Review

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Review

The Sixth Mass Extinction and Amphibian Species Sustainability through Reproduction Biotechnologies, Biobanking, and Conservation Breeding Programs

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Simple Summary: Primary themes in intergenerational justice are a healthy environment, the perpetuation of Earth's biodiversity, and the sustainable management of the biosphere. These goals demand a transformative change in approaches to biodiversity management, especially when considering the predicted sixth mass extinction. Reproduction biotechnologies, biobanks, and conservation breeding programs (RBCs), enabling species restoration, provide a transformative change that can perpetuate biodiversity irrespective of environmental targets, ecosystem collapses, and other sixth mass extinction drivers. Future potentials for RBCs include assisted evolution, species restoration, and humanities extension of the biosphere into space. We provide an overview of RBCs and their future applications, focusing on genetic management, biotechnologies and their applications, and intriguing ethical and philosophical, geopolitical, cultural and social issues. We address these themes with amphibian models to introduce the MDPI Special Issue, The Sixth Mass Extinction and Species Sustainability through Reproduction Biotechnologies, Biobanking, and Conservation Breeding Programs.

Abstract: Primary themes in intergenerational justice are a healthy environment, the perpetuation of Earth's biodiversity, and the sustainable management of the biosphere. However, the current rate of species declines globally, ecosystem collapses driven by accelerating and catastrophic global heating and a plethora of other threats, preclude the ability of habitat protection alone to prevent a cascade of amphibian and other species mass extinctions. Reproduction biotechnologies, biobanking, and conservation breeding programs (RBCs), enabling species restoration, offer a critical transformative change that can economically and reliably perpetuate species irrespective of environmental targets. These capacities extend indefinitely into the future and satisfy humanities needs as the biosphere expands into space. Currently applied RBCs include the hormonal stimulation of reproduction, the collection and refrigerated storage of sperm and oocytes, sperm cryopreservation, *in vitro* fertilisation, along biobanking. Advanced biotechnologies such as assisted evolution and cloning for species adaptation or restoration have yet to be fully realised. We broaden our discussion to include genetic management, broad political and cultural engagement, and future applications. The development and application of RBCs, and particularly assisted evolution and species restoration, raise intriguing ethical, theological, and philosophical issues. We address these themes with amphibian models to introduce the MDPI Special Issue, The Sixth Mass Extinction and Species Sustainability through Reproduction Biotechnologies, Biobanking, and Conservation Breeding Programs.

Keywords: mass extinction; COP 15; COP 28; reproduction technologies; intergenerational justice; de-extinction; climate catastrophe; assisted evolution; terraforming; space colonisation

1. Introduction

The Earths biosphere is under threat from landscape modification, that along with catastrophic global heating and other threats, are driving profound changes in ecosystems globally and accelerating the sixth mass extinction [1]. COP 15, 2022, "Ecological Civilisation: Building a Shared Future for All Life on Earth", focused on direct habitat protection [2,3]. However, habitat modification even in protected habitats, driven by catastrophic global heating, exotic predators and competitors, emergent pathogens, and major losses in prey and other taxa [3,4], will inevitably result in the mass extinction of many amphibian species in the wild [5–8]. Amphibians are the most threatened vertebrate group, where ~65% of populations are decreasing with only ~40% stable; ~530 species are already Critically Endangered and 1,100 species Endangered, with these comprising ~20% of 8,450 described species [9–11]. Rapid declines are also occurring across all terrestrial vertebrates [12].

COP 28, 2023 [13], emphasised the urgency of addressing catastrophic global heating as a major factor in forcing the sixth mass extinction. Catastrophic global temperature increase from pre-industrial levels, based on Intergovernmental Panel on Climate Change (IPCC) estimates, are evidentially conservative. In 2023 the IPPC predicted a 1.10°C increase [1], whereas the actual was 1.46°C [14] and IPPC predictions for 2040 are 1.40°C [1], with alternative models predicting at least 1.85°C [14,15]. These IPPC underestimates could result from IPPC projection models including the reduction of CO_2 emissions and increasing CO_2 capture and disregarding increased heating through crossing global climate tipping points [14,15]. Unfortunately, meeting CO_2 emission reduction targets have failed for most countries with some countries increasing emissions [16]. Amelioration projections for CO_2 removal are also prohibitively costly and depend on speculative technologies and unlikely to be achieved within realistic timeframes [16–18].

Even more alarming are increasing emission of CO_2 from disastrous forest fires rapidly increasing their role as a carbon source rather than a sink [19–21], increasing sea water temperatures and acidity reduced oceanic CO_2 absorption [22,23], and predictions of the potential collapse by mid to late 21st Century of the Atlantic Meridional Overturning Circulation (AMOC) oceanic current affecting weather patterns globally, including possible season reversals in highly amphibian biodiverse major bioregions [24].

Plausibly, more than the current 1650 Endangered or Critically Endangered amphibian species will become extinct in the wild because of catastrophic global heating synergised by other threats [25,26]. Global heating will particularly affect amphibians subject to altitudinal habitat constraints [26–28], dependent on permanent stream flows or wetlands [29], or sensitive to forest destruction through fires [19–21] or elevated temperatures beneath tropical forest canopies [30]. Even under the current low IPPC global heating predictions [1], and with planned habitat protection [13], up to ~70% of remaining native vegetation structures will be significantly modified by 2070, with the loss of faunal suites, and faunal population loss is already occurring in otherwise supposedly pristine and protected ecosystems [4]. For example, bird populations have halved in large and otherwise considered pristine Ecuadorian rainforest reserves [31], and insects and other invertebrates have experiencing major populations declines globally [32]. Because biodiversity is irreplaceable, and its loss so functionally and ethically catastrophic, the precautionary principle directs that we should take the direst global heating predictions as our baseline for an immediate and emphatic response including the general adoption of RBCs for species perpetuation [33].

However, governments have not taken seriously the needs of intergenerational justice that entitles future generations to a healthy environment [34], sustainable biospheric management that includes the perpetuation of Earth's biodiversity [3,35–38], and especially addressing catastrophic global heating [39,40]. Therefore, transformative change and supportive biotechnical, political, and cultural initiatives [41,42] are needed to reduce or prevent biodiversity loss and ameliorate the sixth mass extinction [40,43–45].

These transformative approaches must embrace biotechnological advances rather than relying on optimistic stopgap, adversarial, and traditionalist approaches [3,8,41,42,46,47]. Transformative approaches are slowly gaining traction through the International Geosphere Biosphere Programme [48], the United Nations Convention on Biodiversity [49], the IUCN One Plan Approach to Conservation [50,51] and the 2024 Amphibian Conservation Action Plan [52], Amphibian Ark [53,54], and private carers CBPs [3,55,56]. These transformative approaches include interventional RBC strategies [3] and the promotion of positive societal changes [57]. Futuristic approaches include species perpetuation through long term cell and tissue storage in biobanks that for security may even be eventually secured extra terrestrially [58]. However, the 2024 ACAP was not inclusive and disregarded both the enormous potential of private carers CBPs [56], and the perpetuation of species through biobanking and restoration [3].

Reproduction biotechnologies, biobanking, and conservation breeding programs (RBCs), enabling species restoration, offer a transformative change that irrespective of environmental targets can: 1) reliably and economically maintain genotypic variation within CBPs (Section 2. [10]); 2) produce genetically adaptable individuals for repopulation, augmentation, or supplementation programs [3,10]; 3) integrate *ex situ* and *in situ* conservation [59–66]; and 4) provide the potential for species perpetuation solely in biobanks [3,58,67–73]. These interventional activities can also benefit humanity through cultural and social inclusion, economic and technological advancement, and educational opportunities [3,57,74]. Overall, RBCs offer a significant advancement in the management of biospheric sustainability and the avoidance of mass extinctions by providing a safety net for the perpetuation of Endangered and Critically Endangered species [3,59–66,72,75–77]. Sustainability interventions, including amphibian RBCs, have mainly focused on anurans (frogs and toads) and salamanders. Caecilians (Gymnophiona) are an amphibian order of special concern. Besides a general disregard of caecilians in the public and scientific arena [78], other threats include many data deficient species of unknown conservation status, a lack of autecology knowledge because of their generally subterranean habitats [79], and little research supporting the development of caecilian RBCs [61,80–82].

Two decades have passed since the potentials of amphibian reproduction biotechnologies were first published in Australia in 1999 [60], fertilisation with cryopreserved amphibian sperm was developed at in Russia [83,84], its use for fertilisation in Australia [85,86], and heterocytoplasmic cloning in Japan in 1963 [87,88]. Despite these pioneering achievements, amphibian RBCs are only implemented for a few species, primarily in wealthy western polities [89,90] or through their satellite conservation programs [91].

