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Sperm collection and storage for the sustainable management of amphibian biodiversity

Robert Browne
Sustainable America

Aimee J. Silla
University of Wollongong, asilla@uow.edu.au

Rose Upton
University of Newcastle

Gina Della Togna
Smithsonian Conservation Biology Institute

Ruth Marcec-Greaves
Detroit Zoological Society

See next page for additional authors

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Abstract

Current rates of biodiversity loss pose an unprecedented challenge to the conservation community, particularly with amphibians and freshwater fish as the most threatened vertebrates. An increasing number of environmental challenges, including habitat loss, pathogens, and global warming, demand a global response toward the sustainable management of ecosystems and their biodiversity. Conservation Breeding Programs (CBPs) are needed for the sustainable management of amphibian species threatened with extinction. CBPs support species survival while increasing public awareness and political influence. Current CBPs only cater for 10% of the almost 500 amphibian species in need. However, the use of sperm storage to increase efficiency and reliability, along with an increased number of CBPs, offer the potential to significantly reduce species loss. The establishment and refinement of techniques over the last two decades, for the collection and storage of amphibian spermatozoa, gives confidence for their use in CBPs and other biotechnical applications. Cryopreserved spermatozoa has produced breeding pairs of frogs and salamanders and the stage is set for Lifecycle Proof of Concept Programs that use cryopreserved sperm in CBPs along with repopulation, supplementation, and translocation programs. The application of cryopreserved sperm in CBPs, is complimentary to but separate from archival gene banking and general cell and tissue storage. However, where appropriate amphibian sperm banking should be integrated into other global biobanking projects, especially those for fish, and those that include the use of cryopreserved material for genomics and other research. Research over a broader range of amphibian species, and more uniformity in experimental methodology, is needed to inform both theory and application. Genomics is revolutionising our understanding of biological processes and increasingly guiding species conservation through the identification of evolutionary significant units as the conservation focus, and through revealing the intimate relationship between evolutionary history and sperm physiology that ultimately affects the amenability of sperm to refrigerated or frozen storage. In the present review we provide a nascent phylogenetic framework for integration with other research lines to further the potential of amphibian sperm banking.

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Robert Browne, Aimee J. Silla, Rose Upton, Gina Della Togna, Ruth Marcec-Greaves, Natalia Shishova, Victor Uteshev, Belin Proano, Oscar D. Perez, Nabil Mansour, Svetlana Kaurova, Edith Gakhova, Jacky Cosson, Borys Dyzuba, Ludmila Kramarova, Dale McGinnity, Manuel Gonzalez, John Clulow, and Simon Clulow

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Robert K. Browne [a*], Aimee.J. Silla [b], Rose Upton [c], Gina Della-Togna [d,e], Ruth Marcec-Greaves [f], Natalia V. Shishova [g], Victor K. Uteshev [g], Belin Proaño [h], Oscar D. Pérez [h], Nabil Mansour [i], Svetlana A. Kaurova [g], Edith N. Gakhova [g], Jacky Cosson [j], Borys Dyzuba [j], Ludmila I. Kramarova [k], Dale McGinnity [l], Manuel Gonzalez [m], John Clulow [c], Simon Clulow [c,n]

a *Corresponding author. Sustainability America, Sarteneja, Corozal District, Belize. robert.browne@gmail.com.
b School of Earth, Atmospheric and Life Sciences, University of Wollongong NSW 2522.
c School of Environmental and Life Sciences, University of Newcastle, Callaghan Drive, Callaghan, NSW 2308, Australia.
d Smithsonian Tropical Research Institute, Panama Amphibian Rescue and Conservation Project, Panama City, Panama.
e Universidad Interamericana de Panamá, Dirección de Investigación, Sede Central, Panama.
f National Amphibian Conservation Center Detroit Zoological Society, Detroit, USA.
g Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russia.
h Escuela de Ciencias Biológicas, Pontificia Universidad Católica del Ecuador, Ecuador.
i Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt.
j University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, 38925 Vodnany, Czech Republic.
k Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russia.
l Nashville Zoo at Grassmere, Nashville, TN 37211, USA
m Departamento de Producción Animal, Universidad de Guadalajara, , Guadalajara, Jalisco, Mexico.
n Department of Biological Sciences, Macquarie University, Sydney NSW 2109 Australia.

Corresponding author: R. Browne, PhD, robert.browne@gmail.com.

Abstract

Current rates of biodiversity loss pose an unprecedented challenge to the conservation community, particularly with amphibians and freshwater fish as the most threatened vertebrates. An increasing number of environmental challenges, including habitat loss, pathogens, and global warming, demand a global response toward the sustainable management of ecosystems and their biodiversity. Conservation breeding programs (CBPs) are needed for the sustainable management of amphibian species threatened with extinction. CBPs support species survival while increasing public awareness and political influence. Current CBPs only cater for 10% of the almost 500 amphibian species in need. However, the use of sperm storage to increase efficiency and reliability, along with an increased number of CBPs, offer the potential to significantly reduce species loss. The establishment and refinement of techniques for the collection and storage of amphibian spermatozoa, over the last two decades, gives confidence for their use in CBPs and other biotechnical applications. Cryopreserved spermatozoa has produced breeding pairs of frogs and salamanders and the stage is set for lifecycle proof of concept studies that use cryopreserved sperm in CBPs along with repopulation, supplementation, and translocation programs. The application of cryopreserved sperm in CBPs, is complimentary to but separate from archival gene banking and general cell and tissue storage. However, where possible amphibian sperm banking should be standardised and integrated into other global biobanking projects, especially those for fish, and those that include the use of cryopreserved material for genomics and other research. Research over a broader range of amphibian species, and more uniformity in experimental methodology, is needed to inform both theory and application. Genomics is revolutionising our understanding of biological processes and increasingly guiding species conservation through the identification of evolutionary significant units as the conservation focus, and through revealing the intimate relationship between evolutionary history and sperm physiology that ultimately affects the amenability of sperm to refrigerated or frozen storage. In the present review we provide a nascent phylogenetic framework for integration with other research lines to further the potential of amphibian sperm banking.

