Chapter 12

Access complete book here

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

Natalie E. Calatayud[1,2], Lachlan G. Howell[3,4,5], Rose Upton[3,5], Benjamin Tapley[6], Kevin Johnson[7], Robert Browne[8], Ruth Marcec-Greaves[9], Candace L. Williams[1], David O'Brien[10], Rebecca Hobbs[11], Vance L. Trudeau[12], Deborah S. Bower[14], Simon Clulow[13], John Clulow[3,5] and Gina Della Togna[2,15]

[1] San Diego Zoo Wildlife Alliance, Beckman Center for Conservation Research, Escondido, CA, USA
[2] Amphibian Survival Alliance PO Box 129, Austin, TX 78767 USA
[3] Conservation Biology Research Group, School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia
[4] School of Life and Environmental Sciences, Deakin University Melbourne Burwood Campus, 221 Burwood Highway, Burwood, VIC 3125, Australia
[5] FAUNA Research Alliance, Kahibah, NSW, Australia
[6] Zoological Society of London, Regent's Park, London, UK
[7] Amphibian Ark, Apple Valley, MN, USA
[8] Sustainability America, La Isla Road, Sarteneja, Corozal District, Belize
[9] Honduras Amphibian Rescue and Conservation Center, Tela, Honduras
[10] NatureScot (Scottish Natural Heritage), Inverness, UK
[11] Taronga Institute of Science and Learning, Taronga Conservation Society Australia, Mosman, NSW, Australia
[12] Department of Biology, University of Ottawa, Ontario Canada
[13] Centre for Conservation Ecology and Genomics, Institute for Applied Ecology, University of Canberra, Bruce ACT, Australia
[14] School of Environmental and Rural Science, University of New England, Armidale, NSW, Australia
[15] Smithsonian Tropical Research Institute, Panama Amphibian Rescue and Conservation Project, Gamboa, Panama

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 pengilleyi* 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		*Pelophylax lessonae*	GnRH 0.5 µg/g bw	24% DMFA and 20% sucrose	Intraperitoneal injection	(Uteshev et al., 2013)
		*Rana temporaria*	1.20 µg/g bw; 50 ug 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; Kaurova et al., 2021; Shishova et al. 2011)
		*Epidalea calamita*	hCG 10 IU/g	10% DMFA and 10% sucrose	Intraperitoneal injection	(Arregui et al., 2020)
		*Bufo bufo*	GnRH	N/A	Intraperitoneal injection	(Uteshev et al., 2012)
		*Pleurodeles waltl*	500 IU hCG, 50 µg GnRH total	N/A	Unspecified	(Uteshev et al., 2015)
AFRICA		*Xenopus laevis and X. tropicalis*	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		*Andrias davidianus*	Oxytocin, dosage unspecified	5–25% DMSO	Unspecified	(Peng et al., 2011)
		*Tylototriton kweichowensis*	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		*Amerana (Rana) muscosa*	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)
		*Boreorana (Rana) sylvatica*	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)
		*Rhinella marina*	N/A	Testes macerates: 15% DMSO + 10% sucrose; 20% Glycerol + 10% sucrose	N/A	(Browne et al., 1998)
		*Lithobates pipiens*	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)
		*Anaxyrus boreas boreas*	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		*Anaxyrus americanus*	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)
		*Anaxyrus fowleri*	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)
		*Ambystoma tigrinum*	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)
		*Ambystoma laterale*	GnRH 0.5 µg/g bw	N/A	Intraperitoneal injection; Intramuscular injection	(Marcec pers. comm)
		*Ambystoma mexicanum*	hCG 100–200 IU; Ovopel (GnRH 10–15 µg + 2.5-3 mg MET)	6% DMA	Intramuscular injection	(Mansour et al., 2011l; Gonzalez, 2018; Rivera-Pacheco et al., 2021)
		*Cryptobranchus alleganiensis*	GnRHa 0.4 µg/g bw + MET 10 µg/µL (Amphiplex)	10% DMSO	Intraperitoneal injection	(McGinnity et al., 2022)
		*Necturus maculosus; Necturus beyeri*	1.7–2.3 µg GnRH/g bw	N/A	Intraperitoneal injection	(Stoops et al., 2014; Calatayud et al., 2019)
		*Notophthalmus meridionalis*	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		*Peltophryne lemur*	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		*Rhaebo guttatus*	GnRH 0.4 µg/g GnRH + hCG 7.5, 10 IU/g bw	5% DMFA + 10% Trehalose	Intraperitoneal injection	(Hinkson et al., 2019)
		*Rhinella marina*	N/A	Testes macerates: 15% DMSO + 10% sucrose; 20% Glycerol + 10% sucrose	N/A	(Browne et al., 1998)
		*Atelopus zeteki*	GnRH 4 µg/g bw; GnRHa 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
		*Atelopus limosus*	2.4 µg/g bw GnRH; GnRHa 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
		*Atelopus certus*	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		*Atelopus glyphus*	GnRH 4 µg/g bw	10% DMFA + 10% Trehalose	Intraperitoneal injection	(Della Togna et al., 2020) Image credit: Panama amphibian rescue
		*Atelopus varius*	GnRH 4 µg/g bw	10% DMFA + 10% Trehalose	Intraperitoneal injection	(Della Togna et al., 2020) Image credit: Panama amphibian rescue
		*Strabomantis bufoniformis*	GnRH 4 µg/g bw	10% DMFA + 10% Trehalose	Intraperitoneal injection	(Della Togna et al., 2020) Image credit: Panama amphibian rescue
		*Triprion spinosus*	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
		*Craugastor evanesco*	GnRH 4 µg/g bw	N/A	Intraperitoneal injection	(Otero et al., 2023) Image credit: Panama amphibian rescue
		*Ceratophrys ornata, C. cranwelli*	GnRHa 0.4 µg/g bw + MET 10 µg/g bw (Amphiplex)	N/A	Intraperitoneal injection	(Trudeau et al., 2010)
		*Odontophrynus americanus*	GnRHa 0.4 µg/g bw + MET 10 µg/g bw (Amphiplex)	N/A	Intraperitoneal injection	(Trudeau et al., 2010)
AUSTRALIA		*Pseudophryne pengilleyi*	0.5–2.0 µg/g n RH	N/A	Subcutaneous injection	(Silla et al., 2018)
		*Litoria fallax*	hCG 20 IU/g bw	15% DMSO + 1% sucrose	Subcutaneous injection	(Upton et al., 2018)
		*Ranoidea (Litoria) raniformis*	20 µg Leuprorelin	N/A	Subcutaneous injection	(Mann et al., 2010)
		*Ranoidea (Litoria) aurea*	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)
		*Litoria castanea*	hCG 20 IU/g bw	10% Trehalose + 10% DMFA	Subcutaneous injection	(Hobbs et al., unpublished)
		*Ranoidea (Litoria) caerulea*	hCG 60, 100, 300 IU total	N/A	Subcutaneous injection	(Clulow et al., 2018)
		*Ranoidea (Litoria) booroolongensis*	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		*Mixophyes fasciolatus*	hCG 60, 100, 300 IU total	N/A	Subcutaneous injection	(Clulow et al., 2018)
		*Ranoidea (Litoria) chloris*	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, GnRHa 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).