This geopolitical limitation creates a critical gap in amphibian conservation, as very high biodiversity countries in the global south are not provided with the necessary independent resources to develop the full potentials of RBCs. The development of biobanks representative of all Critically Endangered and Endangered species globally is also particularly lacking [3]. Nevertheless, preventing the immediate extinction of some amphibian species through RBCs has already been achieved. Between 2007 and 2017, there was an increase in amphibian CBPs of ~60%, covering 2.9% of all amphibian species. Half of these CBPs involved repopulation or augmentation, and 70% of these having some success [10,52,92–94]. RBCs using sperm biotechnologies have also benefitted the linking of *in situ* and *ex situ* conservation programs [89,90]

Beyond the geopolitical and technical aspects of amphibian RBCs, cultural factors also play a role in their widespread adoption. Scientific publications and public media should utilize an international, clear, and concise nomenclature system that empowers public engagement and optimizes search engine visibility [3]. At an international level this approach, along with oversight beyond the scientific review process, avoids regionalized jargon, misnomers, and soft euphemisms that hinder the powerful and accurate scientifically based dissemination of information [3,95–97]. These initiatives are now even extending to addressing historical injustices in species nomenclature [98]. Pioneering achievements should be properly attributed to promote global cooperation in a multipolar world (Section 3.7,4.1.).

Intriguing, philosophical and ethical considerations concerning amphibian animal welfare in RBCs have been particularly prominent in respect to the collection of cells or tissues (Section 5.) the release of individuals into potentially hostile ecosystems for repopulation or assisted gene flow (Section 2.4., [93,94]), and advanced reproduction technologies including assisted evolution or species restoration (Section 4). Despite these ethical considerations (Section 5), intergenerational justice and looming sixth mass extinctions [25,26] demand the prioritisation of biobanking to enable the opportunist restoration of species through cloning or other advanced techniques (Section 4.). Furthermore, to ensure the perpetuation of amphibian species through RBCs, a concerted effort towards international democratic engagement within the emerging multipolar world is essential. This collaboration should broadly include local communities, environmental organizations, and private carers [3,55,99–103].

In summary, this review explores the potential of RBCs through exemplifying amphibians through: Section 2) management models for genotypic variation; Section 3) an overview of RBC protocols including their utility, potentials, limitations, and research; Section 4) advanced reproduction technologies including cloning and assisted evolution; and Section 5) the ethical and philosophy aspects of RBCs to foster public trust and support. Finally in Section 6) we summarise current and future applications of amphibian RBCs and particularly the fostering community engagement and international collaboration to secure a future rich in amphibian biodiversity for the foreseeable future [3]. Overall, this review presents a hopeful outlook for the perpetuation of amphibian biodiversity through embracing current and novel biotechnologies, along with fostering international cooperation through exemplary management, and social and cultural sensitivities.

2. Genetic Diversity and Species Management

Reduced genetic diversity can lead to genotypes with reduced potential for environmental adaptation, poor health, and lowered fecundity [104–115]. A goal of the IUCN's One Plan Approach to Conservation is the maintenance of sufficient genotypic variation to provide fitness and evolutionary adaptability. It includes all agencies operating in concert to perpetuate species as both wild and captive populations or biobanked genetic resources of individuals [50,51], in a single genetic management unit called a metapopulation [116]. Therefore, a structured approach using genetic databases of both living amphibians and biobanked material combined with genetic modelling is essential for a scientifically credible RBC program [71,72,89,107,109–112,117].

Capturing the maximum genotypic variation for *ex situ* genetic management [71,89,104–107,118,119] includes the number and proportions of living male, female [110] and biobanked founders [63,66,119]. The IUCN Amphibian Population Management Guidelines recommend 25 founder pairs to represent 99.5% of the target populations genotypic variation (Figure 1. Left Panel, [109,110]). An equal number of founder males and females is preferable; however, a bias toward live females would maximise progeny production for release [120].

The number of founders and initial allelic frequency can be used to calculate the likelihood that an allele will persist in future generations (Figure 1. Right Panel, [82]). However, a greater challenge is the quantification of an allele's potential for future environmental adaptation. For example, a rare allele might be crucial for developing resistance to a new disease [121] or providing camouflage in a habitat with dramatic seasonality in the chromaticism of vegetation structure [122]. For example, the green and golden bell frog, *Litoria aurea*, a sun-basking species, inhabits wetlands subject to seasonal changes in vegetation colour from green to brown (Figure 2), with *L. aurea* varying genotypically throughout its life from pure gold to green, with most individuals a mixture (Figure 2.).

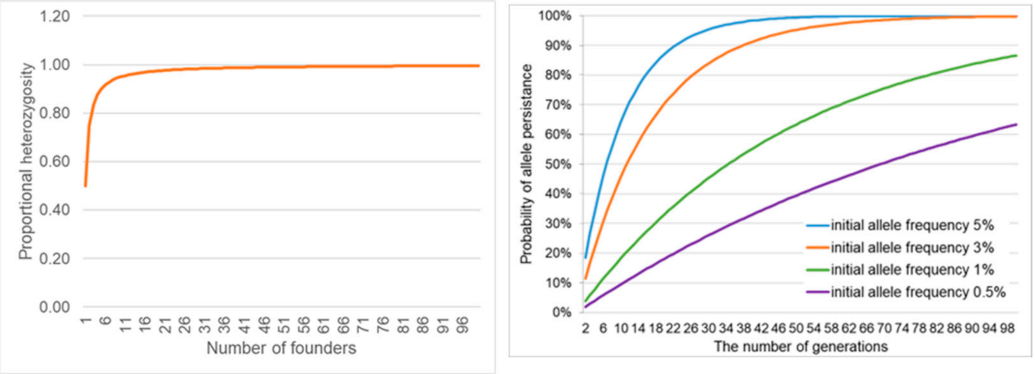

Figure 1. (Left panel) illustrates the proportional heterozygosity/genetic diversity (y-axis) through random sampling of individuals (x-axis) for a founding population. Figure 1 (right panel) shows the probability that an allele will persist in the next generation (x-axis) dependent on the number of equally representative reproducing individuals in the captive population (x-axis).

Figure 2. The green and golden bell frog, *Ranoidea* (*Litoria*) *aurea*, varies in its green and gold colouration in natural populations. Left, most common morph (image-© 2008 Dr. Peter Janzen)); Right green morph (image- © 2004 Graeme R. Gillespie).

Other approaches have presented alternatives for the population type, ratio of males to females, the application, for genetic management through RBCs or assisted gene flow between populations (Table 1. ibid).

Table 1. Other approaches to the numbers of biobanked founder individuals for maintaining *ex-situ* genetic diversity. M = male, F = female.

Population	M	F	Application	Year	Reference
Metapopulation	5	5	27 Threatened species.	2005	[123]
Metapopulation	28	25	CBP	2007	[110]
Metapopulation	25	-	CBP	2012	[124]
Fragmented/sub-pop.	10	-	AGF 4 years, depending on kinship	2022	[125]
Geographic clusters	10	20	Geographic clusters	2022	[120]
Metapopulation	20+	20+	CBP	2024	[108]

Genetic management of mating in CBPs is crucial for preventing inbreeding between closely related individuals [126]. Dependent on the species generation time and lifespan, with an initial population of 25 live males and females, to preserve 90% of genetic diversity in a CBP for 25 years requires the maintenance of a minimum number of 100 individuals with strict studbook pairing, and the 1,600 individuals with group management and random mating (Table 2., [110]). Unfortunately, despite careful studbook pairing to track the breeding history of individuals, unexpected deaths, domestic adaptations favouring survival in captive conditions, and epigenetic effects as changes in gene expression not caused by DNA alterations, can result in the loss of genetic diversity and allelic variation (Figure 1) and progeny poorly adapted to survival in the wild [106,113,114,127].

Table 2. The number of individuals to be housed in a CBP to maintain 90% of the original genetic diversity from 25 male and 25 female founders over programs of 25 years, based on age to maturity, longevity and whether individual studbook management (Individual) or group management (Group). * = Minimum population sizes of more than 100 are recommended to allow for unexpected loss of individuals [110]. # = Only group management because of short longevity.

