryos and also have an adverse effect on embryonic development.

In the GFP-labeled PGC transplantation, PGCs visualized by injecting the GFP-*nos1* 3' UTR mRNA are isolated from segmentation (10- to 15-somite) stage embryos and transplanted individually into sterilized recipient blastulae heterochronically; the single PGC transplantation (SPT) method. Our recently developed technique has a high frequency of germ-line chimera production.

In cryopreservation of teleost fish PGCs, the following three methods have been developed: (1) slow freezing of isolated PGCs, (2) slow freezing of isolated genital ridges, and (3) vitrification of yolk-removed embryos.

Cryopreservation of isolated PGCs seems not to be an option because of the limited number of PGCs (~20 cells/embryo) in segmentation stage zebrafish embryos and inevitable loss of PGCs during isolation. Isolated genital ridges containing PGCs were successfully cryopreserved in order to avoid loss of PGCs in rainbow trout. However, zebrafish PGCs recovered from the genital ridge cannot be used for SPT because PGCs in embryos after the 25-somite stage lose the ability to migrate to the genital ridge in recipient embryos.

In the vitrification of embryos, yolk was removed by aspiration from 14- to 18-somite stage embryos exhibiting GFP-labelled PGCs and the embryos were cooled rapidly by plunging into liquid nitrogen with adequate vitrification solution. This method can diminish the loss of PGCs during cryopreservation procedures and cryopreserve the PGCs with high survivability. Our team successfully produced fertile zebrafish possessing functional gametes that originated from PGCs recovered from vitrified yolk-removed embryos by using SPT method.

In teleost fish, combined with cryopreservation of PGCs, the surrogate production technique would make it possible to preserve genetic resources and to revive extinct species using closely related species as a surrogate when necessary, though further studies are needed to refine the procedures.

## Poster Presentation Abstracts

### 1 The Role of the Frozen Ark Project

Ann Clarke

Frozen Ark, University of Nottingham, UK

The aim of the Frozen Ark Project, an independent registered charity based at the University of Nottingham, is to promote the preservation of genetic resources of endangered wild animal species before the opportunity is lost through extinction. It has grown into an international consortium of 15 institutions, of which 7 are in the UK. It brings together four essential components that have the skills to achieve its aims—the zoos, aquaria, museums and universities.

This organization works to recruit members, run meetings in the UK, oversee the website ([www.frozenark.org](http://www.frozenark.org)), create a database of samples preserved globally, spread information between members and develop protocols for sample collection, preparation and storage.

It is also promoting the formation of Expert Groups designed to oversee specific needs of individual animal taxa. How these groups operate and their affiliations with partners are determined by circumstance and opportunity. In the UK, the first, for land mollusks was set up by curators at the Natural History Museum (NHM, London), biologists at the University of Nottingham and The Wildlife Heritage Trust of Sri Lanka. As represented here, the global Amphibian Ark Biobanking Advisory Committee (ABAC) is a result of a partnership between the Frozen Ark Project and the Amphibian Ark.

The Animal Health and Research Department at Twycross Zoo has trialed protocols for sampling from live and dead zoo animals, temporary storage of samples, their transport to a long-term repository and the collection of necessary data. The World Zoo and Aquarium Conservation Strategy provides a mandate for the collection of samples as an *ex situ* long-term conservation role of zoos.

### 2 Considerations and Screening of Biobanked Amphibian Gametes from Chytrid Positive Animals: Implication for Conservation Programs

Carrie Vance

Mississippi State University, USA

Disease transfer and cross-contamination of biobanked materials is a formidable obstacle to many conservation efforts. For amphibian conservation, chytrid fungus forms the most prevalent threat to many species and their reintroduction programs. Biobanking is urgently needed for endangered amphibians; however, it is important to understand the chytrid presence in these animals to determine if we are simultaneously banking down contaminated gametes or transferring diseases during artificial fertilization procedures for assisted reproduction and colonization. Hence, screening for these infectious diseases is

necessary and quantitative PCR (qPCR) analysis has aided tremendously in this endeavor. In parallel with our qPCR screening of chytrid in our amphibian colonies, we have been developing a novel approach to screening for chytrid fungus in adult amphibians based on Near infrared reflectance spectroscopy (NIRS) and chemometric analysis. Since chytrid manifests itself as a skin disease, our approach is based on variations in light reflectance from the skin surface of individual animals that may be infected. We have been developing this analytical procedure in the Fowler toad (*Bufo fowleri*) and leopard frog (*Rana pipiens*) in conjunction with our sperm banking procedures. Our aim is to be able to screen for chytrid positive gamete donors in the lab, or even in the field, during the biobanking process to identify potential contamination risks. Once infected animals are identified we can then test the gametes and accessory fluid for presence of chytrid using standard qPCR analysis. Best management practices, like disease screening during gene banking, will insure that the risk in amphibian recovery programs is minimized.

### 3 Cryopreservation of Oocytes and Follicular Cells of the Cane Toad (*Bufo marinus*)

John Clulow

University of Newcastle, Australia

Cryopreservation of intact amphibian embryos and large oocytes has not been reported to date. We investigated the feasibility of developing protocols for the slow-cool freezing, storage and retrieval of developmentally competent amphibian ovarian follicles containing Stage I and II oocytes which are much smaller in size than later developmental stages. Ovarian follicles from euthanized cane toads (*Bufo marinus*) were incubated in cryodiluents containing either glycerol or dimethyl sulfoxide (DMSO) to assess cryoprotectant toxicity and response to slow cooling freezing protocols. The fluorescent live cell stain SYBR 14 and its counter stain propidium iodide was used to score the proportion of viable follicle cells before and after cryopreservation. Cryoprotectant type, concentration and exposure time all had significant effects ( $P < 0.05$ ) on the viability of follicle cells, with significant interactions between these variables. Overall, glycerol was less toxic to follicle cells than DMSO. At higher concentrations, glycerol exerted high osmotic stress on oocytes, and there was evidence that DMSO triggered apoptosis in oocytes. The most effective cryopreservation protocol for stage I and II oocyte follicles resulted in a post-thaw recovery of a mean 70% of viable follicular cells. This protocol involved cryopreservation in 15% v/v glycerol, inclusion of seeding and temperature holding periods during cryopreservation, coupled with rapid thawing in a 30°C water bath. The successful cryopreservation of intact follicles in this study indicates the potential to recover functional ovarian tissues post cryopreservation for continuation of amphibian oogenesis *in vitro* or *in vivo*.