1. Introduction

Conservation Breeding Programs (CBPs) are required for the sustainable management of amphibian species threatened with extinction. The use of cryopreserved spermatozoa in CBPs perpetuates male genetic variation, lowers costs, increases biosecurity, reduces the number of required captive individuals, enables the fertilization of a single female's spawn with spermatozoa from many genetically diverse males, and reduces the need for animal transport [1,2,3]. Sperm banks for fishes exist globally for projects ranging from the perpetuation of zebra-fish cell lines [4] to maintaining genetic variation in sport fishing based CBPs and in aquaculture programs [5]. However, the cryopreserved spermatozoa of amphibians has only been practically applied to maintain *Xenopus* transgenic lines for biotechnological research [6]. Techniques for the post-thaw recovery of cryopreserved fishes [7,8] and amphibians [9] oocytes or embryos have not succeeded. However, primordial germ cells from cryopreserved fish embryos have been transplanted to amphibian embryos and then have developed into the gonads of fertile adults [10]. Similar technology for amphibians offers the greatest current potential for the cryopreserved storage of female germplasm. In any case, live females are needed to provide oocytes for *in vitro* fertilization with stored spermatozoa, or to supply larvae for primordial germ cell transplantation [9].

The Amphibian Ark [AArk, [11]) was established as a zoo based organisation to support amphibian CBPs. The AArk Species Conservation Assessments [12] recommends that of the ~570 amphibian species requiring CBPs, that ~500 species or 90% need the support of gene banks including the use of cryopreserved spermatozoa (Supplementary Table 1).

The loss of genetic variation in CBPs can result in poor reproduction, health, and survival [13,14]. Even with large founder populations, genetic selection can occur in a few generations for rapid growth, early maturity, amenability to husbandry or ease of reproduction in captivity [13,15]. Selection toward domestication can be reduced but not eliminated by strict studbook management. However, even strict studbook management is subject to loss of broodstock and difficulties in transporting broodstock between breeding groups. The optimal approach is to use cryopreserved spermatozoa to reduce the effective number of male generations to one, and to provide an easy means of transport [13].

The natural genetic variation of species can be recovered through the use of cryopreserved spermatozoa using oocytes from highly domestic strains (Fig. 1). Consequently, domestic strains of species without studbook requirement, both in aquaculture and in private keeper's collections, could contribute female brood stock to CBPs if adequate stocks of cryopreserved sperm were available to restore the species genetic variation. In the case of *Andrias davidianus*, where genomics recently revealed the taxon consists of 5 component species now mainly as aquaculture hybrids [16], and with all 5 species are functionally extinct in nature [17], these species could be re-established with the use of stored sperm.

Insert Fig. 1.

The spermatozoa of fishes and amphibians can remain viable for days to weeks during refrigerated storage at ~4°C, or indefinitely when cryopreserved in liquid nitrogen at -196°C (Supplementary Tables 2,3. [16,18]). Post-thaw, motile spermatozoa can be used for artificial fertilization [2] and immotile spermatozoa for intracytoplasmic injection into the oocyte (ICSI; [19]). In amphibians, post-thaw spermatozoa from Anurans (frogs and toads) has resulted in reproducing pairs of *Xenopus* [6] and sexually mature males of tree frogs [20], and in Cuadata reproducing pairs of *Salamanders [21], and almost mature Cryptobranchids (Giant salamanders, Dale McGinnity, personal communication).

*Urodeles include all extinct and extant salamanders. The Caudata, Fig. 2. have three main lineages; the Crypotobranchidae, Sirenidae, and "other salamanders". In this review for grammatical simplicity where appropriate we simply use the term ¨Salamander¨ rather than ¨other salamander).

We use studies of freshwater fishes spermatozoa to provide the closest phylogenetic, morphological, and physiological comparisons to amphibian spermatozoa. The parameters used to assess the quality of amphibian spermatozoa include the percentage with flagella movement (percent activation) and velocity (percentage motility), and the velocity and longevity of motile spermatozoa [22]. Membrane integrity, spermatozoa concentration, DNA integrity, and acrosome integrity, and relationship between these and fertilisation and larval growth to adults are also assessed [23].

2. Amphibian phylogenetics, fertilisation history, and reproductive strategies

hylogenetic patterns in the amenability of amphibian species spermatozoa, to the physiological and morphological stressors during storage, could facilitate the development of storage techniques and in general inform amphibian spermatology [2,24,25]. Most Anurans externally fertilise through spermatozoa shed in spermic urine. Spermatozoa is released in semen in the Cryptobranchidae (Cryptobranchids and Hydronobids) and Sirenidae. Fertilization is internal in all Salamanders through the deposition of spermatophores by males that are then picked up by the cloaca of females and all species of Caecilians are internal fertilizers [26].

Of amphibians, the Anurans have the most complicated evolutionary history of fertilisation: primordially with external fertilisation, then internal fertilization as Lissamphibians, external fertilization as Batrachians, a 40 million year period of internal fertilization, then a reversion to external fertilisation from 275 mya to the present [27]. One primitive Anuran retains internal fertilisation [25,28], and less than 15 known species have reverted to internal fertilisation [28]. The Caudata have a less complicated evolutionary history than the Anurans with two families the Sirenidae and the Cryptobranchidae retaining the ancestral Batrachian external fertilisation, and Salamanders readopting internal fertilisation. Caecilians are internal fertilisers ([27] Fig. 2).

Insert Fig. 2.