Examples of Genera	Age to maturity	Longevity	Individual	Group
Acris	< 1 year	1 year	1590	#
Eleutherodactylus, Nectophrynoides, some Hyperoliidae	< 1 year	2-5 year	400	400
Hylidae, some Hyperoliidae, Scaphiophryne	1-5 year	< 5 years	135	265
Dendrobatidae, Typhlonectes, Tylototriton/Echinotriton, Theloderma, Cynops, Leptodactylus, Ceratobatrachus, Mantella, Atelopus	1-5 year	5-15 years	70*	140
Salamandra, some Ambystoma	1-5 year	> 15 years	60*	80*
Cryptobranchus, Andrias	> 5 years	> 15 years	45*	80*

A key role of the biobanking of cells or tissues is to increase the effective population size beyond the limited number of breeding individuals typically available in CBPs (Figure 2.). Sperm storage and artificial fertilisation, and particularly utilising banked cryopreserved sperm, are the only current biotechnologies contributing to the prevention of the gradual loss of genotypic variation in CBPs [61,63,65,66,128]. Biobanked sperm pauses the genetic ageing process within a CBP and provides a reservoir of genetic variation including rare alleles that might be crucial for future adaptation to environmental changes such as disease resistance [121], modified ecosystems [4,6], or predators [122].

The benefits of biobanking sperm are exemplified by real-world examples. Case studies involving three frog species demonstrated how using biobanked sperm for breeding (back-crossing) reduces the required size of the CBPs population, minimize inbreeding, and lowers costs when compared to maintaining large numbers of individuals [129,130]. Biobanked sperm can restore genotypic variation to highly inbred colour morphs irrespective of their genetic diversity, in private carers collections thus avoiding costly intuitional CBPs altogether and encouraging private carers engagement in species perpetuation. For instance, with the popular and highly endangered *Atelopus*, cryopreserved sperm could potentially perpetuate many species potentially through private carers that have already reproduced many species (Figure 3, [55,131]).

Figure 3. Left, *Atelopus bomolocus* (Image – © University of Kansas); Middle, *A. certus* (Image –© 2019 Brian Gratwicke); Right, *A. zeteki* (Image – © Peter Janzen).

Some publications have recommended the biobanking of all threatened amphibian species, Vulnerable, Endangered, and Critically Endangered [118,123]. However, the application of RBCs for species perpetuation should focus on the immediate needs of the current ~1,630 in total Endangered and particularly Critically Endangered species [9–11]. Vulnerable species are not in immediate danger of extinction [9,11,132] and their management should follow autecological recommendations for fieldwork, research and monitoring, and particularly habitat protection including encouraging community engagement [3,9,11,132].

2.3. Genetic Bias in Founder Sperm Collection

Minimising anthropocentric bias, terminal investment effects bias, and sperm yield bias in founder selection for sperm collection ensures the maximum representation the target population's genotypic variation [65,66]. Anthropogenic bias occurs through collectors favouring traits such as vocalisation [133], antagonism and larger size [134,135] and chromaticism [136]. However, these traits may not represent a species' adaptive genotypic variation [137–139] as they weakly associate with mating systems, habitat types, and life history [135]. Although age is associated with size and senescence theory predicts loss of sperm quality and therefore yield, no effect was found by [140].

Evolutionary trait constraint is possible through increased antagonistic inter-male competitive traits [134,135] corresponding with decreased female mate choice, along with ‘sneaker' males and delayed partial clutch fertilisation influencing fertilisation success [141]. Further research is needed to fully reveal the relationships between sperm genotypic variation [142,143], female sperm choice in fertilisation [144], and the fitness of resultant progeny [145].

Biases could also be due to genetically predisposed characteristics toward pathogens, including susceptibility to more overt parasite-mediated behaviour [146], greater size corresponding to the intensity of infection [147], or through terminal investment effects where sick or potentially dying individuals invest larger resources than usual to increase reproductive output [148]. Terminal investment effect was evident through increased spermatogenesis of males, from two anuran families, during hormonal stimulation when infected by the major threatening fungal pathogen of amphibians [148]. Furthermore, infections with parasites [147], from taxonomic groups with endangered species [149–151], also increased spermatogenesis in one species [148,150]. However, in another species male advertisement and mating outcomes were lower in fungal pathogen affected males [152]. Besides potential bias toward parasites, sperm collection through hormonal stimulation includes the possibility of lower sperm yield from less mature or more stress-prone males (See section 4.2. [153]).

2.4. Assisted Gene Flow

Assisted gene flow (AGF), where phenotypic variation is transferred between populations in the wild and in captivity, is an emerging RBC that could potentially increase population fitness but also entails considerable risks. The potential benefits of AGF are the transfer of desirable genotypes between CBPs, biobanks, and wild populations to reduce inbreeding depression [10,65,89,92,154–157]. Reduced inbreeding depression should increase reproductive capacity, evolutionary adaptability, and consequently species survivability [104–114,118,132,158–160]. These advantages are dependent on the target populations size, unique genotypic variation, whether it is a metapopulation including its fragmented sub-populations, or a genetically unique population isolated over geological periods [107,161,162]. AGF strategies toward fragmented subpopulations should utilise pooled genetic diversity from the core population [163] to avoid the diminished genetic diversity of the most divergent or fragmented populations [159,164]. In contrast, AGF should be within isolated population [165], except where historic bottlenecks have reduced genetic diversity to the extent of demonstrably reducing fitness and survivability [166,167]. Any advantages of AGF also depend on natural selective pressures toward genotypic variation that favours survival in the wild irrespective of any loss of genotypic variation [104,107,161,162].

Assisted gene flow toward wild populations can include any life stage, however, various beneficial genetic traits manifest throughout a species' life history [168]; for instance, fungal pathogen lethality varies between the tadpole and the adult stage [169]. Natural selection from early stages favours the retention of endemic genes or beneficial AGF genes along with the loss of detrimental genes [170–175]. Therefore, effective AGF strategies toward advantageous genotypes are through egg masses or early larvae. Oocytes could be sourced through on-site collection from wild gravid females, or females in CBPs, and fertilised with genetically diverse sperm [89].

Small, intermittently fragmented wild populations are targeted for AGF programs [176], with the potential benefits and risks dependent on the populations most recent fragmentation and any subsequent gene flow. However, the genetics of naturally fragmented populations are challenging to profile and are likely naturally trending toward inbreeding rather than outbreeding [177]. Therefore, AGF could reduce both fragmented and isolated population's survival through outbreeding depression and loss of alleles [177,178], the introduction of harmful genes [154,177], influencing male/female incompatibility [179,180], and through pathogen transmission [112–114,132].

Moreover, the impacts of inbreeding depression and the loss of genetic diversity on the extinction risk of amphibian populations, although widely theorised, are not evidenced on amphibians or other taxa’s declines or extinctions through reduced genetic diversity [181], and many species in thrive with very low genetic diversity [182,183]. These include island endemic species with effective population sizes of 500-1,000 individuals [183], a population size close to the theoretical minimum required to maintain genetic diversity [167]. An increasing number of species of amphibians [10,52], and other taxa [184], are also being repopulated in the wild from a few founders. These cases offer optimism for the survival of low populations of amphibians, if a supportive habitat remains for their survival, without the potential risks of AGF-supplemented genetic diversity [169,171,185].

Some large-scale repopulation programs are based on the hope that individuals develop unique genotypes that ameliorate or counteract new ecological realities such as lethal exotic pathogens [186]. However, these programs for toads [93,187] and frogs [94], have yet to result in unaugmented viable populations. Nevertheless, some initially very small natural populations persist despite the prevalence of lethal pathogens [94,169,188]; however, whether this is due to new genotypes, habitat preference or other behaviour at different life stages is uncertain [169,188]. Other programs simply bolster populations through releases with a recent emphasis toward increasing AGF [189].

The potentials for outbreeding depression [177] and pathogen transmission [190,191] should be evidenced before implementing an AGF, and there should be genetic and demographic monitoring both pre- and post-AGF [192,193]. Genetic and demographic monitoring will be a costly process over prolonged periods [167]. A major consideration before implementing costly and potentially risky AGF is that habitat loss may be the overwhelming cause of a species decline. In these cases, habitat protection, amelioration, or provision could maintain species for the time being without the risks and costs of AGF [171,183,185,194], with survival in the wild proving environmental adaptation and genetic fitness [184].

In summary, the benefits of AGF depend mainly on the genetic diversity of the target population, the translocated genotypes, the target population size, proportionate release numbers of individuals with highly beneficial genotypes, the life stage of release, and environmental selection toward the translocated genotypes. The repopulation of captive-bred individuals into the wild can also pose significant risks through outbreeding depression and pathogen transmission. Thorough planning of AGF is essential, including disease risk assessments, genetic management strategies, and post-release monitoring of demographics and population genetics including population viability analysis and computer modeling.