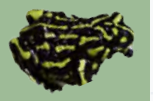
To date, most hormonally-induced sperm and egg collections have been accomplished by the implementation of empirically developed protocols, or replicating those reported successful for other species (Silla & Byrne, 2021), without further exploring if, in fact, those are the optimum protocols for new 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
	*Anaxyrus boreas boreas*	**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
	*Anaxyrus fowleri*	**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 pimozide + 0.15 µg/g P4	Browne et al., 2006a
	*Anaxyrus baxteri*	**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
	*Acris crepitans*	Administered to the water. **Ovulatory dose**: 0.17 µg/g GnRHa + MET 0.42µg/µL			Snyder et al., 2012
	*Ambystoma mexicanum*	**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
	*Ambystoma tigrinum*	**Prime 1**: 1 IU/µg hCG **Prime 2**: 2 IU/µg hCG **Ovulatory dose**: 4 IU/µzg hCG + 0.1µg/g GnRH	0 day 168 hr 24 hr		Marcec, 2016
	*Ambystoma dumerilii*	**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
	*Eleutherodactylus coqui*	**Ovulatory dose**: 10, 20, 50 µg/g GnRHa; 165 IU total hCG			Michael et al., 2004
	*Necturus beyeri*	**Ovulatory dose**: 500 µg GnRHa			Stoops et al., 2014
	*Peltophryne lemur*	**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
	*Amerana (Rana) muscosa*	**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
	*Lithobates sevosa*	**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
	*Lithobates pipiens*	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)		Progesterone, pituitary extract, Testosterone, Corticosterone, Domperidone	Trudeau et al., 2010; Wright, 1961; Fort, 2000
	*Ceratophrys ornata*	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)			Trudeau et al., 2010
	*Ceratophrys cranwelli*	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)			Trudeau et al., 2010
	*Odontophrynus americanus*	0.4 µg/g GnRHa + MET 10 µg/g (Amphiplex)			Trudeau et al., 2010
	*Ranoidea (Litoria) raniformis*	Ovulatory dose: 50 µg des-Gly10, D-Ala6-(LHRH)			Mann et al., 2010
	*Ranoidea (Litoria) aurea*	**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
	*Pseudophryne guentheri*	**Prime 1:** 0.4 µg/g GnRHa **Ovulatory dose:** 0.4 µg/g GnRHa	0 hr 26 hr		Silla, 2010
	*Pseudophryne corroboree*	**Prime 1:** 1 µg/g Lucrin (leuprorelin acetate) **Ovulatory dose:** 1 µg/g Lucrin (leuprorelin acetate)	0 hr 26 hr		Byrne & Silla, 2010
	*Pseudophryne pengilleyi*	**Ovulatory dose:** 0.5 – 2 µg/g Lucrin (leuprorelin acetate)			Silla, 2018
	*Pseudophryne bibronii*	**Prime 1:** 0.4 µg/g GnRHa **Ovulatory dose:** 2 µg/g GnRHa			Silla & Byrne, 2021 Image by Dr. Aimee Silla
	*Pseudophryne coriacea*	**Prime 1:** 0.4 µg/g GnRHa **Ovulatory dose:** 2 µg/g GnRHa			Silla & Byrne, 2021 Image by Dr. Aimee Silla
	*Heleioporus eyrei*	**Prime 1:** 0.4 µg/g GnRHa **Ovulatory dose:** 2 µg/g GnRHa			Silla & Byrne, 2021 Image by Dr. Aimee Silla
	*Lymnodynastes tasmeniensis*	**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
	*Mixophyes fasciolatus*	**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 alleganensis* (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 cryo-preserved 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.

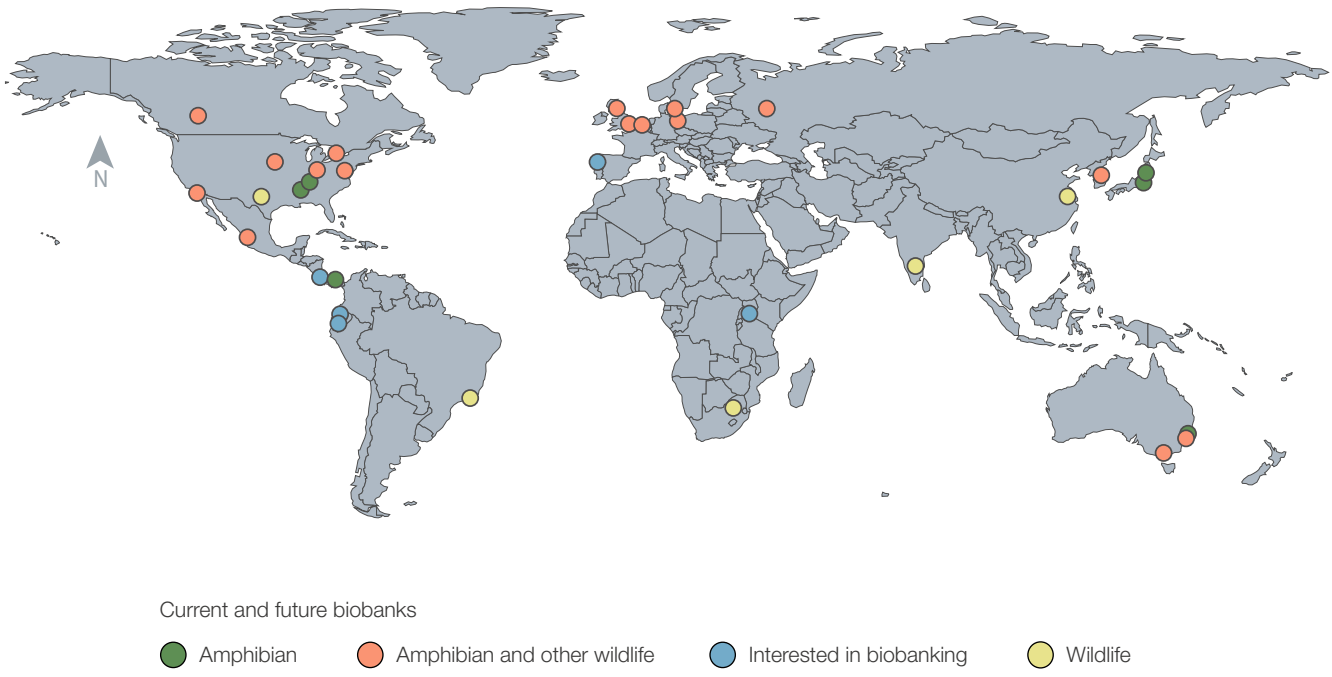


Figure 12.1: Location of known biobanks for wildlife species. Source: Data collected by the ASG Amphibian ARTs and Biobanking Working Group from a survey conducted from 2018-2021.