The three sub-orders of Anurans are the Archaeobatrachia with 4 families and ~27 species, the Mesobatrachia with 6 families and ~168 species, and Neobatrachia with 21 families and the ~5000 species. In Anurans, the spermatozoa of 30 species has been cryopreserved: species in seven Neobatrachia families, two species in one Mesobatrachia family, the Pipidae, which includes *Xenopus*. In anurans, post-thaw assessment of spermatozoa viability as defined by live/dead stains was reported for 12 species, motility for 16 species, and life stages to first cleavage for 4 species, larval development for 7 species, and development to adults for 2 species. In the Caudata the spermatozoa of 5 species have been cryopreserved, 3 salamanders and 2 cryptobranchids. Post-thaw assessment of spermatozoa viability in spermatophores was reported for one species, motility for two species, development to late juvenile/adults with one species, and to fertile adults in one species (Supplementary Table 3).

3. Sperm collection and sperm concentration

Sperm can be collected as testicular macerates or suspensions from any sexually mature male amphibian. Anuran spermatozoa can also be collected through hormonal induction, either as spermic urine through abdominal massage [23] or through cannulation of the cloaca [29,30,31,32,33.34]. Hormonal induction causes internally fertilising salamanders to deposit spermatophores [35] or to express sperm in cloacal fluid (Fig. 3,4 [36,37,38]).

Insert Fig. 3.

Both phylogeny and the environment, especially climate, influence the reproductive strategies of amphibian species and their amenability to hormonal induction [2,22,40]. Hormones can be administered safely and efficiently by injection even with small frogs [31,32,33], and generally most species are amenable to hormonal induction of sperm release with gonadotropin releasing hormones (GnRH) or human chorionic gonadotropin (hCG [2]). GnRH is generally more effective at inducing spermiation than hCG across a wide range of species, however, there are a number of species, mainly from the Bufonidae and Limnodynastidae families, where hCG elicits a stronger response [22,39,41]. Inter-taxon variation also occurs between closely related species, where due to its fertilisation strategy a single species from a family otherwise amenable to hormonal induction responded poorly both hCG and GnRH [2,29]. This and other exceptions may elucidate the specific evolutionary drivers behind reproductive strategies [2].

Hormonal induction depends on the presence of mature spermatozoa in the testes [24]. Seasonal quiescence in spermatozoa maturation can be circumvented through the use of both priming, where sub-inducing doses of hormones, along with dopamine antagonists [42,43], are administered days before the final inducing dose [22,24]. Hormone administration generally induces Anuran spermic urine over periods between 2 -12 h with clear peaks in spermatozoa concentration between 3 and 7 h (Supplementary Table 2. [22,23,24,33,41,44,45]).

The most reliable collection technique for large quantities of mature Anuran spermatozoa is through the maceration of the testes to produce spermatozoa suspensions [6,18]). The concentration of spermatozoa in testicular macerates at ~$10^{8\text{-}9}$/ml is generally one to three magnitudes higher than in

spermic urine (Supplementary Table 2). The high concentration, volume and quality of testicular spermatozoa has resulted in its use in most studies of Anuran spermatozoa cryopreservation until recently, and in the only two studies resulting in mature reproducing pairs [6] or sexually mature males [20]. As well, spermatozoa in suspension from testes have higher refrigerated storage potentials than those stored in intact testes, spermic urine, or in semen (Supplementary Table 2. [6,46]).

Caudata spermatozoa in high concentrations and volumes in semen is easy to collect from seasonally mature or hormonally induced Cryptobranchidae [47,48], and as hormonally induced spermatozoa in cloacal fluid, even from small salamanders approximately 8 g in weight, making the collection of spermatozoa from testes unnecessary unless from recently dead individuals (Ruth Marcec, personal communication). The semen of the cryptobranchid, *A. davidianus*, is collected at concentrations of ~11 x 10^6/mL and up to 12mL/kg of male weight [47,48] with adult males weighing up to 50 kg [49], but in lower absolute and comparative volumes of 2 mL in *Cryptobranchus* (the North American giant salamander clade, Dale McGinnity, personal communication). The comparative differences in sperm numbers between amphibian species may generally relate to different levels of sperm competition [45], including internal competition in Salamanders where the number of spermatozoa per spermatophore varies by more than three magnitudes (Supplementary Table 2. [47]).

Insert Fig. 4.

Processing during cryopreservation, thawing, and recovery can reduce the concentration of spermatozoa to less than <15% of the original [50]. Consequently, in samples derived from spermic urine the post-thaw spermatozoa concentrations could be lower than the fertilization optimum, and even lower than the fertilisation threshold. In Anurans, fertilization rates decline in a sigmoidal curve from the optimum spermatozoa concentration, and concentration three magnitudes lower than the optimum provided only 30% fertilization in one species [51] no fertilisation in another [52]. The optimal spermatozoa concentrations for fertilization may depend on other factors in addition to spermatozoa motility. These include chemo-attractants found in the oocyte gel, oocyte size, or in terrestrial-breeding and foam-nesting Anurans the direct deposition of spermatozoa onto oocytes [2].

Centrifugation is used to concentrate spermatozoa from spermic urine, to remove protein and lipid components from fresh spermatozoa suspensions [44], and post-thaw to remove cryodiluents [20]. The use of centrifugation should be minimised as spermatozoa motility can be reduced by up to 50% through morphological damage [53]. Therefore, in cases where there are surplus amphibians in breeding programs [6], or males can be taken from wild populations [20], the collection of high numbers and concentrations of spermatozoa directly through testes maceration may be the preferred option.

In some fish species the contamination of semen with urine decreases spermatozoa viability [54,55], and in others urine is needed for spermatozoa maturation [56]. Anuran spermatozoa from spermic urine exhibits slightly lower viability than testicular spermatozoa possibly as a consequence of activation in the lower osmolality of urine in comparison to the isotonic testicular environment [45]. Nevertheless, spermatozoa stored in spermic urine at room temperature have been reported to retain similar high levels of motility to testicular sperm for up to 45 m [34]. The effect of urine contamination on Caudata spermatozoa in semen, or when sampled in milt is unknown, but urine contamination of fishes semen negatively affects spermatozoon metabolism [54,55].