3. Reproduction Biotechnologies

Amphibian reproductive modes correlate with those of fish, and amphibian RBCs have reciprocal practical applications in fish reproductive management, biobanking, and CBPs [117,195–200]. Here, we provide an overview of amphibian RBCs including protocols, their application and future directions, along with the practicalities and ethics of sample collection, and address some recent historical and technical misrepresentations (also See section 5.). Amphibian sperm and oocyte collection, their refrigerated or cryopreserved storage, and artificial fertilisation only require basic laboratory facilities and animal handling procedures [201]. Whereas advanced techniques for cell culture and restoration technologies such as cloning, and assisted evolution, require sophisticated laboratory facilities and technical expertise [72]. Webinars describing details of amphibian reproduction biotechnologies (termed assisted reproductive technologies) only for the hormonal stimulation of sperm and oocytes, and *in vitro* fertilisation are available online [202].

3.1. Life Stages and Sample Collection

A generalised caution against using early life stages to provide adults for gamete collection was recently published [89]. However, the males of many anuran species mature in less than one year, and both males and females of most species in two years or less [203,204]. Nevertheless, salamanders have longer maturation periods than anurans of many years [61,205]. Average oocyte numbers show that many Endangered and Critically Endangered anuran species in the wild can provide surplus eggs, larvae, and early juveniles [26], and only a few eggs are needed for biobanking embryonic stem cells for species restoration. Different life stages of many Endangered and Critically Endangered species could also be sourced from zoos, CBPs, and private carers [52,55].

We accessed anuran oocyte numbers in Guirguis et al. [26] taken from IUCN-assessments of 4909 anurans, 575 salamanders and 84 caecilians, that included clutch size of 1611 species in total with 405 threatened species For anurans we added new data of 178 non-threatened species, then categorized the combined data, using IUCN Red List criteria, as species not-threatened, Vulnerable, Endangered, and Critically Endangered (Figure 4). However, further analysis showed that these differences were not consistent within clutch number ranges, particularly as the numbers in each category increase. The inclusion of some very large clutch sizes recorder for non-threatened species could also bias results (Table 3).

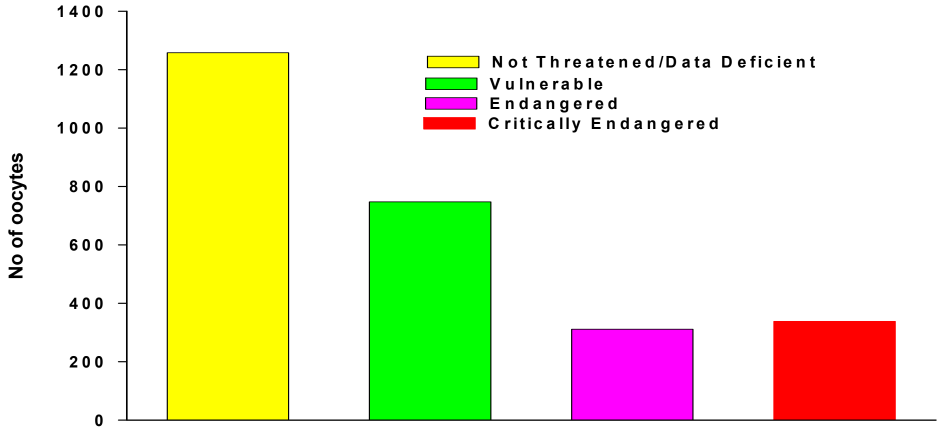

Figure 4. The average oocyte numbers per anuran clutch by IUCN Redlist endangerment status.

Table 3. The average clutch numbers of each IUCN Redlist status by range.

Range	NoT	n	VU	n	EN	n	CE	n
0-10	7	93	4	8	5	8	5	8
10-100	39	361	33	14	27	31	27	14
100-1000	410	571	483	8	394	4	272	14
1000 - 10,000	3013	328	5162	5	2466	5	4278	2
10,000 - 100,000	17980	28	NA	0	NA	0	NA	0

However, fecundity in respect to Redlist status is highly species specific. For example, *Atelopus* that include a very high proportion of Endangered and Critically Endangered species, with many species probably already extinct [206], spawn hundreds of oocytes [26]. In contrast, the 12 *Oophaga* species, with three Endangered, four Critically Endangered, and one Extinct, lay individual eggs (Figure 5., [206–208]). Both *Atelopus* and *Oophaga* species successfully reproduce in captivity and are held in private carer's collections [55]. However, although 96% of *Atelopus* species habits are protected declines and extinctions are continuing [206], management plans do not emphasise the need for biobanking and disregard the potential of private carers collections [207,208]. Nevertheless, a RBC program for *Atelopus* is underway in Ecuador [131].

Figure 5. Left, blue morph; Middle red morph; Right, yellow morph. Different colour morphs of the strawberry poison dart frog, *Oophaga pumilio*, and many other amphibian species are very popular with private carers and have a long history of domestication (Images Peter Janzen).

Vouchering, the process of preserving physical specimens, can provide valuable authenticated sources of biobanked sperm, and other cells or tissues, [209,210] through the taxonomic distinction of metapopulations [116], or genetically divergent isolated populations [194], as distinct management units [211–213]. Vouchering also contributes to amphibian conservation through the epidemiology of amphibian extinctions and declines [186], and by providing life history and ecological information required to facilitate CBPs and release programs [132]. Unfortunately, a decline in vouchering has led to impoverished museum collections that cannot even adequately address even the looming problem of global heating driven mass extinctions [212]. Fortunately, zoos are making significant contributions to biobanking and vouchering [213], but this activity could be more widely extended throughout RBCs.

3.2. Reproduction, Gamete Collection, Donor Stress, and Pathogens

The least stressful reproduction biotechnology is the simulation of their natural reproductive cues to promote reproduction [214,215]. Cues of temperature and humidity are generally intermittently circannual in temperate anurans and continuous in salamanders and tropical anurans [216,217] with precipitation generally triggering reproduction [218]. These are preferrable for animal welfare, and mostly used with most species to produce large numbers of progeny by private carers [55] and in some augmentation or repopulation programs [214]. Temperature regulated brumation can promote gonad maturation, mating and spawning responses to hormonal stimulation [64,214,219], with males and females of at least one salamander requiring different temperatures to promote reproductive maturity [220]. Sex differences in responses to environmental and social breeding cues have also been found in one anuran [221]. An alternative to mating to produce progeny is the collection of sperm to be used fresh, refrigerated for days to weeks, or cryopreserved (Section 3.7.), for the *in vitro* fertilisation of oocytes that may be stored for days to a week (Section 3.4,3.5.,3.6.)

Sperm from testicular tissues has made substantial contributions to both foundational [66,83–85,222] and recent amphibian RBC [76,179,223–225] and has the highest potential for producing large numbers of individuals for repopulation programs. An unsubstantiated bureaucratic impediment toward the use of this technique is found in the IUCN, Amphibian Conservation Action Plan 2024, "*In cases where gamete recovery is part of a conservation strategy euthanasia is not recommended*" [52] (p294).

However, sperm collection from testicular tissue should be the preferred method if males are available as it only requires euthanasia and is the least stressful technique [76,85,86,223,224] with the euthanasia of donors, dissection of testes, and the production of sperm suspensions achieved in a few minutes [179]. Testicular tissue also provides the highest sperm yield in highly concentrated sperm suspensions [61,64,66,76,85,86,222–224]. Sperm collection from testicular tissue is preferable with 1) males taken from the wild without threatening populations, 2) surplus males from CBPs [66,76,223,224], 3) vouchered males section 3.1), 4) the collection of large high-quality sperm yields from small and especially very small amphibians [61,95], 5) mortalities after strict pathogen screening [190,191], 6) the testing of multiple female/male compatibilities [179], 7) to produce quantities of concentrated sperm suspensions of consistent pH and osmolarity [179], and 8) essential for species recalcitrant to hormonal stimulation [10,226].

Hormonal stimulation, (misnamed hormone therapy is some amphibian RBC literature [227]), does not require euthanasia but generally requires injection, and is a more stressful and less efficient alternative to sperm collection from testicular tissue, and has been a popular research topic for more than 40 species [52,65]. In some species hormonal stimulation only yields low sperm numbers, and concentrations, of inferior sperm quality [10,226] further limiting its utility. An instance of hormone stimulation producing extremely low sperm concentrations of poor-quality sperm is found with the Endangered frog, *Leiopelma hamiltoni*, in the phylogenetically basal Anura family Leiopelmatidae, (Figure 6., [226]). A similar response is also likely with *Le. archeyi*, the highest priority amphibian species on the Zoological Society of London's Evolutionary Distinct and Globally Endangered (EDGE) list [196].