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.

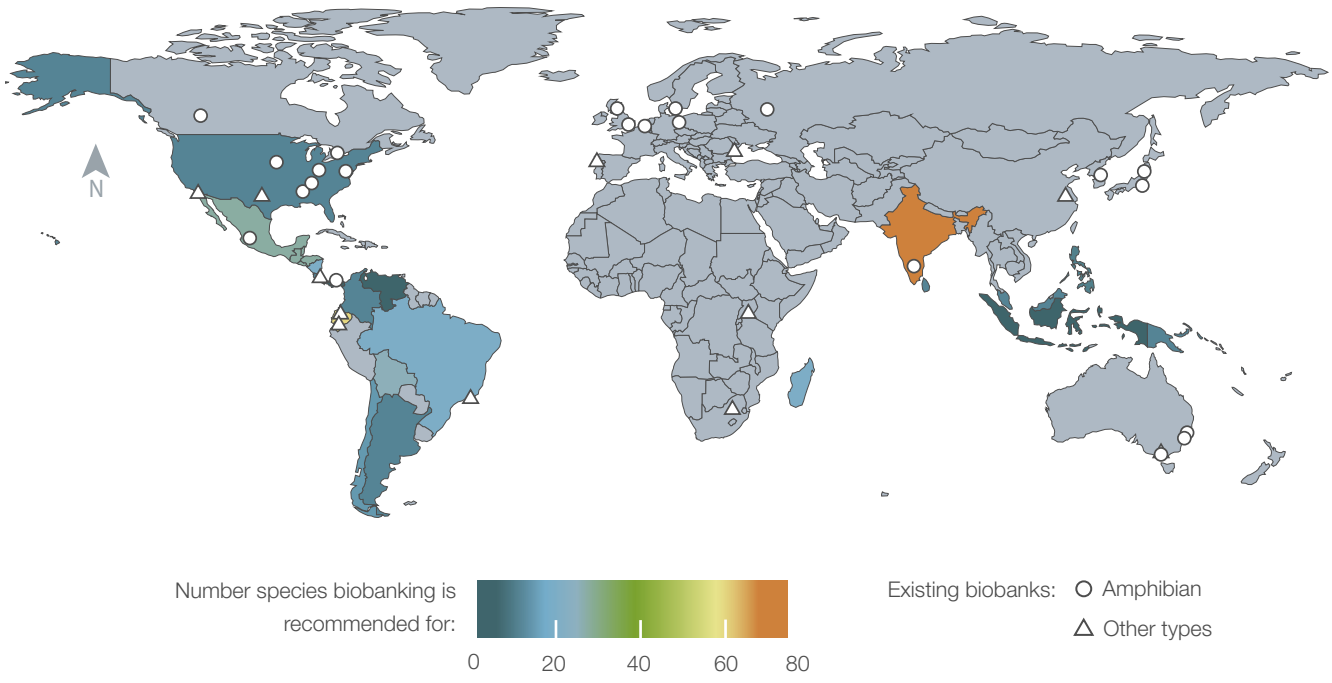


Figure 12.2: Existing biobanks containing general wildlife biomaterials or amphibian-specific samples superimposed over the regions where amphibian biobanking has been recommended by the Conservation Needs Assessments. Countries with biobanks marked with a triangle denote those with wildlife collections 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_t/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_t/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 complemental 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.

References

Amphibian-Ark. (2020). *Species Management*. Species Management. Retrieved 18 November, 2020 from https://www.amphibianark.org/conservation-programs/captive-programs/taxon-management/

Ananjeva, N.B., Uteshev, V.K., Orlov, N.L., Ryabov, S.A., Gakhova, E.N., Kaurova, S.A., ... Browne, R.K. (2017). Comparison of the Modern Reproductive Technologies for Amphibian and Reptiles. *Russian Journal of Herpertology*, *4*(24), 275–290.

Arregui, L., Bóveda, P., Gosálvez, J., & Kouba, A.J. (2020). Effect of seasonality on hormonally induced sperm in *Epidalea calamita* (Amphibia, Anura, Bufonidae) and its refrigerated and cryopreservated storage. *Aquaculture*, *529*, 735677. https://doi.org/10.1016/j.aquaculture.2020.735677

Arregui, L., Kouba, A.J., Germano, J.M., Barrios, L., Moore, M., Kouba, C.K., & Clulow, J. (2021). Fertilization potential of cold-stored Fowler's toad (*Anaxyrus fowleri*) spermatozoa: temporal changes in sperm motility based on temperature and osmolality. *Reproduction, Fertility and Development*, *34*(5), 461–469. https://doi.org/10.1071/rd21037

Beesley, S.G., Costanzo, J.P. & Lee, R.E. (1998). Cryopreservation of spermatozoa from freeze-tolerant and -intolerant Anurans. *Cryobiology*, *37*(2), 155–162. https://doi.org/10.1006/cryo.1998.2119

Bishop, P.J., Angulo, A., Lewis, L.P., Moore, R.D., Rabb, G., & García-Moreno, J. (2012). The Amphibian Extinction Crisis - what will it take to put the action into the Amphibian Conservation Action Plan? *SAPIENS*, *2*(5), 97–111.

Blackburn, H.D. (2017). Biobanking genetic material for agricultural animal species. *Annual Review of Animal Biosciences*, *6*(1), 1–14. https://doi.org/10.1146/annurev-animal-030117-014603

Blesbois, E. (2007). Current status in avian semen cryopreservation. *World's Poultry Science Journal*, *63*(2), 213–222. https://doi.org/10.1017/s0043933907001419

Bronson, E., Guy, E.L., Murphy, K.J., Barrett, K., Kouba, A.J., Poole, V., & Kouba, C.K. (2021). Influence of oviposition-inducing hormone on spawning and mortality in the endangered Panamanian golden frog (*Atelopus zeteki*). *BMC Zoology*, *6*(1), 17. https://doi.org/10.1186/s40850-021-00076-8

Browne, R., Li, H. & Vaughan, M. (2006). Sexually mediated shedding of *Myxobolus fallax* spores during spermiation of *Litoria fallax* (Anura). *Diseases of Aquatic Organisms*, *72*(1), 71–75. https://doi.org/10.3354/dao072071

Browne, R.K., Clulow, J., Mahony, M., & Clark, A. (1998). Successful recovery of motility and fertility of cryopreserved cane toad (*Bufo marinus*) sperm. *Cryobiology*, *37*(4), 339–345. https://doi.org/10.1006/cryo.1998.2129

Browne, R.K., Kaurova, S.A., Vasudevan, K., McGinnity, D., Venu, G., Gonzalez, M., ... Marcec-Greaves, R. (2022). Reproduction technologies for the sustainable management of Caudata (salamander) and Gymnophiona (caecilian) biodiversity. *Reproduction, Fertility and Development*, 34(6), 479-497. https://doi.org/10.1071/rd21356