4. Effects of environmental factors on sperm motility activation and fertilization rate

The major environmental factors affecting spermatozoa motility in externally fertilizing amphibians and fishes are media osmolality, ionic composition, pH, and temperature [25,45]. The motility of spermatozoa in some freshwater fishes is highly influenced by the extracellular concentration of electrolytes [57,58]. The motility of spermatozoa is activated when sperm transition from the high osmolality of the testes to the low osmolality of the freshwater environment, and with tested amphibians is the major factor controlling activation [25,59,60]. With Anuran spermatozoa an osmolality of ~250 $mOsmolkg^{-1}$ prevents activation [25], and dependent on the species osmolalities below 70 $mOsmolkg^{-1}$ [60] to 105 $mOsmolkg^{-1}$ [61] promote activation. Inter-specific variation in the optimal osmolality for fertilization in Anurans was shown where in one species fertility was maintained up to 40 mM [60], in another a steady decline in fertility occurred as osmolalities increased to more than 7 $mOsmolkg^{-1}$ [61], and even intra-specific variation was shown in the optimum osmolality in one species [62].

Anuran spermatozoa show the longest period of sperm motility of all amphibians [25,61] with an average period of motility of 1 h [25]. However, at extremes the spermatozoa of *Xenopus* only maintains

motility for 2 min [63,64] and in one species motility is extended to 7 h [61]. The spermatozoa of Cryptobranchidae and some freshwater fish stays motile for up to 10 min, but with most freshwater fish motility only lasts for seconds to a few min [25]. Higher osmolalities increase the longevity of spermatozoa possibly from less energy being partitioned from motility to maintaining osmotic equilibration [25,65].

The longevity of both fish [65,66,67] and amphibian spermatozoa [68] depends on specific metabolic pathways and the availability of energy substrates [69,70]. Adenosine triphosphate (ATP), adenosine diphosphate, and creatine phosphate [71,72] provide energy for flagella motion and maintain ionic and water balance across plasma membranes [57]. In many fish species increased spermatozoa velocity also positively corresponds with ATP levels [70]. Species variability and specificity of energy metabolism has been shown for fishes [72], however, species specificity has not been shown in the few studies of amphibians. In the Bufonid, *Anaxyrus fowleri*, ATP/adenosine monophosphate over a wide range of concentrations did not affect spermatozoon velocity or longevity (Robert Browne pers. communication).

4.1 Diluents

Diluents are formulated to simulate the cellular concentrations of ions but may also contain organic supplements (Supplementary Table 3 [73,74]). Diluents approximating 220 mOsmolkg^{-1} are generally used as cryodiluents [37,38,73,74], to deactivate spermatozoon motility [74,75], and at low concentrations are used for post-thaw sperm equilibration, activation and fertilization [75]. The ionic composition of diluents and their osmolality for fish are similar to those of blood plasma, and generally include Na^+, Ca^{2+}, K^+, Cl^-, and $H_2CO_3^-$ ions. Other ions that may be formulated in diluents are Mg^{2+}, SO_4^-, and increased relative concentrations of K^+ or Ca^{2+} or $H_2CO_3^-$, however, their benefits are species specific in fish [57].

The formulation of diluents for amphibians were originally taken from physiological salines used for general biology [75], and now include formulations used for cell culture, and for the storage or fertilisation of fish and mammalian spermatozoa [1,2]. However, the K^+ concentration in most of these diluents is only ~2 mM, whereas, K^+ concentrations in anuran testicular plasma is ~70 mM in *Xenopus*

and ~40 mM in *Bufo* [76]. A low K^+ concentration in diluents can produce membrane damage, therefore, a greater knowledge of the role of K+ and other ions in diluents for amphibian spermatozoa would be beneficial.

With some fish species the pH of diluents is a major factor affecting spermatozoon motility, where a pH similar to or higher than that of seminal plasma promotes the activation and longevity of motility [58,77]. Because of few studies, the role of pH in spermatozoa motility in amphibians is not clear. Studies in Anurans show a higher pH 7.1-7.8 in spermic urine than that of urine (pH 6.7-6.8) [34]. In the Caudata, with *A. davidianus* an artificially high pH 7.0-7.5 [47,48] increased refrigerated storage life and low pH inhibited flagella movement, and in contrast the semen of *Cryptobranchus* had a low pH 6.4 (unpublished), and where the highest motility of *Axolotl* spermatozoa was found at pH 10.0-12.0 (Nabil Mansour personal communication).

Diluents are commonly mixed 1:1 by testis weight or semen volume to create sperm suspensions [50]. If spermatozoon suspensions are prepared from non-ionic diluents they will have only 50% of the original concentration of the various ions in testicular tissue, spermic urine, or semen. If sperm suspensions are prepared from ionic diluents the final concentration of ions in the suspensions will depend on the initial concentration of the diluent and the tissues or semen's ionic concentration.

4.2 Stimulants and antioxidants

The stimulants caffeine and/or theophylline have been shown to increase the activation of Anuran spermatozoa [61,78]. The beneficial effects of these compounds on motility are likely the result of phosphodiesterase inhibition increasing levels of cyclic adenosine monophosphate, however, they may also influence spermatozoa motility and longevity as antioxidants by acting as antioxidants, and by suppressing reactive oxygen species formation within the sperm suspension [61]. The effects of other antioxidant compounds on Anuran spermatozoa have also been investigated, however, Vitamin C supplementation was detrimental and Vitamin E supplementation had no effect [79]. Pentoxyfylline did not increase the motility of fresh *Xenopus* spermatozoa [64].

5. Sperm processing and refrigerated storage

Refrigeration at 4°C extends the storage life of spermatozoa by lowering metabolic rates, and most amphibian [53,80,81] and fish [82] spermatozoa largely tolerate refrigeration temperatures to above freezing. Anuran spermatozoa has retained moderate motility (~10-20%) after refrigerated storage of testis in carcasses for ~7 d [83], excised testes for ~14 d [80,83], testicular macerates for between ~14-21 d [80], spermic urine for ~7 d [40], and in the semen of a Cryptobranchid for ~4.5 d [48], and in hormonally induced cloacal fluid from a Salamander for ~3 d (Supplementary Table 2 [38]).

