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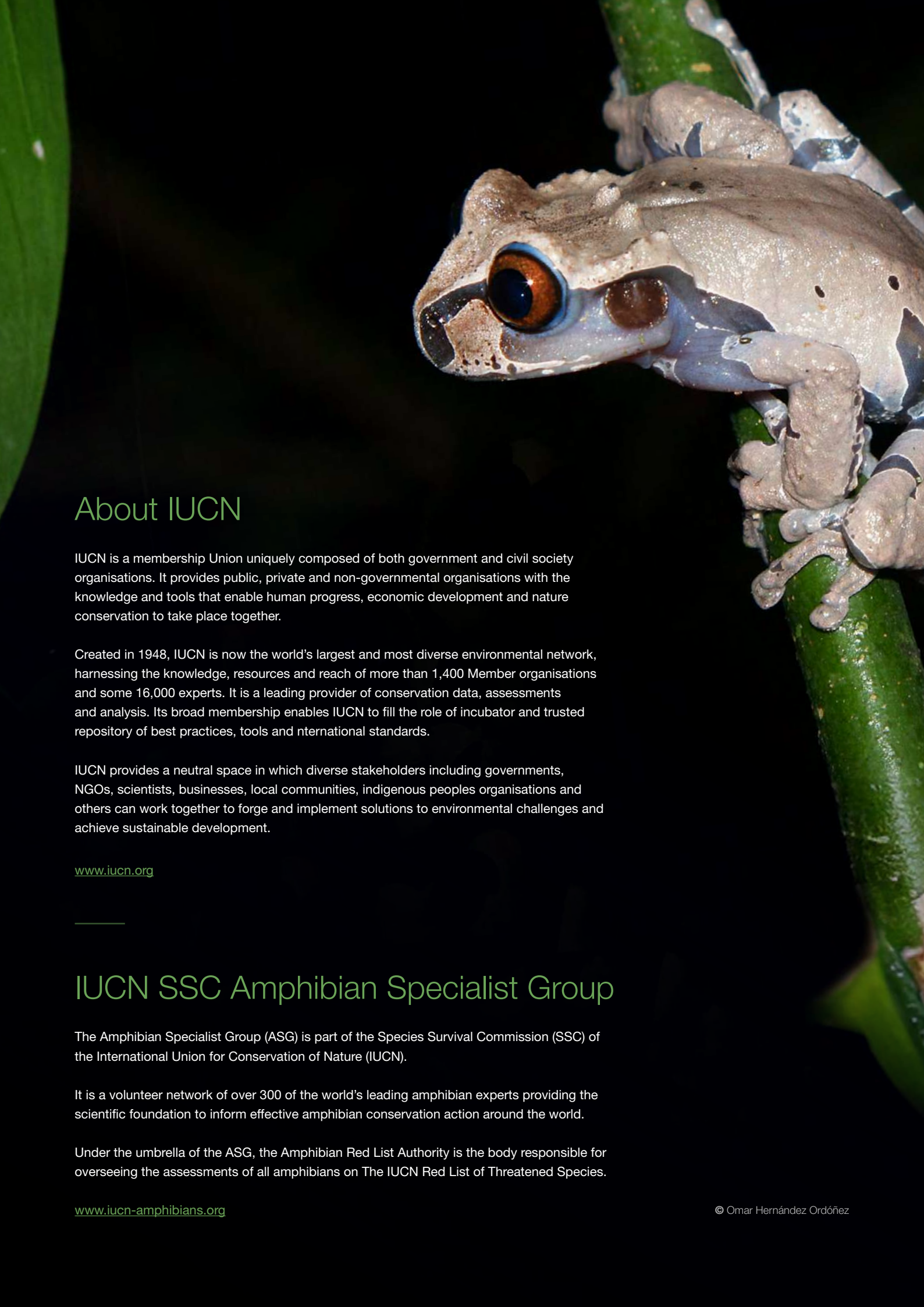
A status review and roadmap for global amphibian conservation

IUCN SSC Amphibian Specialist Group

Edited by: Sally Wren, Amaël Borzée, Ruth Marcec-Greaves and Ariadne Angulo



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Front cover photo: Photograph taken by Kenneth Chin Yu An. *Onychodactylus sillanus*, the Yangsan clawed salamander in situ in Yangsan, Republic of Korea. The species is threatened by habitat loss and climate change; its taxonomic description in 2022 helped citizen science groups and non-governmental organisations to link up with the local government to discuss the development of conservation programs, and a potential update to national environmental regulations.

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Dedication

*For Professor Phil Bishop,
Our mentor, colleague, and friend.
But above all, our inspiration for this
work and our daily endeavours.
Thank you for teaching us to be better
scientists and better people.*

ACAP Editorial Team

Executive summary

As the most threatened vertebrate class on earth, amphibians are at the forefront of the biodiversity crisis, with the recognition of global amphibian declines and extinctions dating back several decades now. The Amphibian Conservation Action Plan (ACAP), the first taxonomic class-level plan of its kind, was first published in 2007 and then updated as a digital resource in 2015, with the goal of acting as a unified global strategy to save these vertebrates. However, although there have been resources allocated to amphibian conservation since the first (2007) ACAP, these have not been nearly sufficient to adequately address the global amphibian crisis.

The current ACAP is adopting two strategies, both novel and alternative to the first ACAP, to address the goal of the amelioration of the amphibian crisis: the development of two complementary documents that work to **1)** synthesise developments in major themes of amphibian conservation over the last 15 years, as in this document, and **2)** summarise the key findings and recommendations to a broader audience in a user-friendly way (a practitioner document that will follow this synthesis document). The purpose is thus to provide the most up-to-date evidence on threats and approaches to amphibian conservation, and from there identify gaps and priorities that can then be disseminated and adopted by stakeholders across the globe.

Each chapter of this status review was developed by the matching Amphibian Specialist Group's (ASG) thematic working groups. Led by 1-3 working group chairs and supported by working group members, chapters have also had the input of professionals outside of ASG with expertise in given themes.

This document consists of two introductory chapters and twelve thematic chapters divided into three sections:

- ➞ **Threats** - Chapters 3-7 on climate change; ecotoxicology; habitat loss; infectious diseases; and trade and sustainable use
- ➞ **Informing decision-making** - Chapters 8-10 on communications and education; conservation planning; and surveys and monitoring
- ➞ **Species management** - Chapters 11-14 on conservation breeding; assisted reproductive technologies and biobanking; genomics; and translocations

In broad terms, each chapter covers the most important knowledge, technological and conceptual developments in a particular theme over the last decade and a half, highlighting knowledge gaps, challenges, needs and opportunities for future conservation action.

Key messages

- 1.** While this document is global in scope it is informed by local and regional realities. Not everything that is in this document will be transferable to every region. However, those aspects that are relevant to a region can be addressed accordingly, and these results can then feedback again into a global strategy, and be readapted in other regions to benefit from the shared experience. Translation from local to global and back to local is crucial to ensure that regional experiences feed into a global framework and that this framework accurately reflects shared patterns and realities so that it can inform international conventions and organisations, especially in view of global environmental change.

2. As a whole there is an enormous deficit in information for most amphibian species, which hampers decision-making and evidence-based, conservation action. Yet, it is urgent that conservation action takes place with the data that are available. More targeted research, undertaken concomitantly with conservation action, is required. Increased collaborations both within and outside the amphibian conservation community are urgently needed to begin to bridge some of the information gaps. Integrating different approaches can help augment information and leverage additional support to amphibian conservation.
3. Relative to the scope of amphibian declines and extinctions, adequate financial and human resources and necessary policy measures have largely lagged behind this decades-long crisis. Should this pattern persist, we can expect to continue losing amphibian populations and species in increasingly large numbers. It is therefore critical that amphibian conservation becomes both an integral and a conspicuous part of the biodiversity conservation agenda of international and national conservation organisations of all sizes, of national and subnational levels of government, of the various institutions that focus on biodiversity education and research, of funding entities, and of organised communities and media.

Resumen ejecutivo

Comprendiendo la clase de vertebrados más amenazada del mundo, los anfibios están en la primera línea de la crisis de biodiversidad, con el reconocimiento de las declinaciones y extinciones de los anfibios remontándose ya varias décadas. El Plan de Acción de Conservación de los Anfibios (ACAP por sus siglas en inglés), el primer plan a nivel de clase taxonómica, fue publicado inicialmente en el 2007 y luego actualizado como un recurso digital en el 2015, con la meta de actuar como una estrategia global unificada para salvar estos vertebrados. Sin embargo, aunque ha habido recursos dirigidos a la conservación de anfibios desde el primer plan, estos no han sido suficientes para abordar la crisis global de los anfibios de forma adecuada.

Este ACAP está adoptando estrategias novedosas y alternativas para aliviar la crisis de los anfibios: el desarrollo de dos documentos complementarios que de manera conjunta **1)** sintetizan los acontecimientos y progreso en temas importantes para la conservación de los anfibios en los últimos 15 años, caso de este documento, y **2)** resumen los principales mensajes y recomendaciones dirigidos a una audiencia amplia de una manera accesible (un documento para implementadores que seguirá este documento de síntesis). El propósito es, por ende, ofrecer la evidencia más actualizada acerca de las amenazas y abordajes en lo que refiere a la conservación de los anfibios, y a partir de ello identificar vacíos y prioridades que pueden luego ser diseminados y adoptados por actores relevantes a lo largo del planeta.

Cada capítulo de este documento fue desarrollado por el respectivo grupo temático del Grupo de Especialistas de Anfibios (ASG por sus siglas en inglés). Liderados por 1-3 presidentes de grupos temáticos y apoyados por miembros de los grupos de trabajo, los capítulos también han recibido el aporte de profesionales fuera del ASG con experiencia en determinados temas.

Este documento comprende dos capítulos introductorios y doce capítulos temáticos divididos en tres secciones:

- ➞ **Amenazas** - Capítulos 3-7 sobre cambio climático; ecotoxicología; pérdida de hábitat; enfermedades infecciosas; y comercio y uso sostenible
- ➞ **Informando la toma de decisiones** - Capítulos 8-10 sobre comunicaciones y educación; planificación de conservación; y muestreos y monitoreo
- ➞ **Manejo de especies** - Capítulos 11-14 sobre reproducción de conservación; tecnologías de reproducción asistida y biobancos; genómica; y translocaciones

En términos generales cada capítulo cubre el conocimiento y desarrollo tecnológico y conceptual más importantes de la última década y media, resaltando los vacíos de conocimiento, retos, necesidades y oportunidades para futuras acciones de conservación.