Unfortunately, many studies of hormonal stimulation to collect sperm do not present the sperm yields as the number of sperm making it difficult to assess the utility of this technique for the target species [52,65]. Nevertheless, even if not generally practicable for large scale progeny production with some species and particularly small amphibians, hormonal stimulation can yield sperm quantities, whether motile or immotile, that are suitable for intracytoplasmic sperm injection (ICSI) [228–232], and low sperm yields of fertile sperm can contribute to biobanks or the production of larvae that then can be raised to maturity to produce large numbers of progeny for release [3].

Figure 6. Hamilton’s frog (*Leiopelma hamiltoni*). Image by Phil Bishop. Attribution ShareAlike 2.5.https://en.wikipedia.org/wiki/Hamilton%27s_frog#/media/File:Leiopelma_hamiltoni02.jpg Phil Bishop, CC BY-SA 2.5 <https://creativecommons.org/licenses/by-sa/2.5>, via Wikimedia Commons.

After hormonal stimulation some anuran species will spontaneously express sperm in urine [233–235] or a cannula or a pipette tip can be inserted into the urethra [10,236]. Salamander sperm can be collected after hormonal stimulation in pre-spermatophores or in gelatinous fluid [237,238], spermic urine [237,238] or semen collected by massaging the lower oviduct and cloaca [205,237,239,240], or through the natural deposition of spermatophores [66]. Very large quantities of hormonally stimulated giant salamanders (Cryptobranchidae, *Cryptobranchus alleganiensis*) hormonally stimulated semen is readily stripped in aquaculture and in the development of RBCs [205].

However, hormonal stimulation using injection is exceptionally stressful to small delicate salamanders [239] including the highly threatened plethodontids that comprise 65% of salamander species [61,239]. Alternative methods for hormonal stimulation of spermiation in small delicate salamanders or anurans include topical or nasal application [61,241,242]. The more robust body shape and consequently lower skin surface to body mass ratio could make small anurans less amenable to the topical or nasal application of hormones than salamanders of the same body weight. On the other hand, anurans have a patch of skin in the ventral pelvic region for absorbing aqueous solutions [243]. Spermiation was stimulated using hormonally injected crickets force fed to salamanders, termed non-invasive oral bioencapsulation [244]. This novel technique offers the potential to avoid the stress of hormone stimulation entirely and worked in one publication for both large and small salamanders [244].

Females of both anurans and salamanders are generally more recalcitrant to hormonal stimulation for oocyte collection, than males for spermiation, and often require priming doses [64,233,237,245–247]. Mature oocytes can then be collected ovarian excision [85], abdominal massage, or cannulation [61–64,237,248,249] Improved targeting of hormonal stimulation has been achieved through ultrasound identifying mature ovaries in both anurans [250] and salamanders [234,237]. Ovarian excision and spawning into physiological saline (Section 3.6.), are low stress techniques for the sampling of large numbers of oocytes [233,247]. Unseasonal hormonal stimulation of oocytes did not affect oocyte quality in one cool-temperate species [251].

An important factor in selecting gamete collection methods is pathogen transmission [190,191]. Testicular sperm may be contaminated by pathogens such as internal parasites and viruses [147,149], whereas hormonally stimulated sperm in various forms may additionally contaminated by bacteria, fungi and external parasites [252].

3.3. Sperm Qualities

Comparative evaluation of spermatological techniques through sperm quality metrics is confounded by inconsistencies in methods and terminology. Key sperm quality metrics are motility, membrane and DNA integrity [65,66,140,195,240,253–257]. Basic metrics of sperm motility are the percentages of immotile sperm, activated sperm as non-swimming motion, swimming sperm, and sperm speed. Sperm motility is subjectively assessed by observation [85], however, meaningful comparative studies need computer-aided sperm assessment (CASA) to give exact percentages and speeds of swimming sperm, along with other motility vectors for more precise analysis [66,258]. Damages to sperm morphology assessed by observation or vital stains are the integrity of acrosomes [176] and mitochondrial collars or sheaths [257,259,260] (misnamed in some in the amphibian RBC literature as ‘mitochondrial vesicles’ [259,261]). DNA integrity is assessed through comet assays and other tests [219,255,256], with other biochemical tests sometimes used for amphibians [253].

3.4. Refrigerated Storage of Sperm

Refrigerated storage at 0-4°C is a useful technique for the short-term storage of amphibian sperm. Refrigerated storage enables delayed *in vitro* fertilisation when there is asynchrony between sperm collection and oocyte availability [253], or for transport between breeding groups [89]. Refrigerated storage for ~10 min. can acclimate sperm to cryoprotectants [257] and be used to delay cryopreservation for days to weeks [66,86]. Refrigerated storage of anuran sperm is highly successful in cadavers [219,235],whole testes [86], macerated testicular tissue [86], and in spermic urine [65]. However, refrigerated salamander sperm only remains motile for a day to several days [61,66], except for weeks when held in spermatophores [262]. Oxygenation [263,264] as well as the addition of antibiotics to reduce bacterial concentrations, improves sperm storage periods while maintaining sperm concentrations and increasing biosecurity [65,263–267].

3.5. Sperm Cryopreservation and Freeze Drying

Cryopreservation is a freezing process that protects sperm using special solutions to create sperm cryosuspension and tailored freezing rates. The cryopreserved sperm can then be held indefinitely in biobanks [65,66,268]. Sperm cryopreservation uses aqueous formulations, termed cryodiluents, that when mixed with sperm to produce cryosuspensions that are then frozen [65,66]. Cryodiluents are formulated from sperm penetrating cryoprotectants such as dimethyl sulphoxide (DMSO, [83,84] or dimethylformamide (DMFA, [219,257]), and non-penetrating saccharides or salts, with supplements such as buffers, fetal bovine serum, and anti-biotics [65,66]. Fertilisation was first achieved with cryopreserved anuran testicular sperm in 1996 using DMSO [83], and with hormonally stimulated sperm in 2011 using DMFA t hen a novel cryoprotectant to amphibians [257]). Sperm of has also been freeze-dried and remain viable, then stored at room temperature [269]., but this technique has not been trailed amphibians either for fertilisation [61,66] or for ICSI [228–231]

A broad canvas of the RBC community in 2019 recommended 5-10% (v/v) DMSO or DMFA and 1-10% (w/v) saccharide as cryoprotectants with slow to moderate cooling rates [66]. Nevertheless, ranges of 12-15% DMSO have proved successful with anurans [83,84,86,219,223,224,257], with 12% DMFA proving superior to DMSO with urinal sperm [257] and consequently used in other studies [258,268,270]. Both fast and slow cooling rates have recently been successful [65,66,258]. Cryosuspension osmolarity on cryopreservation in some studies has shown a significant effect [223] while other studies have shown much less effect [270]. Freezing of sperm over a range of freezing rates can be achieved by constructing a simple and inexpensive device [271], with a comparative description of cooling rate impacts in [258].

The broad differences in successful cryopreservation protocols, even within the same species may be due to subtle variations in techniques for cryoprotectant penetration, freezing regimes, the thawing and the washing of cryoprotectants from cells [65,66], seasonal effects on sperm quality [272], *in vitro* fertilisation techniques lacking meaningful baselines (See 5.9.), unconcise sperm quality metrics (Section 3.3.,4.3.), and sperm genotypic and phenotypic characteristics [115,268].

Little difference was shown between the cryoresistance of motile urinal sperm or sperm from testicular tissues [270].

3.6. Oocyte Storage

The storage of oocytes enables delayed *in vitro* fertilisation if there is asynchrony between the availability of oocytes and sperm [65,66,273]. Because of their high yolk content and large size, the cryopreservation of anuran oocytes is not yet practicable [274]. The viability period of unfrozen oocytes during storage depends on the species' natural spawning temperature, the storage temperature, and the oocytes osmotic, ionic, and gaseous environment [64,86,222,275–277]. Early conventions for oocyte storage assumed that ionic formulations slowed oocyte gel hydration (described as hardening) that blocked sperm penetration [148,276,277], however, research over a broader range of species showed that hydrated oocytes of some species are fertilisable for an hour or more [222]. Unhydrated and refrigerated ovarian oocytes and post ovarian oocytes of cool temperate species have remained viable for many days [275] with a pressurised gaseous environment further extended storage life [278].