5.1 Oxygenation

Anuran spermatozoa uses both aerobic and anaerobic metabolism [67,84,85], whereas fish spermatozoa can only use aerobic metabolism [68,86]. Nevertheless, oxygenation extends the refrigerated storage life of spermatozoa in many fish species [87], however, in some species it decreases storage life and in others has no effect [86]. Increased oxygen concentration was shown to increase the refrigerated storage life of the spermatozoa of two anurans [85,88]. Oxidative damage to fish spermatozoa may be limited by components of seminal fluid [89], and this may also be the case with cryptobranchid spermatozoa in semen.

5.2 Processing Osmolalities

Spermatozoa are affected by varying osmolalities at various stages of processing, storage, activation, and fertilisation [1]. Spermatozoa suspensions whether created from testicular macerates [6,20], Cryptobranchid semen [47], salamander spermatophores [35] or cloacal fluid [37,39], or Anuran spermic urine [73], have high osmolalities. The greatest changes in osmolality between spermatozoa and diluents occur with the processing of spermic urine for refrigerated or cryopreserved storage, and after both refrigerated and cryopreserved storage when spermatozoa are equilibrated to recover motility [90].

5.3 Antibiotics and light

The ability of antibiotics to increase the storage life of refrigerated spermatozoa is a balance between their efficacy and their toxicity with contrasting results in different species and studies. In fish,

antibiotics have been shown to improve the storage of spermatozoa in several species [91,92]. In contrast, the use of antibiotics (penicillin–streptomycin and gentamicin) in diluents reduced motility during the refrigerated storage of Anuran spermatozoa from spermic urine [85] and spermatozoa suspensions from testicular macerates [88]. Gentamicin in concentrations of up to 4 mg mL^{-1} did not affect the refrigerated storage life of spermatozoa in Anuran testicular macerates or in spermic urine, but did reduce bacterial contamination [46]. While there is currently no evidence that antibiotics increase the storage life of amphibian spermatozoa, antibiotics may inhibit bacteria and reduce the risk of pathogen transmission through stored samples [46]. In *A. davidianus* has strong light has been shown to decrease the longevity of fresh sperm [48].

6. Sperm cryopreservation

n response to the amphibian extinction crisis, the cryopreservation of testicular anuran spermatozoa with subsequent fertilization was achieved in the late-1990´s by independent research teams in the Russian Federation [93,94] and Australia [52]. Early studies compared a wide range of penetrating cryoprotectants between species, diluents, and processing methods [52,74,95,96,97,98,99,100,101] focussing on dimethyl sulphoxide (DMSO [52,97]), glycerol [52,97], and methanol [97]. By 2010, DMSO was the most consistent penetrating cryoprotectant in achieving post-thaw motile and fertile spermatozoa. To extend the collection of spermatozoa to non-lethal techniques, research then focussed on techniques for the cryopreservation of hormonally induced spermatozoa in spermic urine. Motility and fertilization with post-thaw spermatozoa from spermic urine was first achieved in 2011, with the novel penetrating cryoprotectant dimethyl formamide (DMFA) giving greater recovery than DMSO [73]. DMFA was then successfully used with four other phylogenetically diverse Anuran species, *Pelophylax lessonae [40]*, *Anaxyrus b. boreas* and *Lithobates sevosa* [100] and *Atelopus zeteki* [101].

Insert Fig. 5.

The preparation of cryosuspensions involves the mixing of spermatozoa samples with cryodiluents (Fig. 4). Compounds in cryodiluents act in synergy to protect spermatozoa during the rigors of freezing and thawing. Cryodiluents are formulated from penetrating cryoprotectants and non-penetrating cryoprotectants, and with amphibian spermatozoa now favour ionic/saccharide non-

375 penetrating cryoprotectants. Both sucrose [40,64] and trehalose [100,101] as saccharide non-penetrating
376 cryoprotectants have provided high post-thaw recovery.
377
378 With fish spermatozoa, non-penetrating cryoprotectants including proteins, lipoproteins and
379 lipids have increased plasma membrane resistance to osmotic stress along with post-thaw recovery
380 [102,103,104]. With amphibians spermatozoa the inclusion of protein/lipids in cryodiluents provided
381 high post-thaw recovery of viability in an Anuran [64], and in Caudata motility in *Cryptobranchus* (Dale
382 McGinnity, unpublished data), and increased fertility in a Salamander [37]. A negative effect of a buffer
383 was shown through a higher post-thaw recovery of the motility of Bufonid spermatozoa using DMSO
384 alone than with the addition of HEPES buffer [40], while the addition of TRIS buffer did not affect the
385 recovery of *Xenopus* spermatozoa [6].
386
387 Concentrations of DMSO or DMFA between 5-10% (v/v) in cryosuspensions have proven the
388 most successful for the cryopreservation of amphibians spermatozoa [6,20,40,52,73] with up to 15%
389 concentration in a Litorid frog [105]. However, high concentrations of up to 15% DMSO have generally
390 proven effective with fish. Glycerol [106], trehalose alone [107], and propylene glycol [108] have
391 proven more effective than DMSO in some fish species and may be suitable for some amphibian
392 species. In some Anurans, even low concentrations of DMSO reduce hatch rates [66], and with
393 spermatozoa from spermic urine reduce fertility and larval survival [83]. In contrast, with *Xenopus*
394 spermatozoa DMSO proved less toxic than glycerol [6]. In one species post-thaw motility was more
395 highly correlated to fertilisation with glycerol in contrast to DMSO [105].
396
397 Cryosuspensions are generally refrigerated for ~10 min before freezing to equilibrate
398 spermatozoa to penetrating cryoprotectants in a low temperature environment that reduces
399 cryoprotectant toxicity [44,73,101]. The penetration rates of penetrating cryoprotectants vary widely,
400 where DMSO reaches equilibrium with fishes spermatozoa within 10 s [109], but with the penetration
401 rate of glycerol being much lower [100,111]. Longer equilibration periods may benefit some Anuran
402 cryopreservation protocols and did not affect post-thaw recovery after 20 min [6], with some sperm
403 recovering motility and fertility after 6 d of refrigerated storage [112]. Consequently, to enable more
404 flexibility in the timing of techniques, and to facilitate the use of some slowly penetrating
405 cryoprotectants, the equilibration period of refrigerated cryosuspensions may extend to 20 min or more.