Mensajes principales

1. Aunque este documento es de alcance global está informado por realidades locales y regionales. No todo lo que está en él será transferible a cada región. No obstante, esos aspectos que sí son relevantes a cada región pueden ser abordados como corresponde, y esos resultados pueden luego retroalimentar

una estrategia global y ser readaptados en otras regiones para lograr un beneficio a partir de la experiencia compartida. La traducción de lo local a lo global y vice-versa es crucial para asegurar que las experiencias regionales puedan alimentar un marco global y que este marco refleje los patrones y realidades compartidos, de manera que pueda informar convenciones y organizaciones internacionales, especialmente dado el cambio ambiental global.

2. Tomado en conjunto, existe un enorme déficit de información para la mayoría de especies de anfibios, lo que dificulta el proceso de toma de decisiones y acciones de conservación basadas en evidencia. No obstante, es urgente adelantar acciones de conservación con la información disponible. Se requiere de investigaciones más enfocadas, adelantadas de manera conjunta con acciones de conservación. Es necesario incrementar las colaboraciones tanto dentro como fuera de la comunidad de conservación de anfibios, para así comenzar a zanjear algunos de los vacíos de información. La integración de abordajes distintos puede ayudar a aumentar la información y catalizar apoyo adicional hacia la conservación de anfibios.
3. En relación a la magnitud de las declinaciones y extinciones de anfibios, los recursos financieros y humanos y las medidas políticas necesarias vienen muy rezagados ante esta crisis de décadas de duración. Si este patrón persiste, podemos esperar la continua pérdida de poblaciones de anfibios y especies en números cada vez mayores. Es por ende crítico que la conservación de anfibios se vuelva un componente tanto integral como conspicuo de la agenda de las organizaciones de conservación internacionales y nacionales de todos los tamaños, de los distintos niveles de gobierno, de las varias instituciones que se enfocan en la investigación y educación de la biodiversidad, de entidades financieras, de comunidades organizadas, y de los medios de comunicación.

Résumé analytique

Etant la classe de vertébrés la plus menacée au monde, les amphibiens sont au premier plan de la crise de la biodiversité, et le déclin et les extinction des amphibiens à l'échelle mondiale sont maintenant compris depuis plusieurs décennies. Le Plan d'Action pour la Conservation des Amphibiens (ACAP en Anglais) est le premier plan d'action pour la conservation des espèces au niveau du rang taxonomique des classes. Il a été publié pour la première fois en 2007, puis mis à jour en tant que ressource numérique en 2015 avec l'objectif de servir à la création d'une stratégie mondiale unifiée pour sauver ces vertébrés. Cependant, bien que des ressources aient été allouées à la conservation des amphibiens depuis le premier ACAP (2007), elles n'ont pas été d'une fraction de l'ordre de grandeur nécessaire pour faire face de manière adéquate à la crise mondiale de la disparition des amphibiens.

La version actuelle de l'ACAP adopte deux stratégies, toutes deux nouvelle et différente du premier ACAP, pour remédier à la crise des amphibiens: la préparation de deux documents complémentaires qui cherchent à **1)** synthétiser les développements dans les principaux thèmes de la conservation des amphibiens au cours des 15 dernières années (une synthèse des développements académiques - ce document), et **2)** résumer les découvertes principales et les recommandations pour le grand public d'une manière accessible (un document de mise en œuvre qui suivra la synthèse des développements). L'objectif est donc de fournir les preuves les plus récentes des menaces et les approches de la conservation des amphibiens, et à partir de là, d'identifier les lacunes et les priorités qui peuvent ensuite être diffusées et adoptées par les acteurs de la conservation à travers le monde.

Chaque chapitre de cette synthèse des développements en conservation a été élaboré par le groupe de travail thématiques travaillant sur le sujet et faisant partie du Groupe des Spécialistes des Amphibiens (ASG en Anglais). Chaque chapitre a été dirigé par 1 à 3 chefs de groupes de travail, en concert avec les membres du groupe. Les chapitres ont également bénéficié de la contribution de professionnels extérieurs à l'ASG et ayant une expertise correspondante.

Ce document se compose de deux chapitres introductifs et de douze chapitres thématiques divisés en trois sections :

- ➞ **Menaces** - Chapitres 3 à 7 sur le changement climatique ; l'écotoxicologie ; la destruction de l'habitat ; les maladies infectieuses ; le commerce et l'utilisation durable
- ➞ **Prise de décisions informées** - Chapitres 8 à 10 sur les communications et l'éducation ; la planification de la conservation ; les enquêtes et le suivi
- ➞ **Gestion des espèces** - Chapitres 11 à 14 sur l'élevage en captivité ; les technologies de procréation assistée et biobanques ; la génomique ; les translocations

En termes généraux, chaque chapitre couvre les connaissances et les développements technologiques et conceptuels les plus importants pour chaque thème développé au cours des quinze dernières années, en mettant en évidence les lacunes, les défis, les besoins et les opportunités pour les futures actions de conservation.

Messages principaux

1. Bien que ce document ait une portée mondiale, il est dirigé par les réalités locales et régionales. Tous les points exposés dans ce document ne seront pas nécessairement transférables à toutes les régions. Cependant, les aspects qui sont pertinents pour une région peuvent être traités en conséquence, et ces résultats peuvent ensuite être réintégrés à la stratégie mondiale, et ensuite être réadaptés dans d'autres régions qui pourront bénéficier de ces expériences. La transformation du point de vue local au point de vue mondial et le retour au point de vue local, est cruciale pour garantir que les expériences régionales alimentent un cadre mondial et que ce cadre reflète correctement les modèles et réalités locales afin d'informer les conventions et organisations internationales, spécialement compte tenu des changements environnementaux mondiaux.
2. Dans l'ensemble, il y a un extraordinaire manque d'informations pour la plupart des espèces d'amphibiens, ce qui entrave la prise de décisions et les actions de conservation basées sur des faits scientifiques. Cependant, il est urgent que les actions de conservation prennent place en tenant en compte des données déjà disponible. Des recherches plus ciblées, entreprises parallèlement aux actions de conservation, sont aussi nécessaires. De nouvelles collaborations au sein de la communauté de conservation des amphibiens, mais aussi avec des personnes externes, sont nécessaires de toute urgence pour commencer à combler ces lacunes en matière d'information. L'intégration de différentes approches peut permettre d'acquérir les informations nécessaires et de tirer parti d'un soutien supplémentaire pour la conservation des amphibiens.
3. En comparaison avec l'ampleur du déclin et l'extinction des amphibiens, les ressources financières et humaines et les mesures politiques nécessaires sont en retard par rapport à cette crise qui dure depuis des douzaines d'années. Si ce schéma persiste, nous pouvons nous attendre à continuer de perdre de plus en plus de populations et d'espèces d'amphibiens. Il est donc essentiel que la conservation des amphibiens devienne à la fois une partie intégrante et visible du programme de conservation de la biodiversité des organisations de conservation internationales et nationales de toutes tailles, mais aussi au niveau des gouvernements nationaux et régionaux, des institutions diverses qui se concentrent sur l'éducation et la recherche sur la biodiversité, des organismes de financement, des associations et des médias.

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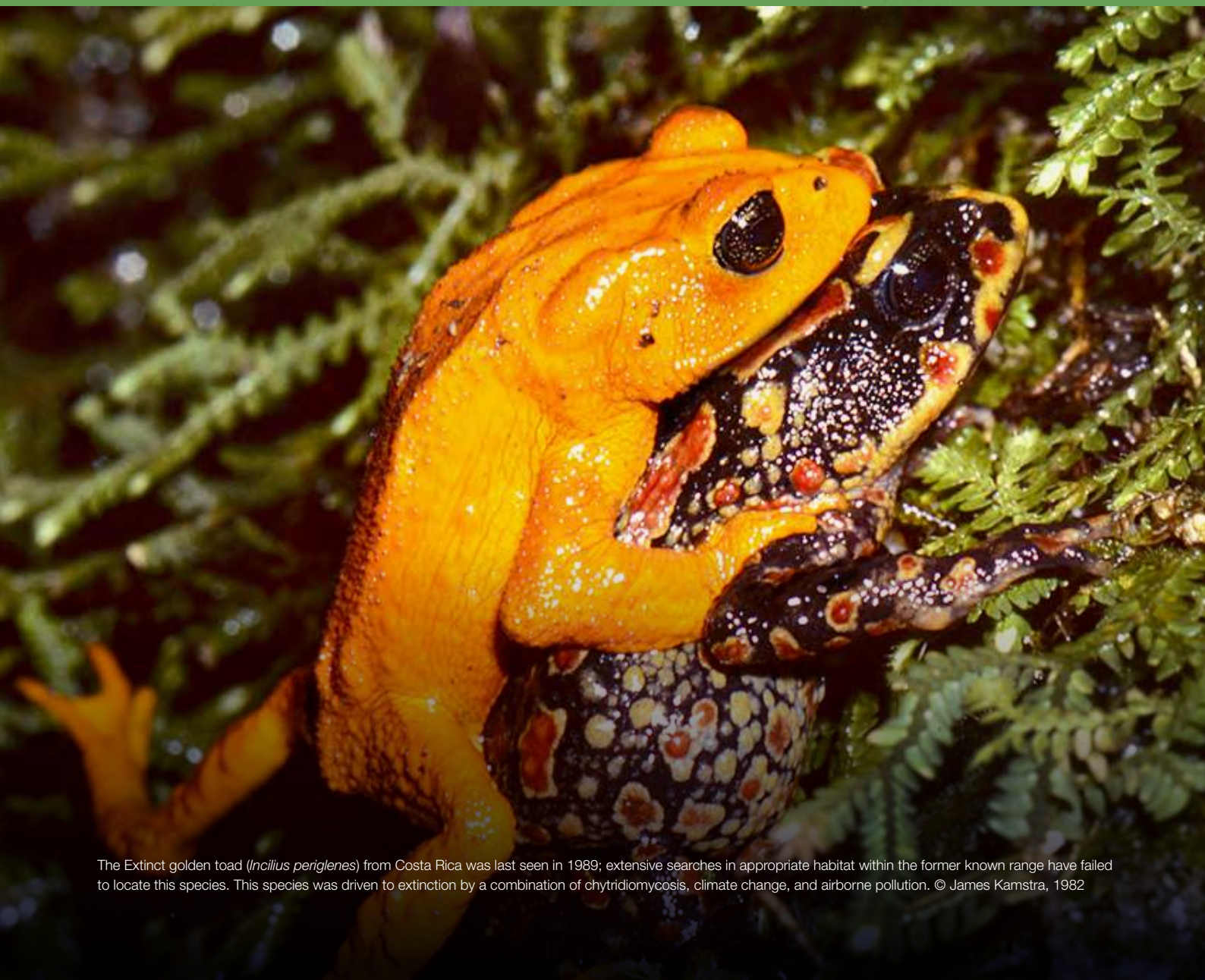
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The ACAP Editorial Team:

Sally Wren, Amaël Borzée, Ruth Marcec-Greaves and Ariadne Angulo



The Extinct golden toad (*Incilius periglenes*) from Costa Rica was last seen in 1989; extensive searches in appropriate habitat within the former known range have failed to locate this species. This species was driven to extinction by a combination of chytridiomycosis, climate change, and airborne pollution. © James Kamstra, 1982

Acronyms and abbreviations

AArk	Amphibian Ark
ACAP	Amphibian Conservation Action Plan
ARLA	Amphibian Red List Authority
ASA	Amphibian Survival Alliance
ASG	IUCN SSC Amphibian Specialist Group
AZE	Alliance for Zero Extinction
Bd	<i>Batrachochytrium dendrobatidis</i>
Bsal	<i>Batrachochytrium salamandrivorans</i>
CBD	Convention on Biological Diversity
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CNA	Conservation Needs Assessment
COVID-19	Coronavirus disease 2019
CPSG	IUCN SSC Conservation Planning Specialist Group
eDNA	Environmental DNA
EDGE	Evolutionarily Distinct and Globally Endangered
GAA	Global Amphibian Assessment
IPCC	Intergovernmental Panel on Climate Change
IUCN	International Union for Conservation of Nature
KBA	Key Biodiversity Area
NGO	Non-Governmental Organisation
SSC	IUCN Species Survival Commission
USFWS	United States Fish and Wildlife Service

Chapter 12



A postmetamorph of *Atelopus varius*, a Critically Endangered species, produced by Artificial Fertilization using hormonally induced eggs and sperm by Dr. Gina Della Togna at the Panama Amphibian Rescue and Conservation Project. © Brian Gratwicke

Chapter 12

Amphibian assisted reproductive technologies and biobanking

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Abstract

Continued amphibian species and population declines have led to the prioritisation of ex situ conservation breeding programme (CBPs) as one of the major strategies to safeguard and mitigate amphibian extinction. In the original version of the *Amphibian Conservation Action Plan* (ACAP), assisted reproductive technologies (ARTs) were incorporated as an appendix of the Captive Breeding Programme's chapter, suggesting their application as an innovative and supplementary approach that could enhance the efficacy of CBPs. This updated version of the ACAP includes, for the first time, an entire chapter dedicated to ARTs and Biobanking exclusively. Created by a group of experts in the field, this chapter describes: **1)** The current state of amphibian ARTs and biobanking, including hormonal stimulation for gamete release and collection, sperm and egg collection from live animals, sperm recovery from carcasses and wild-caught individuals, biobanking success in producing live animals and health and welfare considerations; **2)** The acceptance of ARTs as viable tools for amphibian conservation, their evolution and general recommendations for expanding global amphibian ARTs and; **3)** The incorporation of ARTs into a broader conservation action, describing their application in species conservation needs assessments and the incorporation of ARTs and strategic gamete biobanking into CBP genetic management. The authors of this chapter are optimistic the information relayed here is a great contribution for amphibian conservation since ARTs could facilitate and aid in the preservation of genetic material to manage, augment or rescue populations and species from extinction. As with any ex situ management strategy, ARTs including biobanking, should complement and support CBPs and habitat management programmes in conjunction with efforts to reduce or remove the pressures that initially led to a species' decline. This complementary conservation approach is recommended by the IUCN.

Introduction: statement and actions – the aim of the Working Group

Amphibians are declining at an alarming rate, and the establishment and management of ex situ conservation breeding programmes (CBPs) to safeguard threatened species are a critical component of their sustainable management. In the original version of the *Amphibian Conservation Action Plan* (Gascon et al., 2007) the incorporation of ARTs was proposed to increase the efficacy of CBPs. Assisted reproductive technologies include the use of hormones, gamete storage and biobanking, and in vitro fertilisation to improve reproductive success. Additionally, this chapter will provide: **1)** evidence of the legitimacy and practicality of their applications to amphibian conservation **2)** information concerning the value of ARTs and provide guidance to the broader amphibian conservation community on how ARTs can be incorporated into and complement existing conservation practices (Della Tonga et al. 2020), **3)** the progress of amphibian ARTs to date, **4)** promoting the growth of the ASG Amphibian ARTs and Biobanking Working Group's international community.

Furthermore, it is acknowledged that outside of the immediate biobanking community, the information set out in this chapter should address the concerns and goals of a diverse set of stakeholders, governmental and non-governmental entities, and the conservation, academic and scientific communities. Incorporating ARTs into programmes and policies could help individuals and organisations to make accurate decisions, balancing the risks and costs of implementation.

As mentioned in [Chapter 11](#), in alignment with the IUCN's World Conservation Strategy (Talbot, 1980) and the World Zoo Conservation Strategy (Wheater, 1995), ARTs should not act as the final solution for the management of declining amphibian biodiversity. Rather, ARTs should facilitate and aid the preservation of genetic material to manage, augment, or rescue populations and species from extinction. As with any ex situ management strategy, ARTs including biobanking should complement and support CBPs and habitat management programmes in conjunction with efforts to reduce or remove the pressures that

initially led to a species' decline. This complementary approach is recommended by the IUCN (Gascon et al., 2007). Release of individuals following ARTs should conform to the IUCN's reintroduction and translocation policies (Linhoff et al., 2021).

Many approaches improve the management and success of amphibian CBPs. These include induction of spermiation and ovulation through the use of hormonal stimulation, gamete cryopreservation and refrigerated/cold short-term storage, and artificial fertilisation (Arregui et al., 2021; Browne et al., 2011, 2019; Clulow et al., 2019; Della-Togna, 2015; Della-Togna et al., 2020; Gillis et al., 2021). However, successful genetic management using strategic biobanking can only be accomplished as part of a multidisciplinary approach in collaboration with all institutional, governmental, and private stakeholders. Therefore, the ASG Assisted Reproductive Technologies (ARTs) and Biobanking Working Group aims to coordinate international, regional, and local efforts for the development and implementation of ARTs for at-risk/threatened amphibian populations around the globe.

The current state of amphibian reproductive technologies and gamete banking

Gamete collection is the cornerstone of ARTs. Optimising protocols for gamete collection can improve the fertilisation capability of individuals, facilitate artificial fertilisation, artificial insemination (for internal fertilising species), and cryopreservation of gametes in order to manage and maintain genetic diversity in CBPs (Ananjeva et al., 2017).

In the 1800s, the concepts underlying genome resource banks (GRBs) for cryopreserved gametes were established (Mantegazza, 1866). Commercial needs have driven major advances in ART protocols in fish aquaculture (Tiersch et al., 2007; Walters et al., 2009), agriculture (Day, McLellan & Curry, 1995; Ruta, Lambardi, & Ozudogru, 2020), birds (Blesbois, 2007), mammals (Walters et al., 2009), and humans (Sherman, 1980; Walters et al., 2009). The uptake of GRBs in conservation has been slower, and

despite catastrophic amphibian declines, the utility of biobanks for this class was not acknowledged until recently, where the importance of its development and application has become evident (Gascon et al., 2007). In amphibians, sperm cryopreservation protocols have been applied to accomplish in vitro fertilisation with free swimming sperm (reported for some species, both with fresh or cryopreserved sperm; Browne et al., 2019; Clulow et al., 2019; Della-Togna et al., 2020; Strand et al., 2020) cloning, and intracytoplasmic sperm injection (ICSI), but further refinements and improvements in protocols are needed to complement the conservation efforts. Here we review amphibian ARTs to date.

Hormonal stimulation for gamete release

Several amphibian studies have demonstrated the successful use of exogenous hormones for the collection of sperm from Anura and Caudata. The most utilised hormones include peptides such as Gonadotropin-Releasing Hormone Agonist (GnRH-A [des-Gly10, D-Ala6, Pro-NHEt9]), human Chorionic Gonadotropin (hCG), and combinations of GnRH-A and dopamine antagonists such as metoclopramide, domperidone, or pimozide (Browne et al., 2019; Clulow et al., 2018; Della-Togna et al., 2017; Naranjo et al., 2022; Silla, McFadden, & Byrne, 2019; Vu & Trudeau, 2016). [Tables 12.1](#) and [12.2](#) show some examples of successful hormonal treatments on amphibians (Della-Togna et al., 2020).

The most commonly used methods of hormone administration is via injection, either intraperitoneal, subcutaneous, or intramuscular. These techniques are minimally disruptive and provide the most rapid and effective delivery method reported to date. However, they do require basic training as they are considered “invasive”. In the USA, these procedures are categorised by the Institutional Animal Care and Use Committee (IACUC) as a category “C” as they do not cause more than momentary or slight pain or distress and do not require the use of pain-relieving drugs (Federal Animal Welfare Regulations [CFR Ch.1, 2.36(b) (5-7), (National, 2011)]); however, the categorisation of these types

of procedures will vary globally and even between institutions so it is up to researchers to inform themselves as to local procedural requirements. While injections have been used experimentally on salamanders in general (Guy et al., 2020), they are not suitable for very small amphibians, such as plethodontid salamanders. For these delicate species, topical application provides an ethical alternative for administering exogenous hormones.

In recent years, other forms of hormone administration not requiring injection have been tested. These alternative methods include topical, oral ingestion, and nasal dripping and have proved successful in six anuran species (*Anaxyrus americanus*, *A. baxteri*, *A. valliceps*, *A. fowleri*, *Pseudophryne pengillyi* and *Xenopus laevis*; Obringer et al., 2000; Ogawa et al., 2011; Rowson, Obringer & Roth, 2001; Silla, Roberts & Byrne, 2020). However, it is important to highlight that while hormonal administration through non-injectable methods requires less training and is less invasive, any type of hormonal administration requires a basic knowledge of endocrinology to know how and when to apply these hormones. Furthermore, the success of all these studies have required the use of much higher concentrations of hormones compared to those used through injection and had much lower rates of efficacy compared to injections, most likely due to partial absorbance. The use of highly concentrated or voluminous hormone solutions has added disadvantages as it requires greater financial investment and creates the additional hazard of needing to safely dispose of hormone-contaminated water. Therefore, non-injectable methods are recommended only in instances where there is a restriction to the use of injections because of the size of the animals (Della-Togna et al., 2020). Topical use of GnRH-A has been reported in only two species of caudate (*Eurycea rathbuni* and *E. nana*) with successful increase in gamete production and breeding behaviour from both sexes post application (Browne et al., 2022; Campbell, Anderson & Marcec-Greaves, 2022). One study has successfully collected eggs from *Xenopus laevis* through non-invasive stimulation using progesterone and estradiol dissolved in water (Ogawa et al., 2011).