Browne, R.K., Li, H., Seratt, J., & Kouba, A. (2006). Progesterone improves the number and quality of hormone induced Fowler toad (*Bufo fowleri*) oocytes. *Reproductive Biology and Endocrinology*, *4*(1), 3. https://doi.org/10.1186/1477-7827-4-3

Browne, R.K., Seratt, J., Vance, C., & Kouba, A. (2006). Hormonal priming, induction of ovulation and in-vitro fertilization of the endangered Wyoming toad (*Bufo baxteri*). *Reproductive Biology and Endocrinology*, 4(1), *34*. https://doi.org/10.1186/1477-7827-4-34

Browne, R.K., Silla, A.J., Upton, R., Della-Togna, G., Marcec-Greaves, R., Shishova, N.V., ... Clulow, S. (2019). Sperm collection and storage for the sustainable management of amphibian biodiversity. *Theriogenology*, *133*, 187–200. https://doi.org/10.1016/j.theriogenology.2019.03.035

Browne, R.K., Wolfram, K., García, G., Bagaturov, M.F., & Pereboom, Z.J.J.M. (2011). Zoo-based amphibian research and conservation breeding programs. *Amphibian and Reptile Conservation*, *3*(5), 1–14.

Buck, M. & Hamilton, C. (2011). The Nagoya Protocol on access to genetic resources and the fair and equitable sharing of benefits arising from their utilization to the convention on biological diversity. *Review of European Community & International Environmental Law*, *20*(1), 47–61. https://doi.org/10.1111/j.1467-9388.2011.00703.x

Burger, I.J., Chen, L.D., Lampert, S.S., Kouba, C.K., Barber, D., Smith, D., Cobos, C., Kouba, A.J. (2023) Applying sperm collection and cryopreservation protocols developed in a model amphibian to three threatened anuran species targeted for biobanking management. *Biological Conservation*. Jan 1;277:109850.

Burger, I., Julien, A.R., Kouba, A.J., Barber, D., Counsell, K.R., Pacheco, C., ... Kouba, C.K. (2021). Linking in-situ and ex-situ populations of threatened amphibians through genome banking. *Conservation Science and Practice*, *3*(11). https://doi.org/10.1111/csp2.525

Burger, I.J., Lampert, S.S., Kouba, C.K., Morin, D.J., & Kouba, A.J. (2022). Development of an amphibian sperm biobanking protocol for genetic management and population sustainability. *Conservation Physiology*, *10*(1), coac032-. https://doi.org/10.1093/conphys/coac032

Byrne, P.G., Gaitan-Espitia, J.D., & Silla, A.J. (2019). Genetic benefits of extreme sequential polyandry in a terrestrial-breeding frog. *Evolution*, *73*(9), 1972–1985. https://doi.org/10.1111/evo.13823

Byrne, P.G. & Silla, A.J. (2010). Hormonal induction of gamete release, and in-vitro fertilisation, in the critically endangered Southern Corroboree Frog, *Pseudophryne corroboree*. *Reproductive Biology and Endocrinology*, *8*(1), 144. https://doi.org/10.1186/1477-7827-8-144

Calatayud, N.E., Chai, N., Gardner, N.R., Curtis, M.J., & Stoops, M.A. (2019). Reproductive techniques for ovarian monitoring and control in amphibians. *Journal of Visualized Experiments*, *147,* e58675. https://doi.org/10.3791/58675

Calatayud, N.E., Jacobs, L.E., Williams, C.L., Steiner, C.C., & Shier, D.M. (2022). Recovering an endangered frog species through integrative reproductive technologies. *Theriogenology*, *191*, 141–152. https://doi.org/10.1016/j.theriogenology.2022.07.003

Calatayud, N.E., Langhorne, C.J., Mullen, A.C., Williams, C.L., Smith, T., Bullock, L., ... Willard, S.T. (2015). A hormone priming regimen and hibernation affect oviposition in the boreal toad (*Anaxyrus boreas boreas*). *Theriogenology*, *84*(4), 600–607. https://doi.org/10.1016/j.theriogenology.2015.04.017

Campbell, L.G., Anderson, K.A. & Marcec-Greaves, R. (2022). Topical application of hormone gonadotropin-releasing hormone (GnRH-A) stimulates reproduction in the endangered Texas blind salamander (*Eurycea rathbuni*). *Conservation Science and Practice*, *4*(3). https://doi.org/10.1111/csp2.609

Chai, N. (2016). Surgery in Amphibians. *Veterinary Clinics of North America: Exotic Animal Practice*, *19*(1), 77–95. https://doi.org/10.1016/j.cvex.2015.08.004

Chauvenet, A.L.M., Watson, J.E.M., Adams, V.M., Marco, M.D., Venter, O., Davis, K.J., ... Possingham, H.P. (2020). To achieve big wins for terrestrial conservation, prioritize protection of ecoregions closest to meeting targets. *One Earth*, *2*(5), 479–486. https://doi.org/10.1016/j.oneear.2020.04.013

Childress, W.M., Bosworth, B., Chesney, E., Walter, R.B., & Tiersch, T.R. (2019). On-site capabilities of a mobile laboratory for aquatic germplasm cryopreservation. *North American Journal of Aquaculture*, *81*(4), 349–363. https://doi.org/10.1002/naaq.10099

Childress, W.M., Caffey, R.H. & Tiersch, T.R. (2018). Design and cost analysis of a self-contained mobile laboratory for commercial-scale aquatic species cryopreservation. *Journal of the World Aquaculture society*, *49*(5), 805–826. https://doi.org/10.1111/jwas.12525

Clulow, J. & Clulow, S. (2016). Cryopreservation and other assisted reproductive technologies for the conservation of threatened amphibians and reptiles: bringing the ARTs up to speed. *Reproduction, Fertility and Development*, *28*(8), 1116–1132. https://doi.org/10.1071/rd15466

Clulow, J., Pomering, M., Herbert, D., Upton, R., Calatayud, N., Clulow, S., ... Trudeau, V.L. (2018). Differential success in obtaining gametes between male and female Australian temperate frogs by hormonal induction: A review. *General and Comparative Endocrinology*, *265*, 141–148. https://doi.org/https://doi.org/10.1016/j.ygcen.2018.05.032

Clulow, J., Upton, R., Trudeau, V.L., & Clulow, S. (2019). Amphibian assisted reproductive technologies: moving from technology to application. In P. Comizzoli, J. L. Brown, & W. H. Holt (Eds.), *Reproductive sciences in animal conservation* (pp. 413–463). Cham, Germany: Springer International Publishing. https://doi.org/10.1007/978-3-030-23633-5_14

Costanzo, J.P., Mugnano, J.A., Wehrheim, H.M., & Lee, R.E. (1998). Osmotic and freezing tolerance in spermatozoa of freeze-tolerant and -intolerant frogs. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *275*(3), R713–R719. https://doi.org/10.1152/ajpregu.1998.275.3.r713

Day, J.G., McLellan, M.R. & Curry, M.R. (1995). Cryopreservation and freeze-drying protocols. *Methods in Molecular Biology*, *38*, 189–197. https://doi.org/10.1385/0-89603-296-5:189

Della-Togna, G. (2015). *Structural and functional characterization of the panamanian golden frog (*Atelopus zeteki*) spermatoza - impact of medium osmolality and cryopreservation on motility and cell viability*. University of Maryland, College Park, USA.