406

407 6.1 Cooling rates and cryopreservation

408

409 Samples can be frozen in the field using dry ice or through suspension into LN_2 vapour, and in facilities
410 also using -80°C or programmable freezers. We categorise freezing rates as very slow (10°C/min), slow
411 (30°C/min), moderate (110°C/min), fast (300°C/min), very fast (1200°C/min) from a broad canvassing
412 of the studies in Supplementary Table 2. Stepped freezing rates for amphibians spermatozoa have
413 achieved high post-thaw recovery with testicular spermatozoa [20,99] and with spermatozoa from
414 spermic urine (Supplementary Table 2. [73,100]). The cryopreservation of some fishes [113] and
415 amphibians [52] spermatozoa is affected by changes in cooling rates as low as 5°C/min, and in these
416 cases the use of programmable freezers may be necessary.

417

418 The spermatozoa of several Caudata families have proven amenable to cryopreservation at slow
419 to fast cooling rates. With *Axolotl* spermatophores, cooling rates between ~10°C/min and ~300°C/min
420 did not affect viability [35], and high post-thaw recovery was shown with Salamander sperm using
421 stepped freezing [37]. The sperm of *Cryptobranchus* proved amenable to cryopreservation with the slow
422 lowering of straws into a LN_2 vapour (Dale McGinnity personal communication), but using a similar
423 freezing method with *Andrias* only recovered <10% motility [47]. In fish optimal cooling rates are
424 membrane lipid dependent [114,115] and this is expected to be the case with amphibian sperm.

425

426 **Sperm processing, activation, and fertilisation**

427

428 High levels of motility and fertility are recovered from cryopreserved amphibian sperm when thawed in
429 a wide range of conditions from air at room temperature, to unheated tap water, to immersion into 37°C
430 water baths (Supplementary Table 2, [6,100,101]). The first post-thaw recovery of hormonally induced
431 Anuran sperm was achieved through a four-step osmotic equilibration process at 4°C [83], and with
432 another Anuran the percentage activation, velocity, morphology, longevity, and DNA integrity were
433 higher when spermatozoa were held at 4°C during the post-thaw processing. Therefore, once thawed as
434 shown by the last remaining ice just thawing, cryosuspensions should be held as close to 4°C as
435 possible. The period that post-thaw spermatozoa can be stored and maintain viability is unknown for
436 most species, but with *Xenopus* last 20 min without any effect on fertilisation or embryo survival rates
437 [6,44].

The highest fertilization rates are achieved through high spermatozoa per an oocyte ratios. The general practice of the mixing of cryodiluents with sperm samples at a 1:1 ratio reduces spermatozoa concentration by 50%. For activation, these suspensions are then generally mixed at a ratio of 1:2 or more with water resulting in a further reduction of spermatozoa concentration to ~20% or less of the original sample [51,73]. This lowering of spermatozoa concentrations is particularly significant with low concentrations of hormonally induced spermatozoa and where fertilisation requires high sperm concentrations [73], and also where a significant percentage of sperm lyse or do not recover motility [53]. For the highest fertilisation rates, a process known as dry fertilisation is used where sperm suspensions are deposited directly onto oocytes and then after 5 to 10 min the oocytes are flooded with fresh water [52,75].

7. Morphological integrity of sperm

Many morphological deformations can be found in post-thaw amphibian spermatozoa, such as swelling or rupture of the plasma membrane, loss of the nuclear envelope, fracture of the perforatorium and axoneme, degeneration of the undulating membrane and disappearance of the mitochondrial ridge [48,115]. Morphological damage may be associated with impacts on post-thaw activation mechanisms where fish [116,117] or Anuran [53,112] spermatozoa are intact but unable to activate. The positive correspondence between high post-thaw plasma membrane integrity and fertilization rates was shown with *Silurana tropicalis* in contrast to low membrane integrity and fertilisation rates in *Xenopus laevis* [6,115].

Sperm DNA fragmentation (SDF)

The main objective of spermatozoa storage is to provide unfragmented and viable genetic material to the oocyte upon fertilisation. Sperm DNA fragmentation (SDF) is a highly dynamic process that continues from spermatozoa collection until fertilization. Evolutionary history, morphology and physiology of spermatozoon, and DNA-protein interactions, affect SDF during refrigerated storage, cryopreservation, and post-thaw activation [118].

Sperm DNA fragmentation interferes with syngamy and embryonic development in fish [118]. However, to date there are only seven research publications of SDF in amphibians; *R. temporaria* sperm stored in refrigerated carcasses [83], refrigerated storage in testes or macerates in *X. laevis* and *S. tropicalis* [6,115,119], and the fresh hormonally induced and cryopreserved spermatozoa of *A. zeteki* [44,101] and *Epidalea calamata* [120]. Sperm DNA fragmentation increased and fertilization rates decreased during refrigerated storage of anuran sperm in carcasses [83], in spermic urine [45,101], and in post-thaw spermatozoa [101,115]. However, SDF was not a predictor of survival rates from first cleavage oocytes [6,83]. Sperm DNA fragmentation in post-thaw spermatozoa was higher in seasonally collected spermatozoa than in unseasonal spermatozoa, but also did not correspond to reduced embryo survival [120].

9. Phylogenetic patterns in sperm induction and amenability to storage

Phylogeny and environment interact to mold the reproductive strategies of amphibians [2,24,33]. Most studies of amphibian spermatozoa collection and storage are on Anurans from regions in the temperate zones of Australia, or the cool to warm temperate zones of Europe and North America (Supplementary Table 1, 2). Southern Australia, has a cool to warm climate with stochastic seasonal rainfall and has more studies of Anurans than any other region. In this climate Anurans generally reproduce over extended seasonal periods (Supplementary Table 3. [22]). Recent developments of cryopreservation techniques for fish spermatozoa have also focused on species from temperate climates and with seasonal reproduction, including numerous studies in Brazil [104].