Table 12.1: A summary of exogenous hormone treatments reported in the literature used for the stimulation of spermiation in anurans and caudates

		Species	Hormone induction	Cryopreservation	Stimulation method	References
EUROPE		<i>Pelophylax lessonae</i>	GnRH 0.5 µg/g bw	24% DMFA and 20% sucrose	Intraperitoneal injection	(Uteshev et al., 2013)
		<i>Rana temporaria</i>	1.20 µg/g bw; 50 µg GnRH total	15% DMFA ; 5% glycerol, 2.5% sucrose and 5% egg yolk; 12% DMFA + 10% sucrose	Intraperitoneal injection	(Mansour et al., 2010; Uteshev et al., 2012; Kourova et al., 2021; Shishova et al., 2011)
		<i>Epidalea calamita</i>	hCG 10 IU/g	10% DMFA and 10% sucrose	Intraperitoneal injection	(Arregui et al., 2020)
		<i>Bufo bufo</i>	GnRH	N/A	Intraperitoneal injection	(Uteshev et al., 2012)
		<i>Pleurodeles waltl</i>	500 IU hCG, 50 µg GnRH total	N/A	Unspecified	(Uteshev et al., 2015)
AFRICA		<i>Xenopus laevis</i> and <i>X. tropicalis</i>	N/A	5% DMSO; 20% egg yolk and 0.8 M sucrose + 20 mmol NaHCO ₃	N/A	(Sargent and Mohun 2005; Mansour et al., 2009)
ASIA		<i>Andrias davidianus</i>	Oxytocin, dosage unspecified	5–25% DMSO	Unspecified	(Peng et al., 2011)
		<i>Tylototriton kweichowensis</i>	Prime GnRH 0.025 µg/g bw & spermiation dose GnRH 0.1 µg/g bw (24 hr later)	10% DMSO + BSA 1% + w/wo 10% Trehalose	Intramuscular injection	(Guy et al., 2020) Image credit: Matthew Cohen
NORTH AMERICA		<i>Amerana (Rana) muscosa</i>	0.3–3 µg/g bw GnRH; 5, 10 IU/g bw hCG or combination 0.4, 0.6 µg/g bw GnRH with 5 & 10 IU/g bw hCG	10% Trehalose + 10% DMFA	Intraperitoneal injection	(Calatayud et al., 2022)
		<i>Boreorana (Rana) sylvatica</i>	N/A	Testes macerates: 0.5 M DMSO + 50% FBS v/v; 150 mmol glucose; 0.15–3M glycerol	N/A	(Mugnano et al., 1998; Constanzo et al., 1998; Beesley et al., 1998)
		<i>Rhinella marina</i>	N/A	Testes macerates: 15% DMSO + 10% sucrose; 20% Glycerol + 10% sucrose	N/A	(Browne et al., 1998)
		<i>Lithobates pipiens</i>	0.4 µg/g bw GnRH + 10 µg/g bw MET (Amphiplex)	Testes macerates: 0.5 M DMSO + 50% FBS v/v; 150 mmol glucose; 150 mmol glycerol	Intraperitoneal injection	(Beesley et al., 1998; Constanzo et al., 1998; Trudeau et al., 2010)
		<i>Anaxyrus boreas boreas</i>	10–15 IU/g bw hCG; 6 µg GnRH total; 250–300 IU hCG total	0.5 M Trehalose + 10% DMFA	Intraperitoneal injection	(Roth et al., 2010; Langhorne et al., 2013; Langhorne et al., 2021)

		Species	Hormone induction	Cryopreservation	Stimulation method	References
NORTH AMERICA		<i>Anaxyrus americanus</i>	hCG 300 IU total; 4 µg GnRH total	Testes macerates: 0.5 M DMSO + 50 % FBS v/v	Intraperitoneal injection; Subcutaneous; absorption	(Obringer et al., 2000; Kouba et al., 2012; Beesley et al., 1998)
		<i>Anaxyrus fowleri</i>	hCG 300 IU total; 4 µg GnRH total (injection); 20 µg GnRH total (nasal)	10% DMFA + 10% Trehalose + 0.25% BSA	Nasal & intraperitoneal injection	(McDonough et al., 2016; Julien et al., 2019; Burger et al., 2023)
		<i>Ambystoma tigrinum</i>	hCG 500 IU; priming dose GnRH 0.025 µg/g bw + spermiation dose 0.1 µg/g bw	5% DMSO + 0.5% BSA	Intraperitoneal injection; Intramuscular injection	(Marcec, 2016; Lampert et al., 2022)
		<i>Ambystoma laterale</i>	GnRH 0.5 µg/g bw	N/A	Intraperitoneal injection; Intramuscular injection	(Marcec pers. comm)
		<i>Ambystoma mexicanum</i>	hCG 100–200 IU; Ovopel (GnRH 10–15 µg + 2.5–3 mg MET)	6% DMA	Intramuscular injection	(Mansour et al., 2011; Gonzalez, 2018; Rivera-Pacheco et al., 2021)
		<i>Cryptobranchus alleganiensis</i>	GnRH _a 0.4 µg/g bw + MET 10 µg/µL (Amphiplex)	10% DMSO	Intraperitoneal injection	(McGinnity et al., 2022)
		<i>Necturus maculosus</i> ; <i>Necturus beyeri</i>	1.7–2.3 µg GnRH/g bw	N/A	Intraperitoneal injection	(Stoops et al., 2014; Calatayud et al., 2019)
		<i>Notophthalmus meridionalis</i>	Prime GnRH 0.025 µg/g bw & spermiation dose GnRH 0.1 µg/g bw (24 hr later)	10% DMSO + BSA 1% + w/wo 10% Trehalose	Intramuscular injection	(Guy et al., 2020)
CARIBBEAN		<i>Peltophryne lemur</i>	10 IU/g bw hCG + 0.4 µg/g bw GnRH; 10 µg GnRH total (nasal)	10% DMFA + 10% Trehalose/ 10% DMSO + 10% Trehalose	Intraperitoneal injection; nasal	(Burger et al., 2022) Image credit: JP Zegarra
CENTRAL AND SOUTH AMERICA		<i>Rhaebo guttatus</i>	GnRH 0.4 µg/g GnRH + hCG 7.5, 10 IU/g bw	5% DMFA + 10% Trehalose	Intraperitoneal injection	(Hinkson et al., 2019)
		<i>Rhinella marina</i>	N/A	Testes macerates: 15% DMSO + 10% sucrose; 20% Glycerol + 10% sucrose	N/A	(Browne et al., 1998)
		<i>Atelopus zeteki</i>	GnRH 4 µg/g bw; GnRH _a 0.4 µg/g bw + MET 10 µg/g bw (Amphiplex); 10 IU/g bw hCG	10% DMFA + 10% Trehalose	Intraperitoneal injection	(Della Togna et al., 2015) Image credit: Panama amphibian rescue
		<i>Atelopus limosus</i>	2.4 µg/g bw GnRH; GnRH _a 0.4 µg/g bw + MET 10 µg/µL (Amphiplex); 10 IU hCG; GnRH 4 µg/g bw	10% DMFA + 10% Trehalose	Intraperitoneal injection	(Della Togna et al., 2020) Image credit: Panama amphibian rescue
		<i>Atelopus certus</i>	GnRH 4 µg/g bw	10% DMFA + 10% Trehalose	Intraperitoneal injection	(Della Togna et al., 2020) Image credit: Panama amphibian rescue

		Species	Hormone induction	Cryopreservation	Stimulation method	References
CENTRAL AND SOUTH AMERICA		<i>Atelopus glyphus</i>	GnRH 4 µg/g bw	10% DMFA + 10% Trehalose	Intraperitoneal injection	(Della Togna et al., 2020) Image credit: Panama amphibian rescue
		<i>Atelopus varius</i>	GnRH 4 µg/g bw	10% DMFA + 10% Trehalose	Intraperitoneal injection	(Della Togna et al., 2020) Image credit: Panama amphibian rescue
		<i>Strabomantis bufoniformis</i>	GnRH 4 µg/g bw	10% DMFA + 10% Trehalose	Intraperitoneal injection	(Della Togna et al., 2020) Image credit: Panama amphibian rescue
		<i>Triprion spinosus</i>	2 µg/g bw GnRH; 5, 10 IU/g bw hCG	N/A	Intraperitoneal injection	(Della Togna et al., unpublished) Image credit: Panama amphibian rescue
		<i>Craugastor evanesco</i>	GnRH 4 µg/g bw	N/A	Intraperitoneal injection	(Otero et al., 2023) Image credit: Panama amphibian rescue
		<i>Ceratophrys ornata</i> , <i>C. cranwelli</i>	GnRHα 0.4 µg/g bw + MET 10 µg/g bw (Amphiplex)	N/A	Intraperitoneal injection	(Trudeau et al., 2010)
		<i>Odontophrynus americanus</i>	GnRHα 0.4 µg/g bw + MET 10 µg/g bw (Amphiplex)	N/A	Intraperitoneal injection	(Trudeau et al., 2010)
AUSTRALIA		<i>Pseudophryne pengilleyi</i>	0.5–2.0 µg/g n RH	N/A	Subcutaneous injection	(Silla et al., 2018)
		<i>Litoria fallax</i>	hCG 20 IU/g bw	15% DMSO + 1% sucrose	Subcutaneous injection	(Upton et al., 2018)
		<i>Ranoidea (Litoria) raniformis</i>	20 µg Leuprorelin	N/A	Subcutaneous injection	(Mann et al., 2010)
		<i>Ranoidea (Litoria) aurea</i>	hCG 20 IU/g bw; hCG 60, 100, 300 IU total	15% DMSO + 1% sucrose; 15% DMSO + 10% sucrose	Subcutaneous injection	(Clulow et al., 2018; Upton et al., 2021; Upton et al., 2023)
		<i>Litoria castanea</i>	hCG 20 IU/g bw	10% Trehalose + 10% DMFA	Subcutaneous injection	(Hobbs et al., unpublished)
		<i>Ranoidea (Litoria) caerulea</i>	hCG 60, 100, 300 IU total	N/A	Subcutaneous injection	(Clulow et al., 2018)
		<i>Ranoidea (Litoria) booroolongensis</i>	hCG 20–40 IU/g	10% M Trehalose + 10% DMFA	Subcutaneous injection	(Silla et al., 2019; Hobbs et al., 2023)

		Species	Hormone induction	Cryopreservation	Stimulation method	References
AUSTRALIA		<i>Mixophyes fasciolatus</i>	hCG 60, 100, 300 IU total	N/A	Subcutaneous injection	(Clulow et al., 2018)
		<i>Ranoidea (Litoria) chloris</i>	hCG 60, 100, 300 IU total	N/A	Subcutaneous injection	(Clulow et al., 2018)

Notes: When available, cryopreservation cryodiluents have also been identified. The most commonly reported mode of hormone administration is intraperitoneal injection; however, some species have also achieved gamete release using topical application, subcutaneous and intramuscular injections, as well as nasal and oral administration. Species were assigned to the continent of origin, not the location where the study took place. The table does not show all the species reported in the literature. Image credits: Where image credits are not given, photos were acquired from 'free image' websites. **Abbreviations:** GnRH: Gonadotropin-Releasing Hormone; hCG: Human Chorionic Gonadotropin; µg: microgram; µL: microliter; g: gram; bw: body weight; IU: International Unit; DMSO: dimethyl sulfoxide; DMFA: dimethyl formamide; MET: metoclopramide; M: molar.