Della-Togna, G., Howell, L.G., Clulow, J., Langhorne, C.J., Marcec-Greaves, R., & Calatayud, N.E. (2020). Evaluating amphibian biobanking and reproduction for captive breeding programs according to the Amphibian Conservation Action Plan objectives. *Theriogenology*, *150*, 412–431. https://doi.org/10.1016/j.theriogenology.2020.02.024

Della-Togna, G., Trudeau, V.L., Gratwicke, B., Evans, M., Augustine, L., Chia, H., ... Comizzoli, P. (2017). Effects of hormonal stimulation on the concentration and quality of excreted spermatozoa in the critically endangered Panamanian golden frog (*Atelopus zeteki*). *Theriogenology*, *91*, 27–35. https://doi.org/10.1016/j.theriogenology.2016.12.033

Farquharson, K.A., Hogg, C.J. & Grueber, C.E. (2018). A meta-analysis of birth-origin effects on reproduction in diverse captive environments. *Nature Communications*, *9*(1), 1055. https://doi.org/10.1038/s41467-018-03500-9

Figiel, C.R. (2013). Cryopreservation of sperm from the axolotl *Ambystoma mexicanum*: implications for conservation. *Herpetological Conservation and Biology*, *3*(8), 748–755.

Frankham, R., Ballou, J.D., Briscoe, D.A., & McInnes, K.H. (2002). *Introduction to Conservation Genetics*. (pp. 43–44). https://doi.org/10.1017/cbo9780511808999.004

Gascon, C., Collins, J.P., Moore, R.D., Church, D.R., McKay, J.E., & Mendelson III, J.R. (2007). *Amphibian Conservation Action Plan: proceedings IUCN/SSC Amphibian Conservation Summit 2005*. Gland, Switzerland: IUCN. https://portals.iucn.org/library/node/9071

Gillis, A. (2020). *Assisted reproductive technologies in male* Ambystoma tigrinum *with application to threatened newt species*. Mississippi State University. USA.

Gillis, A.B., Guy, E.L., Kouba, A.J., Allen, P.J., Marcec-Greaves, R.M., & Kouba, C.K. (2021). Short-term storage of tiger salamander (*Ambystoma tigrinum*) spermatozoa: The effect of collection type, temperature and time. *PLoS One*, *16*(1), e0245047. https://doi.org/10.1371/journal.pone.0245047

González, M. (2018). *Desarrollo de técnicas de criopreservación para espermatóforos y embriones de Ambystoma mexicanum para su uso en reproducción asistida*. Universidad de Guadalajara. México.

Graham, K.M., Langhorne, C.J., Vance, C.K., Willard, S.T., & Kouba, A.J. (2018). Ultrasound imaging improves hormone therapy strategies for induction of ovulation and in vitro fertilization in the endangered dusky gopher frog (*Lithobates sevosa*). *Conservation Physiology*, *6*(1), coy020-. https://doi.org/10.1093/conphys/coy020

Green, S.L., Parker, J., Davis, C., & Bouley, D.M. (2007). Ovarian hyperstimulation syndrome in gonadotropin-treated laboratory South African clawed frogs (*Xenopus laevis*). *Journal of the American Association for Laboratory Animal Sciences*, *46*(3), 64–67.

Gurdon, J.B. (1968). Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. *Development*, *20*(3), 401–414. https://doi.org/10.1242/dev.20.3.401

Gurdon, J.B. & Byrne, J.A. (2002). Principles of Cloning. *Part iii: cloning by species*, *Proceedings of the National Academy of Sciences.381952*, 281–286. https://doi.org/10.1016/b978-012174597-4.50016-8

Guy, E.L., Gillis, A.B., Kouba, A.J., Barber, D., Poole, V., Marcec-Greaves, R.M., & Kouba, C.K. (2020). Sperm collection and cryopreservation for threatened newt species. *Cryobiology*, *94*, 80–88. https://doi.org/10.1016/j.cryobiol.2020.04.005

Guy, E.L., Martin, M.W., Kouba, A.J., Cole, J.A., & Kouba, C.K. (2020). Evaluation of different temporal periods between hormone-induced ovulation attempts in the female Fowler's toad *Anaxyrus fowleri*. *Conservation Physiology*, 8(1), coz113. https://doi.org/10.1093/conphys/coz113

Harley, D., Mawson, P., Olds, L., McFadden, M., & Hogg, C. (2018). The contribution of captive-breeding in zoos to the conservation of Australia's threatened faunad. In S. Garnett, P. Latch, D. Lindenmayer, & J. Woinarski (Eds.), *Recovering Australian Threatened Species: A Book of Hope* (pp. 281–294). Clayton South, Australia: CSIRO Publishing.

Hewitt, R. & Watson, P. (2013). Defining Biobank. *Biopreservation and Biobanking*, *11*(5), 309–315. https://doi.org/10.1089/bio.2013.0042

Hinkson, K.M., Baecher, J.A. & Poo, S. (2019). Cryopreservation and hormonal induction of spermic urine in a novel species: The smooth-sided toad (*Rhaebo guttatus*). *Cryobiology*, I, 109–111. https://doi.org/10.1016/j.cryobiol.2019.05.007

Hobbs, R.J., Upton, R., Calatayud, N.E., Silla, A.J., Daly, J., McFadden, M.S., O'Brien, J.K. (2023) Cryopreservation Cooling Rate Impacts Post-Thaw Sperm Motility and Survival in *Litoria booroolongensis*. *Animals*. Sep 25;13(19):3014.

Holt, W.V., Pickard, A.R. & Prather, R.S. (2004). Wildlife conservation and reproductive cloning. *Reproduction*, I(3), 317–324. https://doi.org/10.1530/rep.1.00074

Hopkins, B.K. & Herr, Ch. (2007). Cryopreservation of frog (*Rana pipiens*) sperm cells collected by non-lethal methods. *Proceedings of the Annual Conference of the International Embryo Transfer society*, *20*, 120. https://doi.org/10.1071/rdv20n1ab78

Howell, L.G., Frankham, R., Rodger, J.C., Witt, R.R., Clulow, S., Upton, R.M.O., & Clulow, J. (2021a). Integrating biobanking minimises inbreeding and produces significant cost benefits for a threatened frog captive breeding programme. *Conservation Letters*, *14*(2). https://doi.org/10.1111/conl.12776

Howell, L.G., Mawson, P.R., Frankham, R., Rodger, J.C., Upton, R.M.O., Witt, R.R., ... Clulow, J. (2021b). Integrating biobanking could produce significant cost benefits and minimise inbreeding for Australian amphibian captive breeding programs. *Reproduction, Fertility and Development*, *33*(9), 573. https://doi.org/10.1071/rd21058

Hu, Y., Fan, H., Chen, Y., Chang, J., Zhan, X., Wu, H., ... Wei, F. (2021). Spatial patterns and conservation of genetic and phylogenetic diversity of wildlife in China. *Science Advances*, *7*(4), eabd5725. https://doi.org/10.1126/sciadv.abd5725

IUCN. (2023). *The IUCN Red List of Threatened Species*. Version 2022-2. Retrieved November 18, 2023 from https://www.iucnredlist.org.