More studies over a wider range of families, and species within families, are needed to reveal phylogenetic patterns in species amenability to spermatozoa cryopreservation Spermatozoa cryopreservation has been trialed in 2 Bufonid species and 6 Ranid from the cool to warm temperate zones of Eurasia and North America; and in 3 Bufonid species, 2 Hylid and 1 Eleutherodactylid from the tropical and subtropical zones of South and Central America (Supplementary Table 3). Although Bufonidae and Hylidae are sister clades, there were different responses to similar protocols, showing high recovery for Bufonid spermatozoa [44] and low recovery for Hylid spermatozoa (Belin Proaño and Oscar D. Pérez, personal communication). In contrast, the spermatozoa of both Ranids and Bufonids, which diverged ~170mya [121], are amenable to cryopreservation. However, Pelodryadid sperm showed greater amenability to cryopreservation than Myobatrachid sperm [98], where Pelodryadids diverged

from the Myobatrachids ~140 mya [122,123], and with no difference between spermatozoa from two Myobatrachid subfamilies that diverged 70 mya [99,123].

The Pipidae genera *Silurana* and *Xenopus* diverged only ~20-40 mya [124], however, post-thaw *S. tropicalis* sperm retains higher motility, membrane and DNA integrity than *X. laevis* sperm [15]. The two species of Pipidae (sub-order Mesobatrachia) in which cryopreservation has been trialed, are phylogenetically distant from other trialed anurans which are all from the Neobatrachidae, and in contrast to the spermatozoa of Neobatrachia the spermatozoa of both Pipidae species successfully cryopreserves in an ionic/sucrose diluent alone [6].

Insert Fig. 6.

A possible relationship between amphibians climatic range and the amenability of Anuran spermatozoa to cryopreservation was shown where the spermatozoa of freeze-tolerant wood frogs *Rana sylvatica* had a much higher post-thaw recovery than the more temperate climate leopard frogs *R. pipiens* and American toads *A. americanus* (Fig. 6, [125]) but this concept remains to be investigated over a wider range of species. Caudate spermatozoa from two distantly related families has been successfully cryopreserved in the Cryptobranchidae (Dale McGinnity personal communication, [46]) and the Ambystomatida [35,36]. Phylogenetic atterns of spermatozoa cryopreservation still need to be established in the remaining eight Caudate families.

10. Evaluation of techniques and their standardization

The development of techniques for the collection, processing, and storage of amphibians spermatozoa depend on assessing spermatozoa quality through standardised metrics. In fishes, percentage motility and velocity [82], and plasma membrane integrity [115,126], have mainly been used as metrics of spermatozoa quality, with far fewer studies extending to fertility and development [127]. Research on Anurans spermatozoa has generally used percentage motility and velocity as a metric [61,87,128], though a number of studies have used live/dead (viability) stains [27,29,31], and to a lesser extent fertility and development depending on the study goals and the availability of oocytes [34,73,83,100]. With Anurans, two recent Proof of Concept Studies used cryopreserved spermatozoa to produce mature

adults [6,20]. The post-thaw recovery of Caudate spermatozoa from semen has been assessed by fertility and development to mature adults (Dale McGinnity personal communication, [37]), and from spermatophores by live/dead stains [35]. However, vital stains may not always be reliable when assessing the membrane integrity of spermatozoa in spermatophores (Manuel Gonzalez pers. communication. Fig. 7.).

Insert Fig. 7.

To further the development of both research and practical application, at each stage of processing the quality of spermatozoa should be assessed by standardized methods for percentage of activation, velocity, period of motility, concentration and volume. Any procedures to induce spermatozoa or oocytes should be recorded including the body length, weight, age, and reproductive condition of males, and testes weight when collecting testicular spermatozoa. Spermatozoa suspensions can be measured for pH, osmolality, and ionic composition. If a study includes spermatozoa morphology, where possible the cataloguing of images from both stained slides and electron microscopy should be undertaken.

Some studies have shown an unexpected lowering of spermatozoa concentration during processing and storage [24] and a better understanding of the extent and nature of these lysed and missing spermatozoa is needed. Fertilization techniques should be quantified in terms of the number of spermatozoa used for specific numbers of oocytes (e.g. sperm-to-oocyte ratio), the associated water volumes and concentrations, gamete holding times, and protocols used for activation of the gametes.

The percentage activation, motility, and also the velocity of spermatozoa can be assessed by observers using phase contrast microscopes, or more accurately percentage motility and velocity by Computer Assisted Sperm Analysis (CASA). CASA objectively measures the percentage of motile spermatozoa and their various types of velocity [82] and analyses the resultant data with a sophisticated statistical programs. In almost all cases, CASA systems rely on head movement of spermatozoa, and free software developed for fish spermatozoa is also suitable for amphibian spermatozoa as a plug-in of ImageJ software [129].

11. Application of amphibian sperm banks

Amphibians produced by *in vitro* fertilisation have been released in supplementation programs (Robert Browne, personal communication), and refrigerated spermatozoa transported between facilities to successfully fertilise oocytes [130], but no amphibians from cryopreserved spermatozoa have been released. In contrast, fish aquaculture has a long history with the use of *in vitro* fertilization since 600 BC in mainland China (Fan-Li The Art of Fish-Breeding 600 BC), and has been widely used globally for the restocking of fish since the mid-20th century [131].

Although the spermatozoa of 200 aquaculture and 60 threatened species has been cryopreserved [132] it has only had limited use in practice especially for threatened populations [5,133]. For example, cryopreserved spermatozoa have been used in a CBP for marble trout (*Salmo marmoratus*) to maintain pure strains [5], and pallid sturgeon (*Scaphirhynchus albus*) juveniles included in general releases (William Wayman, personal communication).