Gamete collection

• Sperm and egg collection from live animals

Exogenous hormonal stimulation for gamete collection via injection has been successfully implemented in several amphibian species (Table 12.1; Table 12.2). This includes using hCG, GnRH, GnRH combined with hCG, and GnRH combined with dopamine antagonists (such as in the Amphiplex mixture). These methods have been effective in many anuran species, some of which are listed in Table 12.1, and in a smaller number of Caudata species. Non-invasive methods such as oral, dermal, or topical administration have also resulted in the successful collection of gametes for six anuran species using hCG and GnRH (Julien et al., 2019; Obringer et al., 2000; Rowson, Obringer & Roth, 2001; Silla, Roberts & Byrne, 2020). Additionally, oocyte collection has been more challenging than sperm, but, nevertheless, successful collections have occurred with the use of different concentrations of hCG, GnRH, and GnRH with hCG, GnRH with metoclopramide (Amphiplex), Follicle-stimulating hormone (FSH), pituitary extract (PE), pregnant mare serum gonadotropin (PMSG), Testosterone (T), corticosterone (C), Domperidone (D), Pimozide (P) and Lucrin to name a few (Table 12.2).

To date, most hormonally-induced sperm and egg collections have been accomplished by

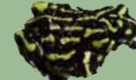
the implementation of empirically developed protocols, or replicating those reported successful for other species (Silla & Byrne, 2021), without further exploring if, in fact, those are the optimum protocols for new target species. Ideally, protocols should be standardised in a species-specific manner (for males and females) through experimentation, identifying the most efficacious hormones and concentrations that yield high quantity, quality and viability of gametes and characterises peaks of sperm concentration and oviposition timepoints as species characterisation metrics (Della-Togna et al., 2020). When achieving full optimisation protocols for a species is not possible due to extreme circumstances such as natural disasters, the application of developed protocols for closely related species to new species is recommended.

• Sperm recovery from carcasses

Testicular sperm sampling is usually achieved by euthanasia, followed by maceration of the testes, sperm analysis, and storage (either refrigerated or cryopreserved) for its immediate or later use. In cases where gamete recovery is part of a conservation strategy, euthanasia is not recommended; however, opportunistic sperm collection may be possible in instances where animals have died naturally, for vouchering purposes or

Table 12.2: A summary of amphibian species and corresponding exogenous hormone protocols used to induce ovulation

	Species	Exogenous hormone protocols used to induce ovulation	Prime timing	Alternative hormone	References
	<i>Anaxyrus boreas boreas</i>	Prime 1: 3.7 IU/g hCG Prime 2: 3.7 IU/g hCG Ovulatory dose: 13.5 IU/g hCG	0 hr 96 hr 24 hr	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)	Calatayud et al., 2015 ; Calatayud pers. comm
	<i>Anaxyrus fowleri</i>	Prime 1: 500 IU hCG + 0.4 µg/g LHRHa Prime 2: 100 IU hCG + 0.8 µg/g LHRHa Ovulatory dose: 500 IU hCG + 0.4 µg/g LHRHa	0 hr 96 hr 24 hr	0.61 µg/g LHRHa + 0.0076 µg/g pimozone + 0.15 µg/g P4	Browne et al., 2006a
	<i>Anaxyrus baxteri</i>	Prime 1: 500 IU hCG + 0.4 µg/g LHRHa Prime 2: 100 IU hCG + 0.8 µg/g LHRHa Ovulatory dose: 500 IU hCG + 0.4 µg/g LHRHa	0 hr 96 hr 24 hr	Alternative method excludes the first prime and relies on a single prime (prime 2 dosage)	Browne et al., 2006b. Image by Joel Santore
	<i>Acris crepitans</i>	Administered to the water. Ovulatory dose: 0.17 µg/g GnRHa + MET 0.42µg/µL			Snyder et al., 2012
	<i>Ambystoma mexicanum</i>	Ovulatory dose: 500 IU total hCG OR 1,000 IU total hCG No priming required if given during reproductive season		400IU total FSH	Gonzales, pers comm; Trottier & Armstrong, 1974
	<i>Ambystoma tigrinum</i>	Prime 1: 1 IU/µg hCG Prime 2: 2 IU/µg hCG Ovulatory dose: 4 IU/µg hCG + 0.1µg/g GnRH	0 day 168 hr 24 hr		Marcec, 2016
	<i>Ambystoma dumerilii</i>	Ovulatory dose: 500 IU total hCG; 1,000 IU total hCG No priming required if given during reproductive season			Gonzales, pers comm Image by Dr. Manuel Gonzalez
	<i>Eleutherodactylus coqui</i>	Ovulatory dose: 10, 20, 50 µg/g GnRHa; 165 IU total hCG			Michael et al., 2004
	<i>Necturus beyeri</i>	Ovulatory dose: 500 µg GnRHa			Stoops et al., 2014
	<i>Peltophryne lemur</i>	Prime 1: 1.5 IU /g hCG Prime 2: 1.5 IU/g hCG Ovulatory dose: 0.2 µg GnRHa; 4 IU hCG; 0.5 µg GnRHa + 4 IU hCG	0 hr 48 hr		Burger et al., 2021
	<i>Amerana (Rana) muscosa</i>	Prime 1: 0.4 µg/g GnRHa Ovulatory dose: 0.4 µg/g GnRHa + 10 µg/g MET	0 hr 24 hr		Calatayud et al., 2015 Image by Dr. Spencer Siddons
	<i>Lithobates sevosa</i>	Prime 1: 3.7 IU/g hCGg/g MET Prime 2: 3.7 IU/g hCG Ovulatory dose: 13.5 IU/g hCG	0 hr 24 hr 96 hr	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)	Graham et al., 2018
	<i>Lithobates pipiens</i>	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)		Progesterone, pituitary extract, Testosterone, Corticosterone, Domperidone	Trudeau et al., 2010; Wright, 1961; Fort, 2000
	<i>Ceratophrys ornata</i>	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)			Trudeau et al., 2010
	<i>Ceratophrys cranwelli</i>	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)			Trudeau et al., 2010
	<i>Odontophrynus americanus</i>	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)			Trudeau et al., 2010
	<i>Ranoidea (Litoria) raniformis</i>	Ovulatory dose: 50 µg des-Gly10, D-Ala6-(LHRH)			Mann et al., 2010
	<i>Ranoidea (Litoria) aurea</i>	Prime 1: 10 µg GnRH Ovulatory dose: 20 µg GnRH + 300 IU hCG	0 hr 72 hr		Clulow et al., 2018

	Species	Exogenous hormone protocols used to induce ovulation	Prime timing	Alternative hormone	References
	<i>Pseudophryne guentheri</i>	Prime 1: 0.4 µg/g GnRHa Ovulatory dose: 0.4 µg/g GnRHa	0 hr 26 hr		Silla, 2010
	<i>Pseudophryne corroboree</i>	Prime 1: 1 µg/g Lucrin (leuporelin acetate) Ovulatory dose: 1 µg/g Lucrin (leuporelin acetate)	0 hr 26 hr		Byrne & Silla, 2010
	<i>Pseudophryne pengilleyi</i>	Ovulatory dose: 0.5 – 2 µg/g Lucrin (leuporelin acetate)			Silla, 2018
	<i>Pseudophryne bibronii</i>	Prime 1: 0.4 µg/g GnRHa Ovulatory dose: 2 µg/g GnRHa			Silla & Byrne, 2021 Image by Dr. Aimee Silla
	<i>Pseudophryne coriacea</i>	Prime 1: 0.4 µg/g GnRHa Ovulatory dose: 2 µg/g GnRHa			Silla & Byrne, 2021 Image by Dr. Aimee Silla
	<i>Heleioporus eyrei</i>	Prime 1: 0.4 µg/g GnRHa Ovulatory dose: 2 µg/g GnRHa			Silla & Byrne, 2021 Image by Dr. Aimee Silla
	<i>Lymnodynastes tasmaniensis</i>	Prime 1: 0.49 – 1.2 µg/g GnRHa + Pimozide 10 µg/g Ovulatory dose: Pituitary extract; 100 IU hCG; GnRH 0.9 – 1.2 µg/g GnRHa + Pimozide 10 µg/g	0 hr 20 hr		Clulow et al., 2018
	<i>Mixophyes fasciolatus</i>	Prime 1: 25, 50 IU PMSG; 100 IU hCG Prime 2: 25, 50 IU PMSG; 100 IU hCG Ovulatory dose: 100 IU hCG	0 hr 96;144 hr 24 hr 24 hr		Clulow et al., 2018

Abbreviations: GnRH (LHRH): Gonadotropin-Releasing Hormone; hCG: Human Chorionic Gonadotropin; µg: microgram; µL: microliter; g: gram; bw: body weight; IU: International Unit; MET: metoclopramide; M: molar. Image credits: Where image credits are not given, photos were acquired from 'free image' websites.

have had to be euthanised for medical reasons. Researchers must ensure dead animals are sufficiently intact and fresh, to ensure that an adequate quality sample can be obtained. A study by Shishova et al. (2013) on sperm recovery from 0 to 7-day old refrigerated carcasses of the species *Rana temporaria* showed that sperm quality in terms of motility, forward progressive motility, DNA integrity and fertilisation capacity decreased over time. Therefore, regardless of the environment and situation, this approach requires rapid detection and processing of the carcass to yield the highest quality gametes possible. However, it is important to note that the quantity and quality of the cells could be compromised depending on the situation surrounding the death and the latency with which testes are recovered. Therefore, where samples may be collected opportunistically due to the death of a captive

animal, a high degree of coordination between institutional departments (i.e., husbandry, reproductive biology, and pathology staff) is required to ensure timely processing and successful gamete recovery. We recommend establishing these communications before embarking on any collection, thereby ensuring all internal and external permitting and bureaucratic requirements are cleared, since any delay related to this process may result in the loss of valuable viable cells. In addition to opportunistic collection of testes from recently deceased animals, it is recommended that coordination with researchers for planned euthanasia also occurs. For example, programmed euthanasia of healthy type specimens or other common species used in approved research are sources of concentrated gametes, typically of high quality, that could be collected (Kaurova et al., 2021).