Oocyte storage is highly dependent on the target species natural spawning temperature, where the oocytes of species spawning in cold water may remain viable for many days [275] and tropical or subtropical species only for hours [86,276,277]. Studies with a single species showed that a percentage of post-ovarian oocytes undergo a very rapid loss of fertility [179]; possibly a female sperm choice mechanism for greater progeny heterozygosity with polyandrous species [279], or to maintain sub-population environmental adaptability where female/male genetic incompatibility lowered fertilisation rates in anurans with little dispersal ability [179,180]. In other species, some oocytes resist fertilisation during spawning, leaving fertilisable oocytes for later fertilisation [141]. However, the physiological or genetic mechanisms behind selective oocyte fertility loss over time, and any subsequent genetic biases toward progeny, are undetermined. Therefore, besides species specific temperature effects, the mechanisms for the loss of oocyte viability in aqueous solutions generally appears to be through the diffusion of ions or proteins from the oocyte gel needed for sperm motility or for oocyte metabolism [280–283] rather than gel hydration [276,277]. Specific genotypic mechanisms also influence the storage period and even extend to individual oocytes [179].

The cryopreservation of amphibian oocytes or early embryos has no parallel with techniques used for mammalian oocytes, which are very small, ~0.08-0.20 mm diam., and have a miniscule amount of egg yolk [284]. However, the cryopreservation of amphibian oocytes or embryos includes many parallels with fishes [197,198,274] due to both possessing highly fatty and structured egg yolk and oocytes sizes. Nevertheless, the success of cryopreservation techniques for fish embryos up to 0.8 mm diam. [197] are challenging to apply to the large size of more than 95% of amphibian oocytes that are over 0.9 mm diam. (Figure 7.). Therefore, the currently most the most promising techniques to perpetuate the amphibian female genome is using cryopreserved biomaterial for heterocytoplasmic cloning (Table 4, ibid) or stem cells to generate ovaries in surrogate species (Section 5. [274]).

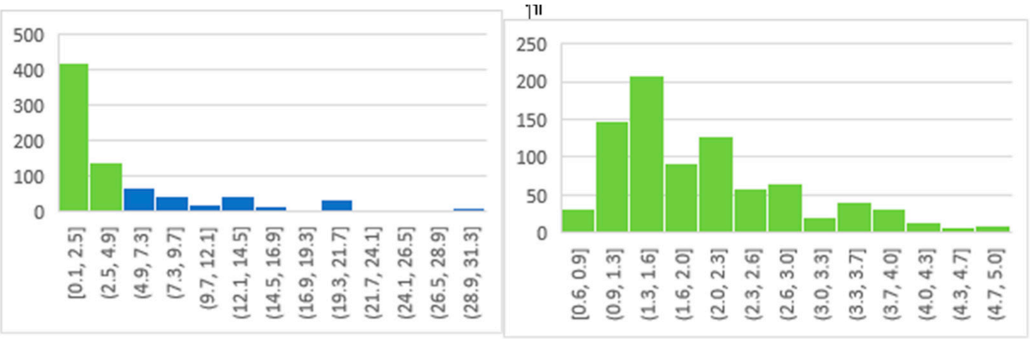

Figure 7. The x-axis shows the number of anuran species, and the y-axis shows oocyte volumes (mm3). Left. 805 species with oocyte diameters to 4.9 mm and volumes to 31.3 mm^3. (b) Right. ~430 of these 805 species from the first two bars in the left figure with oocyte diameters ≤ 1.8 mm and volumes ≤ 5.0 mm^3.

3.7. Artificial Fertilisation; Sperm Concentrations, Fertilisation Periods, and Rates

The critical end point of artificial fertilisation comparing sperm concentrations and quality, and fertilisation rates, are subject to major limitations in techniques, terminologies, and in historic attribution. The term “artificial fertilisation" is generic and not only includes *in vitro* fertilisation where sperm is placed over oocytes as presented in [179,273,285], but also artificial insemination with sperm placed internally [286], and intracytoplasmic sperm injection (ICSI) with sperm placed into an oocyte [228–231]. *In vitro* fertilisation is the critical end point of much of amphibian RBC research and has been achieved with scores of anuran species, and tens of salamander species [52], but artificial insemination has been trialed with only one salamander species [286].

The first book devoted to amphibian RBCs published in 2022 in "*Historical Perspectives on the Development of Amphibian Reproductive Technologies for Conservation*” ([273], p1) stated that *in vitro* fertilisation in amphibian RBCs was ‘dry fertilisation’, and attributed “dry fertilisation” as a pioneering experimental achievement, to Rugh, 1961, in the USA [287]. Historically, dry fertilisation was pioneered in 1856 in Russia where inactivated fish sperm was mixed with oocytes then after a short period the sperm was activated with water to achieve fertilisation [288]. In contrast, Rugh first published the placement of activated sperm on anuran oocytes in 1934 [289]. This technique was then furthered in other early studies [290,291], first used in amphibian RBCs in Russia in 1996 [84] and subsequently generalised in amphibian RBCs [219,233–235,247].

Despite being subject to so many publications [52] our knowledge of *in vitro* fertilisation is limited by a lack in publications of comparable sperm quality metrics (Section 4.3.), sperm concentrations and fertilisation periods [85,222,257,292,293], and sperm activation and fertility in different osmotic environments [65,66,225,240,293,294]. The effect of osmolarity can even extend to osmotic environment of donor males [294]. Fertilisation curves are the end point of sperm quality; however, few publications have included amphibian fertilisation curves; first presented in the general scientific literature in 1975 [292] and the RBC literature in 1998 [86]. Furthermore, the fertilisation period in most amphibian studies is 10 min. and refers to concentrated sperm suspensions [86]. However, comprehensive fertilisation curves at different sperm concentrations show that fertilisation rates with *Rhinella (Bufo) arenarum* increased for at least 30 min. post application, with post application period more influential than linear sperm concentrations on fertilisation rates (Figure 8. [292]).

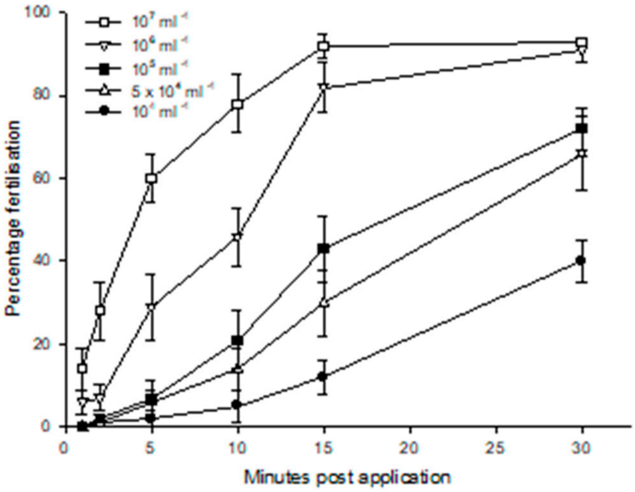

Figure 8. *Rhinella (Bufo) arenarum* (Bufonidae) fresh testicular sperm concentrations, the percentage in vitro fertilisation, and minutes post-application before washing with fresh water [292].

Other fertilisation curves show saturated fertilisation with sperm from testicular tissue at concentrations of 2.5 x 10^5 ml^{-1} after 60 min. [293], ~10^4 ml^{-1} after 60 min. [222], ~10^6 ml^{-1} after 10 min. [86]), and 10^{5-7} ml^{-1} after 60 min. [295]. Fertilisation rate in general will also depend on the relatively short motility period, and lower quality, of cryopreserved sperm compared with fresh sperm [296]. For instance, after 15 min. application *Rana temporaria* spermic urine at ~10^6 ml^{-1} provided ~45% fertilisation while testicular sperm provided only ~2% fertilisation. Spermic urine achieved saturated fertilisation at ~10^7 ml^{-1} but testicular sperm only at ~10^8 ml-1 [257].

The need for robust fertilisation curves to compare fertilisation rates was exemplified through much higher fertilisation rates than expected with cryopreserved urinal *Anaxyrus (Bufo) fowleri* sperm. Control fresh swimming sperm provided ~85% saturated fertilisation at ~4.2 × 10^6 ml^{-1} concentration, whereas after cryopreservation swimming sperm at only ~2% concentration of the control provided ~20% fertilisation [297], even though the fertility of cryopreserved sperm is lower than fresh sperm [257,270]. A robust fertilisation curve could explain this apparent enigma, and in general provide baselines for meaningful comparisons between all studies.

4. Advanced Reproduction Biotechnologies (aARBs)

Advanced Reproduction Biotechnologies (aARBs) can perpetuate species through the cryopreservation of cultured or uncultured somatic cells [298] with subsequent cloning [89,198,274,284,299,300], or stem cells used for the generation of gametes in surrogate species [67,70,71,274]. The generation of gametes includes the implantation of cryopreserved immature ovarian follicles, primordial germ cells, or induced pluripotent stem cells to generate chimaeras [63,67,274]. These techniques were first conceptually introduced to the amphibian RBCs literature in 1999 [60].