Johnson, K. & Carrillo, L. (2022). Amphibian Conservation Needs Assessments. *WAZA News Magazine*, *22*(1), 13–16.

Julien, A.R., Kouba, A.J., Kabelik, D., Feugang, J.M., Willard, S.T., & Kouba, C.K. (2019). Nasal administration of gonadotropin releasing hormone (GnRH) elicits sperm production in Fowler's toads (*Anaxyrus fowleri*). *BMC Zoology*, *4*(1), 3. https://doi.org/10.1186/s40850-019-0040-2

Kamau, E., Fedder, B. & Winter, G. (2010). The Nagoya Protocol on Access to Genetic Resources and Benefit Sharing – what is new and what are the implications for provider and use countries and the scientific community? *Law Environment and Development Journal*, *3*(6), 248–263.

Kaurova, S.A., Uteshev, V.K., Gapeyev, A.B., Shishova, N.V., Gakhova, E.N., Browne, R.K., & Kramarova, L.I. (2021). Cryopreservation of spermatozoa obtained postmortem from the European common frog *Rana temporaria*. *Reproduction, Fertility and Development*, *33*(9), 588. https://doi.org/10.1071/rd20336

Kouba, A.J., delBarco-Trillo, J., Vance, C.K., Milam, C., & Carr, M. (2012). A comparison of human chorionic gonadotropin and luteinizing hormone releasing hormone on the induction of spermiation and amplexus in the American toad (*Anaxyrus americanus*). *Reproductive Biology and Endocrinology*, *10*(1), 59. https://doi.org/10.1186/1477-7827-10-59

Kouba, A.J., Lloyd, R.E., Houck, M.L., Silla, A.J., Calatayud, N., Trudeau, V.L., ... Della-Togna, G. (2013). Emerging trends for biobanking amphibian genetic resources: The hope, reality and challenges for the next decade. *Biological Conservation*, 164, 10–21. https://doi.org/10.1016/j.biocon.2013.03.010

Kouba, A.J. & Vance, C.K. (2009). Applied reproductive technologies and genetic resource banking for amphibian conservation. *Reproduction, Fertility and Development*, *21*(6), 719–737. https://doi.org/10.1071/rd09038

Langhorne, C. J. (2016). *Developing assisted reproductive technologies for endangered North American amphibians*. Mississippi State University.