• Sperm collection from wild-caught individuals

An important conservation strategy, particularly in the management of ex situ populations, is maintaining genetic variation. A strategy to accomplish this without increasing the number of individuals in CBPs, would be the introduction of diverse genes into ex situ populations through in situ sperm collection from donor individuals. This approach can improve the resilience of the rescued population without increasing the number of individuals in it (Howell et al., 2021a, 2021b). The use of refrigerated spermatozoa for in vitro fertilisation within days, can be complemented by cryopreserving spermatozoa in the field as a low-cost, spatially conservative, and long-term strategy to manage genetic variation in CBPs for biobanking. Equipment and resources that are cost-effective and are adaptable are optimal for use in the field and methods should include some key considerations:

- 1) Knowledge of the best timing of when samples should be collected (i.e., peak concentration time points and sperm quality parameters).
- 2) Use of effective and established cryopreservation protocols that have been pre-tested on the target species or a close relative).
- 3) Knowledge of field site accessibility to inform whether the operation requires a fully independent mobile laboratory facility, reduced capacity mobile laboratory facility or a field-kit only approach (see Della-Togna et al., 2020 for specifics).
- 4) Access to cryogenic agents (i.e., Liquid Nitrogen).
- 5) Implementation of established biosecurity protocols.

Biobanking success: producing viable offspring

Biobanking is a multi-decadal strategy that has been used to store biological samples for research and conservation of genetic information for a number

of taxonomic groups by cryopreservation (Hewitt & Watson, 2013). To date, technologies for cryopreservation of amphibian cells and tissues remain limited mostly to spermatozoa (Browne et al., 1998, 2019; Della-Togna et al., 2020; Kouba & Vance, 2009; Naranjo et al., 2022; Shishova et al., 2011; Shishova et al., 2013) and cell lines because of the composition, and large surface area and volume of oocytes, eggs, and embryos. Further technologies have been proposed to tackle the logistical challenges facing cryopreservation of the maternal lineage but will not be expanded upon in this chapter and we refer the reader to some of the following references for more detail (Browne et al., 2019; Clulow et al., 2019; Clulow & Clulow, 2016; Kouba et al., 2013; Strand et al., 2020; Zimkus, Hassapakis & Houck, 2018).

Since the ACAP was published in 2007, papers citing sperm cryopreservation have been published for 41 species (35 Anurans and 6 Caudata; Table 12.1). Six species and one sub-species of the 41 biobanked species known to us represent salamanders, and include: *Cryptobranchus alleganiensis* (McGinnity et al., 2022; Unger, Mathis & Wilkinson, 2013), *Ambystoma mexicanum* (Figiel, 2013), *Ambystoma tigrinum* (Gillis, 2020; Gillis et al., 2021; Marcec, 2016), *Ambystoma dumerilii* (González, pers. comm.), *Notophthalmus meridionalis*, *Tylototriton kweichowensis* (Guy, Gillis, et al., 2020) and *Andrias davidianus* (Browne et al., 2019; McGinnity et al., 2022; Peng, Xiao & Lui, 2011). No caecilian species have been reported in biobanks to date (Table 12.1).

Few publications report post-thaw artificial fertilisation (Burger et al., 2021; Langhorne, 2016; Marcec, 2016; McGinnity et al., 2022; Upton et al., 2018, 2021) and truly demonstrate the biological competence of frozen amphibian sperm with the production of viable F1 (first filial generation of offspring) individuals. Studies which reportedly produced offspring that successfully metamorphosed after artificial fertilisation include: *Anaxyrus boreas boreas*, *Lithobates sevosa* and *Anaxyrus fowleri* (Langhorne, 2016) and *Ambystoma tigrinum* (Marcec, 2016), *Ranoidea (Litoria) aurea* (Upton et al., 2021), *Litoria fallax* (Upton et al., 2018), *Cryptobranchus alleganiensis* (McGinnity et al., 2022), *Andrias davidianus* (Peng, Xiao & Lui, 2011)

and *Peltopryne lemur* (Burger et al., 2021). Yet, fewer studies have demonstrated the reproductive fitness of those offspring; *Ranoidea (Litoria) aurea* and *L. fallax* males produced by cryopreserved sperm reached sexual maturity and were capable of sperm production while ultrasounds showed that the two *Ranoidea (Litoria) aurea* females produced had reached sexual maturity and were gravid (Upton et al., 2018, 2021). *Peltopryne lemur* produced with cryopreserved sperm reached sexual maturity, producing 46 viable F2 individuals, of which 14 were released into the wild; of those remaining in captivity (24), one produced F3 tadpoles that were subsequently reintroduced into the wild (Burger et al., 2021).

Health and welfare considerations

The health of an animal must be taken into consideration when preparing for ARTs and ethical animal use permissions sought with the appropriate local or state authority. Certain species may be unable to withstand the stress of procedures such as sperm or egg collection. Although at present, there is no evidence that the principal hormones used in ART directly cause toxicity or health complications in amphibians, the application of exogenous hormones should be done under careful consideration and consultation with trained personnel. Since hormonal control of amphibian reproduction is often species-specific (Norris, 2004; Ogielska & Bartmanska, 2009) caution is recommended when applying hormones to any species for the first time (Clulow et al., 2019; Silla, Calatayud & Trudeau, 2021). To date, a few studies suggest that collection frequency can affect sperm quality in at least one anuran species (Guy, Martin, et al., 2020; McDonough et al., 2015). The benefits that ARTs can provide to amphibian conservation currently outweigh any reported side effects on animal health (Chai, 2016). In a captive setting, full or partial egg retention (dystocia) may occur in female amphibians when husbandry parameters are not ideal. Egg retention that does not resolve may follow in a multitude of secondary health complications that may result in death. However, in the event of egg retention, exogenous hormones can be administered

to promote egg deposition (Bronson et al., 2021; Calatayud et al., 2019; Chai, 2016; Wright & Whitaker, 2001).

The use of cryopreservation in conjunction with hormone-induced gamete collection can replace the need for extirpation of animals from the wild or movement between breeding colonies, which reduces transport-induced stress and potentially life-threatening situations (Della-Togna et al., 2020; Langhorne, 2016). ARTs also allow for improved long-term management of genetics and the prevention of inbreeding (Byrne, Gaitan-Espitia & Silla, 2019; Howell et al., 2021b; Silla, Calatayud & Trudeau, 2021) while offering greater potential of good health and high survivability in offspring.

Acceptance of ARTs as viable conservation tools

Advance of ARTs as possible amphibian conservation tools

It is beyond the scope of this document to present information on the technical details of the emerging technologies that could be applied to amphibian conservation, such as cloning. A number of approaches have been reviewed by other authors and are referenced in this section. The future of ARTs relies on how these technologies will overcome the difficulties conservationists face with managing amphibian genome resources while preserving the highest genetic diversity (Clulow et al., 2019; Holt, Pickard & Prather, 2004; Mastromonaco & Songsasen, 2020). Cloning (somatic cell nuclear transfer) is probably the best-known technology resulting in the production of live young, but despite its success, it has not been incorporated into amphibian conservation. First described in an amphibian species, *Lithobates pipiens* and later *Xenopus laevis*, cloning was implemented to explore the fundamentals of developmental biology (reviewed by Gurdon & Byrne, 2002). Reproductive cloning followed shortly after when Gurdon (1968) reported the production of normal adult clones (individuals derived from nuclear transplantation that

are identical to the parent). A suite of approaches is now available to support conservation across a number of taxonomic groups, particularly mammals (Mastromonaco & Songsasen, 2020). Cell transplantation (primordial and spermatogonial) may provide alternate sources of genetic material of a wild or threatened species compared to sperm and oocytes alone. Through reprogramming and regeneration, cells can diversify into renewable and operational genetic material of infinite potential (Clulow & Clulow, 2016; Mastromonaco et al., 2014; Mastromonaco & Songsasen, 2020). Somatic cell technologies also offer promise since their use precludes the need for viable gametes, thereby enabling genetic contribution of individuals that are reproductively dysfunctional or perish before reaching sexual maturity and fail to contribute to the gene pool (Mastromonaco et al., 2014).

General recommendations for expanding global amphibian ARTs

The COVID-19 pandemic revolutionised work practises and has transformed the delivery of training to a wide and diverse group of users. However, in many cases,

developing a project involving ARTs will still require trained personnel and adequate resources (Della-Togna et al., 2020). The basic requirements for the development and implementation of ARTs globally are:

- 1) The provision of protocols for gamete collection, gamete storage, and the subsequent recovery of individuals from cryobanked materials for a broad range of taxa.
- 2) Establishment of biobanks or partnering with biobanks that have secured long-term funding in different regions of the globe (Figure 12.1).
- 3) International Nagoya Protocol (Buck & Hamilton, 2011; Kamau, Fedder & Winter, 2010) and national laws and policies that allow and facilitate the collection of gametes from existing CBPs or from the wild, transportation, and storage of biological materials.
- 4) Access to collection sites using local knowledge and expertise, taking into account that many species are located in or near indigenous communities and protected areas, each with particular restrictions.

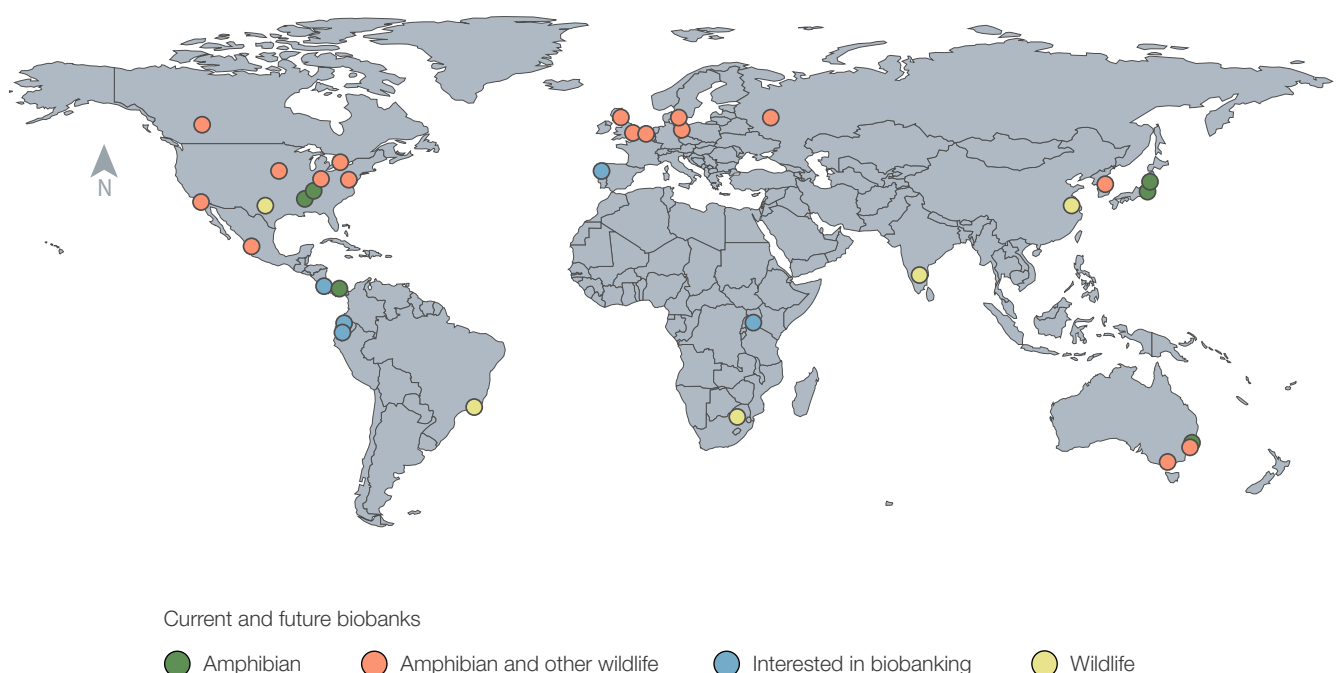


Figure 12.1: Location of known biobanks for wildlife species. Source: Data collected by the ASG Amphibian ARTs and Biobanking Working Group from a survey conducted from 2018-2021.