4.1. Cloning

Heterocytoplasmic cloning enables species restoration solely from biobanked nuclei through surrogate species [89,198,274,284,299,300], and the critical need to develop heterocytoplasmic cloning has not received the attention it deserves in amphibian RBCs. To perpetuate amphibian biodiversity, heterocytoplasmic clones must develop to reproductively mature and fertile females to produce oocytes for fertilisation with cryopreserved sperm. Homocytoplasmic anuran clones from embryonic cells developed to late blastula as early as 1952 [301], with heterocytoplasmic cloning first accomplished with anurans in Japan and with salamanders in France (Table 5., ibid). Heterocytoplasmic clones then developed to the gastrula stage in 1957 [302], early neurula in 1958

[303], adults in 1961 [304], with naturally mating and spawning adults producing viable offspring in 1963 [87,88,305], second and third generations from 1963 to 1972 [306,307], and from as early as 1971/1972 adult heterocytoplasmic clones were produced from a wide range of other anurans [308,309], and also from salamanders (*Pleurodeles* sp.) (Table 4. ibid; [310,311]. In 1998 nuclei from cryopreserved totipotent cells developed to the gastrula stage [300] using the techniques of [299]. These foundational studies and the extraordinary rate of the development of de-extinction (species restoration) projects for other taxon relying on cloning [312], herald a new age in amphibian cloning that will lead species perpetuation solely from cryopreserved nuclei. This will greatly reduce the need for CBPs for species unable to survive in the wild, or CBPs for species of little cultural interest. This advance will not only relieve some of the financial burden through maintaining live amphibians but also have considerable animal welfare benefits.

Table 4. Heterocytoplasmic SCNT cloning in amphibians.

Nucleus donors	Recipients	Results	Year	Ref
Rana pipiens	*Aquarana* (*Rana*) *catesbeiana*	Died late blastula	1952	[301]
R. n. brevipoda	*R.n. nigromaculata*	Metamorphs	1957	[302]
R. pipiens	*R. sylvatica*	Late blastula/early neurula	1958	[303]
R. n. nigromaculata	*R. n. brevipoda*	Adults	1961	[304]
R. pipiens	*R. palustris*	Post-neurula	1963	[305]
R. n nigromaculata	*R. n. brevipoda*	Adults –F1 reproduction	1963	[87,88]
R. japonica	*R. ornativentris*	F2	1963	[306]
R. nigromaculata *R. temporaria*	*R. brevipoda* *R. japonica*	F3.	1972	[307]
R. japonica *R. temporaria*	*R. temporaria* *R. japonica*	Adults	1972	[308]
R. brevipoda *R. plancyi* *R. brevipoda* *R. esculenta*	*R. plancyi* *R. brevipoda* *R. esculenta* *R. brevipoda*	Adults	1972	[309]
Pleurodeles waltlii *P. poireti*	*P. poireti* *P. waltlii*	Adults	1971	[310]
P. waltlii *P. poireti*	*P. poireti* *P. waltlii*	Adults	1972	[311]

The first book devoted to amphibian RBCs published in 2022 in "*Historical Perspectives on the Development of Amphibian Reproductive Technologies for Conservation*" ([273], p1) attributed "*one of the most noteworthy and groundbreaking accomplishments toward amphibian reproductive technologies*" to Gurdon's, 1962, publication of amphibian homocytoplasmic cloning in the USA [313], with this accomplishment was also attributed to Gurdon receiving a Noble Prize in 2012 [314]. However, Gurdon's homocytoplasmic cloning was not pioneering and extended Briggs and King's homocytoplasmic cloning of amphibians in 1952 [301], neither concerned heterocytoplasmic cloning a technique essential for amphibian species perpetuation [273]. Furthermore, the 2012 Nobel Prize for Physiology or Medicine was awarded jointly between John Gurdon and Shinya Yamanaka [314], not for cloning, but for the reprogramming of nuclei to pluripotency [314], as discussed by Gurdon in [315] his acceptance speech "The Egg and the Nucleus: A Battle for Supremacy" [316].

4.2. Assisted Evolution

Traditional methods of assisted evolution are the selection of desirable traits in domestic species [317] as found in amphibians with private carers coloured morphs [55]. Other assisted evolution techniques with amphibians include the natural selection of beneficial traits through mass releases [93,94], and approaches toward genetic engineering to provide pathogen immunity or adaptability to the climate crisis [52,119,318,319]. In contrast to most amphibians, the benefits of assisted evolution in mammals are challenged by their low reproductive rates [72]. Nevertheless, assisted evolution is a crucial strategy to select or increase the amphibian genotypic variation or for species restoration as amphibians are highly amenable to RBCs [3,52,67].

5. Ethics and Communication

By addressing ethical principles, communication challenges, and cultural normalization, we unlock the tremendous potential of RBCs to fulfil our obligations toward intergenerational justice [3,35–37]. However, there are few publications concerning the ethical standards of amphibian RBCs [52,320]. The ultimate standard for the ethical treatment of all animals is "*The physical and psychological well-being of an animal* (sic, in captivity). *It is good or high if the individual is fit, healthy, free to express natural behavior, free from suffering and in a state of wellbeing*." [215].

Ethical principles dictate that RBC practitioners are moral agents responsible for the wellbeing of their moral subjects, the species of concern [321,322]. Practitioners' ethical motivation towards amphibian RBCs is driven by a responsibility to perpetuate amphibian biodiversity while avoiding unnecessary harm [52]. Practitioners are guided by the precept, "*How would I like it if I were them?*" an ethical principal present in all cultures with ethical traditions [322]. RBC animal ethics standards include amphibians possessing sentience through the cognition of stimuli without association or interpretation [322–324], standard that can even extend to invertebrates [325].

In practice, the ethical use of RBCs depends on a balance between 1) the sentience of the target individuals [321,326], 2) the species' role as a biospheric entity thereby benefiting other moral subjects [327]; 3) support for intergenerational justice toward the environment [35–37], and 4) the principle of the greatest good for the greatest number as applied to the species perpetuation [328]. Ethical considerations depend on the reason for conducting the technique, its efficiency, general applicability, and associated stress to the targeted individuals (Table 6., ibid). Once an RBC protocol or program using animals is justified the three basic animal ethics principles of refinement, reduction, and replacement, in research or application can be applied [329–331].

Refinement can be achieved through procedures that minimize stress and pain during techniques to collect sperm and oocytes (Section 3.2, [244,320]), and avoiding hormonal stimulation by providing habitat simulations in CBP to promote mating and spawning [201,215]. Research regarding amphibian stress during confinement and handling will further guide the research and application of amphibian RBCs [153]. Reduction achieved through reducing the number of individuals in CBPs through using stored sperm, and by avoiding extensive hormonal stimulation trials to optimise hormonal sperm collection, when alternatively large yields of sperm could be obtained from testicular tissues (Section 3.2.). Replacement by programs maintaining genotypic variation through biobanking of sperm from any Endangered or Critically Endangered species for later use (Section 3.2., [3,65,66]). Replacement can also be achieved by genetic and demographic modelling to effectively reduce the required number of individuals to manage CBPs, biobanking, supplementation, repopulation, or assisted gene flow programs (Section 2.4.).

Biotechnologies for sperm collection have mainly focused on hormonal stimulation of sperm or oocytes, with research involving more than 40 species [52,320]. This high stress technique, and especially with small amphibians, generally only yields low sperm numbers and concentrations [226]. Furthermore dose-response curves for two hormones are recommended to optimise this technique [10], requiring at least 32 individuals being subject to hormonal stimulation, and the use of cannulation to collect sperm with most species. However, if mature males are available high sperm yields can be collected through the reliable low stress technique of testicular tissue to provide the sperm numbers for the fertilisation of large numbers of oocytes (Section 3.2). In either case even a few biobanked sperm can provide genotypic variation or produce mature adults to provide large numbers of progeny.

Nevertheless, the collection of spermic urine through cannulation is critical for the progress of the basic science of spermatology to provide sperm that are known to be equivalent to those expressed in natural spawning [257]. The comparative quality in amphibian RBCs is dependent on the basic science of the sperm structure, physiology and motility mechanisms particularly in respect to viscosities found in internally fertilising amphibians [61] and the physical structure of oocyte gel and female sperm choice mechanisms [279]. In many cases these advances can only be achieved using sperm from spermic urine, and cannulation is the best procedure to obtain this sperm in many species. Institutional animal ethics requirements should be sympathetic toward this critical need for the perpetuation of amphibian biodiversity.