Starkville, USA.
Langhorne, C.J., Calatayud, N.E., Kouba, C.K., Willard, S.T., Smith, T., Ryan, P.L., & Kouba, A.J. (2021). Efficacy of hormone stimulation on sperm production in an alpine amphibian (*Anaxyrus boreas boreas*) and the impact of short-term storage on sperm quality. *Zoology*, *146*, 125912. https://doi.org/10.1016/j.zool.2021.125912
Linhoff, L.J., Soorae, P., Harding, G., Donnelly, M.A., Germano, J.M., Hunter, D.A., ... Eckstut, M.E. (2021). *IUCN Guidelines for amphibian reintroductions and other conservation translocations*. Gland, Switzerland: IUCN.
Mann, R.M., Hyne, R.V. & Choung, C.B. (2010). Hormonal induction of spermiation, courting behavior and spawning in the southern bell frog, *Litoria raniformis*. *Zoo Biology*, 29(6), 774–782. https://doi.org/10.1002/zoo.20331
Mansour, N., Lahnsteiner, F. & Patzner, R.A. (2009). Optimization of the cryopreservation of African clawed frog (*Xenopus laevis*) sperm. *Theriogenology*, *72*(9), 1221–1228. https://doi.org/10.1016/j.theriogenology.2009.07.013
Mansour, N., Lahnsteiner, F. & Patzner, R.A. (2010). Motility and cryopreservation of spermatozoa of European common frog, *Rana temporaria*. *Theriogenology*, *74*(5), 724–732. https://doi.org/10.1016/j.theriogenology.2010.03.025
Mansour, N., Lahnsteiner, F. & Patzner, R.A. (2011). Collection of gametes from live axolotl, *Ambystoma mexicanum*, and standardization of in vitro fertilization. *Theriogenology*, *75*(2), 354–361. https://doi.org/10.1016/j.theriogenology.2010.09.006
Mantegazza, P. (1866). Fisiologia sullo sperma umano. *Rendic reale instit lomb*, *3*, 183–186.
Marcec, R. (2016). *Development of assisted reproductive technologies for endangered North American salamanders*. Mississippi State University. Starkville, USA.
Mastromonaco, G.F., González-Grajales, L.A., Filice, M., & Comizzoli, P. (2014). Reproductive sciences in animal conservation, progress and prospects. *Advances in Experimental Medicine and Biology*, *753*, 385–427. https://doi.org/10.1007/978-1-4939-0820-2_16
Mastromonaco, G.F. & Songsasen, N. (2020). Reproductive technologies for the conservation of wildlife and endangered species. In G. A. Presicce (Ed.), *Reproductive Technologies in Animals* (pp. 99–117). Cambridge, USA: Academic Press. https://doi.org/10.1016/b978-0-12-817107-3.00007-2
McDonough, C.E., Martin, M.W., Vance, C.K., Cole, J.A., & Kouba, A.J. (2015). Frequency of exogenous hormone therapy impacts spermiation in male Fowler's toad (*Bufo fowleri*). *Reproduction, Fertility and Development*, *28*(7), 995–1003. https://doi.org/10.1071/rd14214
McGinnity, D., Reinch, S.D., Schwartz, H., Trudeau, V., & Browne, R.K. (2022). Semen and oocyte collection, sperm cryopreservation and IVF with the threatened North American giant salamander *Cryptobranchus alleganiensis*. *Reproduction, Fertility and Development*, *34*, 470–477. https://doi.org/doi.org/10.1071/RD21035
Mendelson III, J.R., Lips, K.R., Gagliardo, R.W., Rabb, G.B., Collins, J.P., Diffendorfer, J.E., Daszak, P. D., Ibáñz D., R., Zippel, K.C., Lawson, D.P., Wright, K.M., Stuart, S.N., Gascon, C., da Silva, H.R., Burrowes, P.A., Joglar, R.L., La Marca, E., Lötters, S., du Preez, L.H., Weldon, C., … Brodie Jr., E.D. (2006). Confronting Amphibian Declines and Extinctions. *Science*, *313*(5783), 48–48. https://doi.org/10.1126/science.1128396
Michael, S.F., Buckley, C., Toro, E., Estrada, A.R., & Vincent, S. (2004). Induced ovulation and egg deposition in the direct developing anuran *Eleutherodactylus coqui*. *Reproductive Biology and Endocrinology*, *2*(1), 6. https://doi.org/10.1186/1477-7827-2-6
Mugnano, J.A., Costanzo, J.P., Beesley, S.G., & Lee, R.E. (1998). Evaluation of glycerol and dimethyl sulfoxide for the cryopreservation of spermatozoa from the wood frog (*Rana sylvatica*). *Cryo Letters*, *4*(19), 249–254.
Naranjo, R.E., Naydenova, E., Proaño-Bolaños, C., Vizuete, K., Debut, A., Arias, M.T., & Coloma, L.A. (2022). Development of assisted reproductive technologies for the conservation of *Atelopus sp*. (*spumarius* complex). *Cryobiology*, *105*, 20–31. https://doi.org/10.1016/j.cryobiol.2021.12.005
National, R.C. (2011). *Guide for the care and use of laboratory animals 8th Edition* (N. R. Council, Ed.). Washington, USA: The National Academies Press.
Norris, K. (2004). Managing threatened species: the ecological toolbox, evolutionary theory and declining-population paradigm. *Journal of Applied Ecology*, *41*(3), 413–426. https://doi.org/10.1111/j.0021-8901.2004.00910.x
Obringer, A.R., O'Brien, J.K., Saunders, R.L., Yamamoto, K., Kikuyama, S., & Roth, T.L. (2000). Characterization of the spermiation response, luteinizing hormone release and sperm quality in the American toad (*Bufo americanus*) and the endangered Wyoming toad (*Bufo baxteri*). *Reproduction, Fertility and Development*, *12*(2), 51–58. https://doi.org/10.1071/rd00056
Ogawa, A., Dake, J., Iwashina, Y., & Tokumoto, T. (2011). Induction of ovulation in *Xenopus* without hCG injection: the effect of adding steroids into the aquatic environment. *Reproductive Biology and Endocrinology*, *9*(1), 11. https://doi.org/10.1186/1477-7827-9-11
Ogielska, M. & Bartmanska, J. (2009). Oogenesis and female reproductive systems in Amphibia. In M. Ogielska (Ed.). *Reproduction of Amphibians* (pp. 153–272). Enfield, USA: Science Publishers.
Otero, Y, Calatayud, N.E., Arcia, I.D., Mariscal D., Samaniego, D., Rodríguez, D., Rodríguez, K., Guerrel, J., Ibáñez, R., Della Togna, G. (2023) Recovery and Characterization of Spermatozoa in a Neotropical, Terrestrial, Direct-Developing Riparian Frog (*Craugastor evanesco*) through Hormonal Stimulation. *Animals*. Aug 22;13(17):2689.
Peng, L., Xiao, Y. & Lui, Y. (2011). Effect of cryopreservation and short-term storage of Chinese giant salamander sperm. *Acta Hydrobiologica Sinica*, *2*(35), 325–332. https://doi.org/10.3724/sp.j.1035.2011.0
Ralls, K., Ballou, J.D. & Templeton, A. (1988). Estimates of lethal equivalents and the cost of inbreeding in mammals. *Conservation Biology*, *2*(2), 185–193. https://doi.org/10.1111/j.1523-1739.1988.tb00169.x
Rivera-Pacheco, J., Herrera-Barragán, J., León-Glaván, M., Ocampo-Cervantes, J., Pérez-Rivero, J., & Gual-Sill, F. (2021). Criopreservación espermática de *Ambystoma mexicanum* (Shaw & Nodder, 1798). *Abanico Veterinario*, *11*, 1–14. https://doi.org/10.21929/abavet2021.12
Roth, T.L., Szymanski, D.C. & Keyster, E.D. (2010). Effects of age, weight, hormones, and hibernation on breeding success in boreal toads (*Bufo boreas boreas*). *Theriogenology*, *73*(4), 501–511. https://doi.org/10.1016/j.theriogenology.2009.09.033
Rowson, A.D., Obringer, A.R. & Roth, T.L. (2001). Non-invasive treatments of luteinizing hormone-releasing hormone for inducing spermiation in American (*Bufo americanus*) and Gulf Coast (*Bufo valliceps*) toads. *Zoo Biology*, *20*(2), 63–74. https://doi.org/10.1002/zoo.1007
Ruta, C., Lambardi, M. & Ozudogru, E.A. (2020). Biobanking of vegetable genetic resources by in vitro conservation and cryopreservation. *Biodiversity and Conservation*, *29*(13), 3495–3532. https://doi.org/10.1007/s10531-020-02051-0
Sargent, M.G. & Mohun, T.J. (2005). Cryopreservation of sperm of *Xenopus laevis* and *Xenopus tropicalis*. *Genesis*, *41*(1), 41–46. https://doi.org/10.1002/gene.20092
Sherman, J.K. (1980). Human artificial insemination and semen preservation. In G. David & W. S. Wide (Eds.), *Human artificial insemination and semen preservation* (pp. 95–105). Boston, USA: Springer. https://doi.org/10.1007/978-1-4684-8824-1_16
Shishova, N.R., Uteshev, V.K., Kaurova, S.A., Browne, R.K., & Gakhova, E.N. (2011). Cryopreservation of hormonally induced sperm for the conservation of threatened amphibians with *Rana temporaria* as a model research species. *Theriogenology*, *75*(2), 220–232. https://doi.org/10.1016/j.theriogenology.2010.08.008
Shishova, N.V., Uteshev, V.K., Sirota, N.P., Kuznetsova, E.A., Kaurova, S.A., Browne, R.K., & Gakhova, E.N. (2013). The quality and fertility of sperm collected from European common frog (*Rana temporaria*) carcasses refrigerated for up to 7 days. *Zoo Biology*, *32*(4), 400–406. https://doi.org/10.1002/zoo.21070
Silla, A.J. (2010). Effects of luteinizing hormone-releasing hormone and arginine-vasotocin on the sperm-release response of Günther's Toadlet, *Pseudophryne guentheri*. *Reproductive Biology and Endocrinology*, *8*(1), 139. https://doi.org/10.1186/1477-7827-8-139
Silla, A.J. & Byrne, P.G. (2021). Hormone-induced ovulation and artificial fertilisation in four terrestrial-breeding anurans. *Reproduction, Fertility and Development*, *33*(9), 615. https://doi.org/10.1071/rd20243
Silla, A.J., Calatayud, N.E. & Trudeau, V.L. (2021). Amphibian reproductive technologies: approaches and welfare considerations. *Conservation*

Physiology, *9*(1), coab011. https://doi.org/10.1093/conphys/coab011

Silla, A.J., McFadden, M. & Byrne, P.G. (2018). Hormone-induced spawning of the critically endangered northern corroboree frog *Pseudophryne pengilleyi*. *Reproduction, Fertility and Development*, *30*(10), 1352. https://doi.org/10.1071/rd18011