- 5) Country policies on access to genetic resources allow such large-scale operations and have sustainable funding in place for long-term preservation of the collections.

Incorporating ARTs into broader conservation action: Informing effective management

Conservation Needs Assessments

The Amphibian Ark works with a range of amphibian field biologists and other experts to develop Conservation Needs Assessments (CNAs) for amphibian species, which in turn generate a range of recommended strategies, including ex situ conservation actions (Johnson & Carrillo, 2022). This evaluation and prioritisation help conservation managers to maximise the impact of limited conservation resources by identifying which amphibian species are most in need and are likely to receive the most benefit from particular types of conservation action. Biobanking is one of the conservation actions and is recommended for species which are under

imminent danger of extinction (locally or globally) because the threats they face cannot or will not be reversed in time to prevent likely species extinction. They, therefore, require ex situ management, or rescue, as part of an integrated programme to ensure their survival. To date, 4,200 CNAs have been completed for 3,544 amphibian species in 47 countries, out of which 398 have been recommended for biobanking (Johnson & Carrillo, 2022; Figure 12.2). CNAs are one of the few conservation assessment tools which generate prioritised lists of species for biobanking, and as such, provide not only a logical and transparent procedure for guiding amphibian conservation activities within a country or region, but also a good reference for those involved with ARTs when considering species which should be targeted for biobanking (Figure 12.2). The Amphibian Ark recommends that detailed and collaborative species actions plans be co-developed by all relevant stakeholders for species considered for ex situ rescue (Amphibian-Ark, 2020), and the use of ARTs and gamete cryopreservation should be considered when developing these action plans. Further detail on planning can be found within Chapter 9.

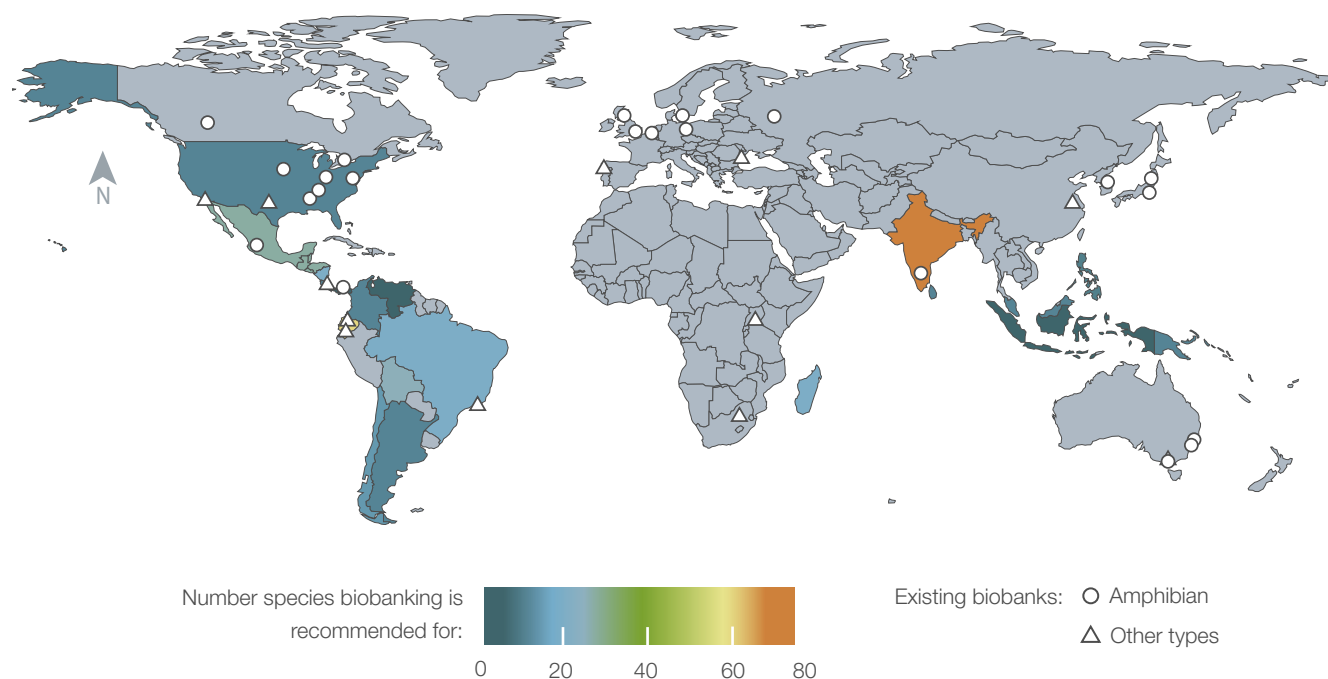


Figure 12.2: Existing biobanks containing general wildlife biomaterials or amphibian-specific samples superimposed over the regions where amphibian biobanking has been recommended by the Conservation Needs Assessments. Countries with biobanks marked with a triangle denote those with wildlife collections but that do not currently hold amphibian material or for which there has been no confirmation of banked amphibian samples. Circles denote areas where current amphibian-specific biobanks are located. Source: Data collected by the ASG Amphibian ARTs and Gamete Biobanking Working Group.

Table 12.3: Summary of some of the institutions holding amphibian samples around the world which are linked to CBPs.

Institution	Country	Number of species	Core Biobanking Facility	Affiliated Biobanking Facility	Personal communication
San Diego Zoo Wildlife Alliance's Frozen Zoo		26/31	Spermatozoa/ cell lines		Dr. Barbara Durrant and Ms. Marlys Houck personal communication
Mississippi State University (MSU) (Amphibian Genome Resource Bank)		16		Spermatozoa	Dr. Carey Vance-Kouba personal communication
Toronto Zoo		2		Spermatozoa/ cell lines	Dr. Gabriela Mastromonaco & Ms. Paula Mackie personal communication
Cincinnati Zoo and Botanical Gardens		5		Spermatozoa	Dr. Terri Roth personal communication
Conservation Biology Research Group, University of Newcastle		30		Spermatozoa	Associate Professor John Clulow & Dr. Rose Upton personal communication
Taronga Conservation Society		12	Spermatozoa		Dr. Rebecca Hobbs personal communication
Smithsonian Tropical Research Institute Panama Amphibian Rescue and Conservation Project (PARC)		7	Spermatozoa		Dr. Gina Della Tonga personal communication
Institute of Cell Biophysics, Russian Academy of Sciences		5	Spermatozoa, embryonic cells		Dr. Victor Uteshev personal communication

Notes: Biobanks are designated as 'core' if they are standalone institutions that are, **1.** not reliant on short-term grant funding, **2.** have self-reliant infrastructure, **3.** backed by one or more replicated collections and/or are, **4.** part of a consortium of several biobanks that operate as insurance collections; **5.** Alarmed security measures in case of failures. 'Affiliated' banks are those that are, **1.** funded on a short-term, grant-dependent basis, **2.** do not have an insurance collection and/or, are, **3.** dependent on the infrastructure of an external institution i.e. Academic Institution

Feasibility and design (strategy): Incorporation of ARTs and strategic gamete biobanking into CBPs

The integration of biobanking and ARTs into genetic management programmes has long been realised for agriculturally important animal and plant species (Blackburn, 2017) yet continues to lag for wildlife species. This is likely due to a multitude of factors that differ between conservation management programmes and these for-profit industries, including: access to sustainable financial resources and infrastructure, clear species prioritisation, need for taxa- or species-specific protocols, coordinated stakeholder engagement, and government support. Wildlife biobanking is a long-term genetic management strategy that requires all of these factors to work in concert and be dynamic and responsive to evolution in needs, technologies and management strategies over long-time scales; timescales that may reflect many generations of the target species.

Current and emerging ARTs can be integrated within CBPs, where they will be of greatest benefit when combined with a multifaceted conservation action plan (Table 12.3). As such, biobanking strategic goals and decision frameworks are likely to reflect those for establishment of CBPs and may include additional considerations, i.e., do gamete collection protocols exist for this species? Is there a suitable model or subspecies should hybridisation be the only available sample end-use? Are there existing in situ programmes that offer potential for opportunistic collection? Albeit collection of samples from species where data remains deficient or development of a CBP has not been forecast are not necessarily excluded, but this runs the risk of these resources becoming nothing more than a museum serving to support phylogenetic analysis and taxonomy, but little else.

One of the most significant driving factors of the poor representation of amphibians across CBPs is high costs. The proposed budget for the development of CBPs in the original ACAP document was US\$ 120,000 per year per species, with estimates of US\$12.5 million to captive-manage 100 species

each for one year (Gascon et al., 2007). Outdated by over a decade and lacking detail, these costs are likely highly conservative. Estimates from 2018 in Australia suggest CBPs cost on average around US\$ 136,567 per year per species, often for many years or even decades (Harley et al., 2018). Despite these high costs, there is emerging evidence of the cost-reductions and efficiencies that are possible when integrating biobanking technology and ARTs into CBPs as practical support tools. Economic and genetic modelling using real-world data for the CBP for Oregon spotted frogs (*Rana pretiosa*) suggests that biobanking technology could lower the size of the live colony required to be held in captivity, substantially lowering the costs of CBPs, as well as reducing inbreeding of output amphibians from these programmes (Howell et al., 2021a). This modelling provides an examination of programme costs and captive genetic diversity (heterozygosity H_i/H_o values derived from inbreeding rates) when a simple low-cost biobanking set-up (consisting of basic additional freezing infrastructure; e.g. freezers and liquid nitrogen dewars) is integrated into an established amphibian CBP. In hypothetical captive colonies designed to meet the same genetic retention target (90% of source population heterozygosity for a minimum of 100 years, in line with proposed global genetic benchmarks; Soulé et al., 1986), there was a 26-fold cost reduction in populations with biobanking integrated compared to populations under conventional CBPs conditions (Howell et al., 2021a). This means that 26 species could be captive bred for the price of one in programmes designed to meet globally accepted genetic retention targets under the with- and without-biobanking scenarios.