Table 5. Ethical considerations with techniques for the natural or induced mating and spawning, and the collection of sperm or oocytes. .

Technique	Injection	Application stress	Collection technique	Collection stress	*Yield	Donor Size limitation	References
			Mating and spawning				
Natural mating and spawning	None	None	Fertilised eggs	None	Very high	None	[214,215]
Hormonally induced mating and spawning	Yes	Moderate	Fertilised eggs	None	Very high	None	[64,214]
			Oocyte collection				
Hormonal stimulation oocytes with spawning into solutions	Yes	Moderate	Spawning into physiological saline	Low	High to very high	None	[233,247]
Hormonally stimulated ovarian oocytes	Yes	Moderate	Abdominal massage or cannulation	High	Low	Avoid for small species	[64,233,237,245–247]
Excision of hormonally stimulated ovarian oocytes	Yes	Moderate	Direct sampling	None	Very high	None	[233,247].
			Sperm collection				
Collection from testicular tissue	Yes	Moderate	Macerating testicular tissue	None	Very High	None	[61,64,66,76,85,86, 222–224]
Hormonal stimulation sperm	Yes	Moderate	Urination	Moderate	High to none	Avoid for small species	[233,247]
Hormonal stimulation sperm	Yes	Moderate	Cannulation	High	Moderate - very low	Avoid for small species	[52,65]
Hormonal stimulation sperm - topical, nasal, oral, or food item	None	Low	Cannulation	High	Moderate - very low	Possibly for small species	[61,241,242]

Besides animal welfare, the ethics of amphibian RBCs extend to human/animal interactions through emotional and social engagement and the broad ecological impacts of repopulations or translocations [3,71,72,323,324,332]. Furthermore, speciesism, where one species is placed as morally more important than other species is shown where target species are selected on political or institutional rather than phylogenetic grounds [196]. There is considerable engagement of the theological community in the need to support biodiversity conservation [333] and avoiding language that may be theologically misinterpreted such as resurrection [75] for species restoration, and playing God helps to ensure broader public support [75,334–336]. Theologies can also contribute to biospheric sustainability through social and ethical models that respecting nature and consequently the activities of their members [337]. Although subject to ethical scrutiny the popularity of species restoration is shown by the global reach of the Colossus de-extinction project [311], with its potential for biospheric sustainability and profound community outreach [3,332].

6. Current and Future Application

The development of amphibian RBCs has progressed to the collection and storage of anuran and salamander sperm and oocytes and their use for *in vitro* fertilisation to produce sexually mature adults [61,76,77,224]. These RBCs can support either institutional or private CBPs, along with repopulation or translocation programs [3], and assisted gene flow (Section 2.4.) However, costly programs for wild populations should be highly targeted toward ecologically, phylogenetically, or culturally significant species that are likely to eventually independently survive in the wild for at least decades [52,116,196]. This targeting is particularly relevant in consideration of the rapid and accelerating anthropogenic modification of the biosphere (See Introduction). Species not expected to survive in the wild, irrespective of the environmental targets of COP 15 or COP 28, should be subject to CBPs supported by biobanked sperm or perpetuated solely in biobanks (See section 3.). Only through addressing these issues will the amphibian RBC community satisfy their ethical responsibilities toward providing reliable and cost-effective intergenerational justice. Caecilians offer an exceptional needs and opportunities for the development and application of RBCs, because of caecilians internal fertilisation, and in many species' internal development, and maternal care in all terrestrial species [61,79–81].

The high costs of implementing recovery-based conservation are attributed to maintaining or providing specialized habitats, extensive research and monitoring, conservation breeding and reintroduction programs, and the need for professional management [3,338]. The financial burden of meeting these global biodiversity conservation targets through reducing extinction risk through interventions along with establishing and maintaining protected areas is substantial, with estimates of billions a year [339], with mammals and birds typically requiring the most resources [340]. To address these challenges, a globally inclusive approach is needed, focusing on developing highly cost-efficient RBC facilities in regions with high amphibian diversity, that welcome community engagement and international collaborations [3,341].

The short-term prevention of the extinction of amphibian species through RBCs to maintain, repopulate, or translocate amphibian populations in the wild [3], has already been reliably and economically demonstrated. Between 2007 and 2017, there was a ~60% increase in amphibian CBPs, including 80 species, or 2.9% of all species, with half of these CBPs involving repopulation or augmentation and 70% having some success [10,52]. However, these approaches must recognise a future of dramatic ecosystem collapses driven by global heating leading to mass extinctions [13,38–40,45]. The establishment of biobanks of amphibian cells and tissues will provide cost effect and reliable species perpetuation [3,58,67–73].

Exciting opportunities for amphibian RBC research and development include cloning and somatic cell techniques for species restoration (de-extinction) [312]), extraterrestrial biotechnologies [3,342,343], and terraforming for colonisation [3,342]. Terraforming conceptually includes the past, current, and future anthropogenic modification of Earth's biosphere as extended to extraterrestrial ecosystems along with their biodiversity [3,342].

Public education and outreach for amphibian conservation and include educational programs, community engagement, media campaigns, and interactive experiences [344]. These initiatives can foster empathy, promote citizen science, and encourage sustainable practices [3,52,55,345]. Perceptions of conservation success can vary but are generally perceived in terms of species and habitat improvements, effective program management, outreach initiatives, and the application of science-based conservation including RBCs [52,345]. The 2024 Amphibian Conservation Action Plan (ACAP) provides a guide for capturing outcomes, identifying gaps, and measuring progress in conservation efforts [52].

However, the 2024 ACAP [52] framework was limited by the mandate of supporting '*Amphibians thriving in nature*' (in the wild) and largely disregards the transformational changes to species management when considering of the sixth mass extinction [3]. Chapter 12 '*Amphibian assisted reproductive technologies and biobanking*', in the '*Priorities and recommendations*' section, does not mention species perpetuation through biobanking, cloning, and species restoration, with similar viewpoints by the Amphibian Ark in neglecting species that cannot foreseeably be returned to the wild [346]. Furthermore, the ACAP '*Chapter 11. Conservation breeding*' is not inclusive of private carers potential to contributions the perpetuation of amphibian biodiversity [55]. The disregard on many internet sources for the benefits of the biotechnical aspects of RBCs and biobanking [347], also shows the need and the challenges to the effective advocation and popularisation of the full potentials of RBCs.

The advocation and popularisation of amphibian RBCs includes engaging cultural discourses involving assisted evolution [348], cloning and species restoration [72,349], synthetic biology [342,350,351], and theological debates about humanities relationship to nature through synthetic biology, resurrecting species, and playing God [348–351]. Support for these initiatives includes standardised scientifically based terminologies popularised through the public media [3,95–97,259,352]. Furthermore, an emphatic effort must be made to remove anachronistic bureaucratic impediments including policy and media [52].

The development of solid RBC career streams will attract ambitious and talented researchers and activists to build RBC projects by competing for influence and resources [3,95]. Initiatives should focus on species perpetuation to ameliorate amphibian mass extinction and be inclusive of the broadest global community [3,333]. Community popularisation of advanced reproduction technologies is exemplified by the highly successful Colossal project, where their central theme of de-extinction (species restoration) is extended for their target species to community-based field conservation projects [312,332], advocating bioengineering as an initiative to help heal the Earth [353,354].

In summary, we have shown that the perpetuation of amphibian biodiversity, rather focusing on the limited goals of the 2024 ACAP, other institutions, and media sites of amphibians thriving in the wild should recognise the community's responsibility to adapt to the environmental and cultural realities of the Anthropocene [355,356] including ecosystem modification's, collapses and the sixth mass extinction [6,7,15,38,355]. Besides current initiatives, this transformation should harness the potential of biobanking and species restoration to perpetuate species and be inclusive of all global participants including private carers CBPs [3,55]. A greater focus is also needed to garner global support and international engagement toward developing RBCs in highly biodiverse regions, especially developing countries, and the provision of independent finance. An independent, democratic, and inclusive global organisation representing an increasingly assertive multipolar world is needed to advocate these needs [3].

Finally, will humanity over the coming millenniums look back to this century relegating plausibly thousands of amphibian species that will not continue to exist in the wild to extinction as promoted in [346,357]. Or alternatively will we face the challenges of the Anthropocene presented by inevitable biospheric modification and especially through global heating [39,40], and perpetuate species through realist innovative, cost effective, and reliable biotechnologies [3] supported by broader community inclusion in a multipolar world [3,44].

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