Silla, A.J., McFadden, M.S. & Byrne, P.G. (2019). Hormone-induced sperm-release in the critically endangered Booroolong frog (*Litoria booroolongensis*): effects of gonadotropin-releasing hormone and human chorionic gonadotropin. *Conservation Physiology*, *7*(1), coy080. https://doi.org/10.1093/conphys/coy080

Silla, A.J., Roberts, J.D. & Byrne, P.G. (2020). The effect of injection and topical application of hCG and GnRH agonist to induce sperm-release in the roseate frog, *Geocrinia rosea*. *Conservation Physiology*, *8*(1), coaa104. https://doi.org/10.1093/conphys/coaa104

Snyder, W.E., Trudeau, V.L. & Loskutoff, N.M. (2012). A Noninvasive, transdermal absorption approach for exogenous hormone induction of spawning in the northern cricket frog, *Acris crepitans*: a model for small, endangered amphibians. *Reproduction, Fertility and Development, 25*(1), 232-233. https://doi.org/10.1071/rdv25n1ab168

Soulé, M., Gilpin, M., Conway, W., & Foose, T. (1986). The millenium ark: How long a voyage, how many staterooms, how many passengers? *Zoo Biology*, *5*(2), 101–113. https://doi.org/10.1002/zoo.1430050205

Stoops, M.A., Campbell, M.K. & Dechant, C.J. (2014). Successful captive breeding of *Necturus beyeri* through manipulation of environmental cues and exogenous hormone administration: a model for endangered *Necturus*. *Herpetological Review*, *2*(45), 251–256.

Strand, J., Thomsen, H., Jensen, J.B., Marcussen, C., Nicolajsen, T.B., Skriver, M.B., ... Pertoldi, C. (2020). Biobanking in amphibian and reptilian conservation and management: opportunities and challenges. *Conservation Genetics Resources*, *12*(4), 709–725. https://doi.org/10.1007/s12686-020-01142-y

Talbot, L.M. (1980). The World's conservation strategy. *Environmental conservation*, *7*(4), 259–268. https://doi.org/10.1017/s0376892900007955

Tiersch, T.R., Yang, H., Jenkins, J.A., & Dong, Q. (2007). Sperm cryopreservation in fish and shellfish. *Society of Reproduction and Fertility Supplement*, *65*, 493–508.

Trottier, T.M. & Armstrong, J.B. (1975). Hormonal stimulation as an aid to artificial insemination in *Ambystoma mexicanum*. *Canadian Journal of Zoology*, *53*(2), 171–173. https://doi.org/10.1139/z75-021

Trudeau, V.L., Somoza, G.M., Natale, G.S., Pauli, B., Wignall, J., Jackman, P., ... Schueler, F.W. (2010). Hormonal induction of spawning in 4 species of frogs by coinjection with a gonadotropin-releasing hormone agonist and a dopamine antagonist. *Reproductive Biology and Endocrinology*, *8*(1), 36. https://doi.org/10.1186/1477-7827-8-36

Unger, S., Mathis, A. & Wilkinson, R. (2013). A comparison of sperm health in declining and stable populations of hellbenders (*Cryptobranchus alleganiensis alleganiensis* and *C.a. bishopi*). *The American Midland Naturalist*, *170*(2), 382–392. https://doi.org/10.1674/0003-0031-170.2.382

Upton, C. (2020). Conserving natures? Co-producing payments for ecosystem services in Mongolian rangelands. *Development and change*, *51*(1), 224–252. https://doi.org/10.1111/dech.12549

Upton, R., Clulow, S., Calatayud, N.E., Colyvas, K., Seeto, R.G.Y., Wong, L.A.M., ... Clulow, J. (2021). Generation of reproductively mature offspring from the endangered green and golden bell frog *Litoria aurea* using cryopreserved spermatozoa. *Reproduction, Fertility and Development*, *33*(9), 562. https://doi.org/10.1071/rd20296

Upton, R., Clulow, S., Mahony, M.J., & Clulow, J. (2018). Generation of a sexually mature individual of the Eastern dwarf tree frog, *Litoria fallax*, from cryopreserved testicular macerates: proof of capacity of cryopreserved sperm derived offspring to complete development. *Conservation Physiology*, *6*(1), coy043-. https://doi.org/10.1093/conphys/coy043

Uteshev, V.K., Kaurova, S.A., Shishova, N.V., Stolyarov, S.D., Browne, R.K., & Gakhova, E.N. (2015). In Vitro fertilization with hormonally induced sperm and eggs from sharp-ribbed newts *Pleurodeles waltl*. *Russian Journal of Herpetology*, *22*(1), 35–40.

Uteshev, V.K., Shishova, N.V., Kaurova, S.A., Manokhin, A.A., & Gakhova, E.N. (2013). Collection and cryopreservation of hormonally induced sperm of pool frog (*Pelophylax lessonae*). *Russian Journal of Herpetology*, *2*(20), 105–109.

Uteshev, V.K., Shishova. N.V., Kaurova, S., Browne, R.K., Gakhova, E.N. (2012). Hormonal induction of spermatozoa from amphibians with *Rana temporaria* and *Bufo bufo* as anuran models. *Reproduction, Fertility and Development*, *24*(4):599-607.

Vu, M. & Trudeau, V.L. (2016). Neuroendocrine control of spawning in amphibians and its practical applications. *General and Comparative Endocrinology*, *234*, 28–39. https://doi.org/10.1016/j.ygcen.2016.03.024

Walters, E.M., Benson, J.D., Woods, E.J., & Critser, J.K. (2009). *Sperm Banking*. 1–17. Cambridge, UK: Cambridge University Press. https://doi.org/10.1017/cbo9781139193771.002

Wheater, R. (1995). World Zoo Conservation Strategy: a blueprint for zoo development. *Biodiversity & Conservation*, *4*(6), 544–552. https://doi.org/10.1007/bf00222513

Wright, K.M. & Whitaker, B.R. (2001). *Amphibian medicine and captive husbandry* (Wright, K. M., & Whitaker B. R., Eds.). Malabar, USA: Krieger Publishing Company.

Zhang, Z., Kass, J.M., Mammola, S., Koizumi, I., Li, X., Tanaka, K., ... Usio, N. (2021). Lineage-level distribution models lead to more realistic climate change predictions for a threatened crayfish. *Diversity and Distributions*, *27*(4), 684–695. https://doi.org/10.1111/ddi.13225

Zimkus, B.M., Hassapakis, C.L. & Houck, M.L. (2018). Integrating current methods for the preservation of amphibian genetic resources and viable tissues to achieve best practices for species conservation. *Amphibian & Reptile Conservation*, *2*(12), 1-27 (e165).

Zippel, K., Johnson, K., Gagliardo, R., Gibson, R., McFadden, M., Browne, R., ... Townsend, E. (2011). The Amphibian Ark a global community for ex situ conservation of amphibians. *Herpetological Conservation and Biology*, *3*(6), 340–352.

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