This research is further supported by recent modelling in two Australian species, the orange-bellied frog (*Geocrinia vitellina*) and white-bellied frog (*Geocrinia alba*), where similar proportionate cost-reductions and genetic benefits were exhibited (Howell et al., 2021b). This study modelled the genetic and cost benefits of incorporating ARTs and biobanking into CBPs of *G. vitellina* and *G. alba* at Perth Zoo, Australia. To meet the 90% heterozygosity retention target in conventional CBP conditions would require

400 live *G. vitellina* and *G. alba*, costing US\$ 732,755 and US\$ 478,289 in year one and US\$ 310.4 million and US\$ 189.2 million across 100 years respectively, compared to just 17 live individuals for each species, costing US\$ 45,300 and US\$ 32,000 in year one, and US\$ 14 million and US\$ 8.7 million across 100 years in CBPs integrating ARTs and biobanking. The study also revealed that world-first ambitious targets of 95% and 99% H_i/H_o retention may be possible in amphibian CBPs under realistic cost frameworks.

The integration of frozen founder spermatozoa would also provide significant genetic benefits. Conventional CBPs have various challenges with genetic diversity which can compromise the value of captive-bred animals for release to the wild, including inbreeding depression in unavoidably small captive colonies (Ralls, Ballou & Templeton, 1988), reduced reproductive fitness (Farquharson, Hogg & Grueber, 2018), and domestication and adaptation to captivity (Frankham et al., 2002). Biobanking and ARTs would reduce the rate of inbreeding in amphibian CBPs, and biobanked males would not be subject to domestication and adaptation to captivity, which would generally make animals produced using ARTs and biobanking better suited for release to the wild (Howell et al., 2021a, 2021b). Ultimately, these studies reveal a promising and potentially feasible model; the integration of ARTs and low-cost additional biobanking infrastructure into existing amphibian CBPs globally to achieve cost and genetic outcomes for species, institutions and end-users. Given the generally poor understanding and transparency around the costs associated with amphibian biobanking, the slow progress towards a viable funding mechanism for amphibian biobanking, and the limited funding landscape for amphibian conservation efforts, this is likely the most feasible model for the integration of biobanking and ARTs into CBPs (Della-Togna et al., 2020).

The implementation of this model requires first:

1) building the case for amphibian biobanking using economic and genetic arguments, **2)** secure accessible founder populations of broodstock in CBPs, **3)** establishing financial planning and funding mechanisms for long-term biobank sustainability,

4) leverage existing CBP infrastructure through partnerships and secure additional biobanking infrastructure.

Amphibian conservationists and ART practitioners should focus on developing examples of this model in practice. Howell et al. (2021b) provide a broad pathway of actions required to transition ARTs and biobanking into Australian CBPs under this model to produce practical examples. Since the model described above would be highly transferable, the pathway may also provide a feasible strategy to transition ARTs and biobanking into CBPs globally. The strategy is provided in more detail in Howell et al., (2021b), but would involve various key steps, including: **1)** Continue building the case for amphibian biobanking using economic and genetic arguments; **2)** Secure captive colonies of target species, through partnership with captive institutions or development of novel amphibian CBPs; **3)** Financial planning and funding mechanism development (focussing on long-term biobank sustainability, understanding long-term required costs and the applicability of biobank funding mechanisms developed for biobanks in other sectors; **4)** Leverage existing CBP infrastructure through partnerships and secure additional biobanking and freezing infrastructure. Additionally, it is important to consider that broader amphibian conservation programmes depend on biopolitical, biogeographical, and phylogenetic factors and they should be considered when developing strategies to implement ARTs and biobanking. This model of integrating additional biobanking infrastructure into established programmes will be a low-cost option, e.g. around US\$ 9,300 for basic freezing infrastructure as modelled in Howell et al. (2021b), which are supported by estimates of US\$ 14,600 to incorporate basic biobanking capacity into CBPs at Zoos Victoria (Della-Togna et al., 2020) and the low-cost self-contained mobile laboratories for aquatic species cryopreservation presented in Childress, Caffey & Tiersch (2018) and Childress et al. (2019); **5)** Close species-specific knowledge gaps in target amphibian species in order to develop optimised species-specific biobanking protocols. This will require applied research effort, access to

colony animals and skilled labour, and access to significant research funding, up to US\$ 2.2 million in targeted applied research funds per species across 5-year research programmes, as estimated in Howell et al., (2021b).

Priorities and recommendations

- » Increase membership of CBP and GRB personnel to the ASG Assisted Reproductive Technologies (ARTs) and Biobanking Working Group to expand networking, capacity building and promote equitable exchange of knowledge.
- » Strengthen the communication and collaboration among the IUCN Animal Biobanking for Conservation Specialist Group, the ASG Assisted Reproductive Technologies (ARTs) and Biobanking Working Group, the ASG Genomics Working Group, ASG Captive Breeding Working Group and the IUCN Conservation Genetics Specialist Group to promote strategic alignment of actions for amphibian conservation.
- » Develop a stronger partnership between the ASG Assisted Reproductive Technologies (ARTs) and Biobanking Working Group and the Amphibian Ark to reach captive breeding programmes that could benefit from the application of ARTs and Biobanking.
- » Incorporate the application of ARTs in the Amphibian Ark's Conservation Needs Assessments questionnaire with the purpose of gathering information on existing banked biomaterials and needs and advances in the development of such technologies.
- » Strengthen the online presence of the ASG Amphibian Assisted Reproductive Technologies (ARTs) and Biobanking Working Group to create a web-based platform to:
 - Increase communication
 - Educate and improve awareness
 - Provide resources for capacity building
 - Connect practitioners at a regional level
- » Identify CBPs holding endangered species that:
 - Have faced challenges or unsuccessful reproductive attempts
 - Could immediately benefit from the development and application of ARTs
 - Genetic rescue is recommended and urgent

Such are the cases of the species *Mantella cowani* (Madagascar), *Conraua derooi* (Ghana & Togo), *Telmatobius dankoi* (Chile), and many of the 382 species recommended for ex situ conservation programmes and the 399 recommended for genome resource banking (CNA, 2022).

- » Integration of ARTs and Biobanking in CBPs as complementary and powerful tools to strengthen reproductive success and genetic management.
- » Identify wild and micro-endemic species such as *Melanophryniscus admirabilis* for which CBPs have not been established and could benefit from in situ application of ARTs and gamete collection for biobanking.
- » Development of species-specific hormonal stimulation, gamete characterization, artificial fertilisation and cryopreservation protocols.

Conclusions and future directions

With more than 900 amphibian species likely requiring some form of ex situ insurance population (Zippel et al., 2011), predictions that global resources needed to sustain amphibian CBPs are extremely limited and are already around a decade old (Bishop et al., 2012). The reality is that the situation has worsened and continues to highlight the poor representation of amphibians in global CBP efforts. As we enter the Anthropocene and recognise our global mandate for the sustainable management of biodiversity, a correspondence of biotechnical options, including the use of ARTs and biobanks, are being offered to assist in achieving realistic and potentially promising goals for the perpetuation of threatened species. Therefore, maximising the global impact of amphibian gene

banking is now at its most critical point. Strategies for the best way to implement ARTs into broader amphibian conservation programmes depend on biopolitical, biogeographical, and phylogenetic targeting. Biopolitical targeting should be designed and executed to target the obvious and publicly accessible benefits of safeguarding the target species. This will reciprocally garner greater public influence and political support leading to further resource allocation. The development of techniques for amphibian ARTs has almost exclusively been in moderate to high-income industrialised countries. Yet most amphibian species, except South-East Asia, North America and eastern Australia, occur in the low to moderate-income countries within Central and South America, New Guinea, and Africa (Figure 12.2). Most threatened Anura come from Central and South America, Caudata from Asia and North America, and Gymnophiona (caecilians) from India and Africa.

As we enter the new age of the sustainable management of biodiversity, increasingly technical options, such as the merging of CBPs and ARTs, are being offered to assist in achieving realistic goals. However, despite their application and reliability, financial constraints still pose a major obstacle. Generally, CBPs have been largely financed and supported by zoos as part of their conservation work; however, over the last two decades private groups, supported by seed grants or ongoing finance from various amphibian conservation organisations, have established private amphibian CBPs. When these are located in a priority region, they provide the ideal opportunity to begin the merging of CBPs with gene banking of tissue, gamete or cell lines.

Biogeographically, emphasis should be on CBP facilities that are located in the regions predicted to suffer the most loss of amphibian phylogenetic biodiversity. With the appropriate training, in-country CBPs can easily maintain fully genetically varied populations of species through broodstock management that incorporates sperm collected from individuals in their CBPs and in the field. However, this will require securing access to liquid nitrogen supply or to a local or regional biobank storage

facility and the adequate representation of experienced personnel on the ground willing to exchange, support and train in-country researchers, especially in instances where no technical expertise exists. For this, we propose the establishment of regional teams, led by one or more personnel specifically trained in ART procedures to be funded and willing to support any area where immediate intervention is required. The IUCN, the ASG and other large entities should help facilitate funding avenues to sustain this strategy if there is to be a long-term commitment to the preservation of amphibian species and the incorporation of ARTs into mainstream amphibian conservation strategies. Thus, biobanking can become incorporated into associated fieldwork for the species including maintaining or increasing suitable habitat. These works contribute not only to the perpetuation of amphibian species but also to global sustainability.

Prioritisation of regions for amphibian CBP ARTs should address the urgency for conservation but should also take into account the practicality of conserving species based on their intrinsic value to the ecosystem and not on a singular species criterion. Second, determining what species to biobank should also consider the available, biogeographical patterns in genetic and phylogenetic diversity (Hu et al., 2021; Upton, 2020) predictions of future habitat loss through vegetation destruction or through changes in global temperatures (Zhang et al., 2021), and from recommendations generated by Conservation Needs Assessments (Johnson & Carrillo, 2022) and IUCN's amphibian Red List (IUCN 2023). By prioritising resources to maximise conservation efficiency toward the protection of ecoregions closest to meeting targets, there can be a doubling benefit to cost, whilst excluding some areas of high biodiversity for species of particular taxon including amphibians (Chauvenet et al., 2020). Upton (2020) showed that up to 40% of amphibian phylogenetic diversity could be protected by increasing protection of 1.9% of global terrestrial area. Thus, the targeting of CBPs and/or ARTs should also be focused on these regions both in terms of their biodiversity but also in terms of increased risk to amphibian species.

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A scanning electron microscope image of mountain yellow-legged frog sperm (*Rana muscosa*), taken at the Electron Microscopy Facility at San Diego State University (SDSU). © Ingrid Niesman



Research has established hormone protocols for the stimulation of both spermiation and ovulation in the Australian frog *Ranoidea (Litoria) aurea*, which is classified as Near Threatened. © Jodi Rowley