The concept of sperm banks to support CBPs for select species is separate from archival genetic resource banking (AGRB) for the widest range of possible species for taxonomic and other purposes. The banking of amphibian spermatozoa requires cryopreservation, whereas, AGRB for taxonomic purposes requires the storage of samples, preferably including the whole specimen, at room temperature. In contrast to the indefinite storage period of AGRB, the storage period and the use of cryopreserved spermatozoa must be defined within a Sustainable Management Plan. Limited resources must be focused on species where the programs goal is the reestablishment of genetically varied populations in nature, with these examples then extending to the broader amphibian CBP community. Not all CBPs will require the use of cryopreserved spermatozoa [104] including those where genetically varied populations may be rebuilt without the use of cryopreserved spermatozoa. At an extreme, access to cryopreserved spermatozoa alone is useless if females are not available.

There are many fish sperm banks in Europe, Brazil, Mexico, USA, and Canada that are mostly dedicated to commercial fish, but between them and others globally house sperm samples from hundreds of threatened species. Some of these collections include spermatozoa from species that were common at the time of collection but are now endangered or extinct (see review [104]). Sperm banking should be based on forming links and partnerships, between the target CBP and other participating entities

595 including civil and governmental institutions, within a framework of overlapping and shared interests.
596 The establishment of communication networks and information portals, along with standardization of
597 terminologies and lexicon provide for efficient communication [104].
598
599 Future expansion of the use of cryopreserved spermatozoa for aquatic species in aquaculture and
600 will mostly be based on advances in high-throughput cryopreservation and commercial-scale application
601 [134,135]). With amphibians the optimal cryopreservation protocol will vary dependent on the species,
602 however, as the field develops greater standardization of protocols for at least each species will be
603 advantageous [104,134], through increasing offspring production, and minimizing variability and the
604 waste of samples (Fig. 8).
605
606 Insert Fig. 8.
607
608 Lifecycle Proof of Concept studies (Fig. 8. [18]) should now be integrated within select CBPs;
609 based on the species conservation status, CBP facilities ability to complete the lifecycle, and the
610 potential for release into their natural environment. Complete integration requires three stages, the
611 development of appropriate technologies, funding for the establishment and maintenance of sperm
612 banks, and the integration of sperm banks into CBPs.
613
614 The minimum number of males needed for CBP to maintain 90% of a species genetic variation
615 in a 55 year CBP is 75 males, with numbers dramatically increasing with shorter generation times and
616 lower longevity [136]. The cost of sperm banking depends on the scale where the minimum of one
617 cryostorage container can house many hundreds of samples, and as storage capacity increase the storage
618 costs per sample lowers and costs for other capital items such as a microscope stay static. The location
619 of facilities in the low income regions of the highest amphibian biodiversity will generally lower labour
620 costs [104]. The estimated total costs for sperm processing, cryopreservation, and storage of each
621 sample in the USA, based on Caffey and Tiersch [135], is ~$5.00 USD for the first year and less for
622 subsequent years. The initial part of this cost for processing will be species specific, but in any case the
623 cost of sperm banking is one to two orders of magnitude less than keeping live males and more secure.
624

The development of techniques for the cryopreservation of amphibian sperm have almost exclusively been in moderate to high income industrialised countries, except recently for three anuran species in Ecuador (Belin Proaño and Oscar D. Pérez, personal communication, [44]). Yet most amphibian species, except SE North America and eastern Australia, are found in the low to moderate income countries of Central and South America, SE Asia, New Guinea, Africa and Madagascar (Fig. 9). Most currently threatened amphibians come from Central and South America (Fig 9. [137]).

Insert Fig. 9.

Sperm banks and CBPs are ideally be located within species range, where males can be sampled opportunistically, cumbersome legislation regarding spermatozoa transport between facilities does not apply, and the CBP relates to the general sustainable management of the regional environment. However, the number of institutionally supported CBPs in or out of range countries can only support about 10% of species in need of conservation actions. Private keepers CBPs in or out of range offer an opportunity to prevent the extinction of the 90% of neglected amphibian species, along with supporting in range CBPs, habitat protection and restoration, and increasing public perception and political influence for the sustainable management of amphibian biodiversity [138].

12. Conclusion and future directions

There is an increasing need for the storage of spermatozoa in the sustainable management of amphibian biodiversity. Techniques for the use of cryopreserved spermatozoa are developed and there is no technical reason that sperm banking cannot be implemented for many species programs. Considering the depth of the amphibian conservation crisis it is imperative to develop ¨Proof of Concept Projects¨ for the use of cryopreserved spermatozoa in CBPs, and that broadly engage the global amphibian conservation community and promote the sustainable management of the environment in general.

Techniques for the collection and refrigerated storage of amphibian spermatozoa are well advanced in the Anura and Caudata, but neglected in the Sirenidae and Caecilians. Sperm banking must embrace the diversity of reproductive modes in amphibians, and further develop techniques to optimize the cryopreservation of their spermatozoa. Patterns between species phylogeny, their evolutionary

history, and reproductive modes will lead to more generalized concepts regarding the cryopreservation of amphibian spermatozoa. This progress will be furthered by a greater understanding of the critical components of protocols, and a greater standardization of methods to enable more meaningful comparisons between studies and to focus on critical points in the cryopreservation process.

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Legends for Figures

Fig. 1. The rapid effects of selection in captive breeding are shown in these colour varieties of the Chinese giant salamander (*Andrias davidianus*) which now has more than 12×10^6 individuals in aquaculture, mostly hybrids between 5 recently revealed cryptic species. *Image Robert Browne*.

Fig. 2. The evolutionary relationships between major amphibian clades, orders, families and species with respect to major parameters informing an understanding of sperm storage and cryopreservation. Studies may have included more than one species, and the assessment methods are for all studies of each species (see Supplementary Table 2,3 for more detail). Blue = External fertilisation, Red = Internal fertilisation.

Fig. 3. Hormonal stimulation and sperm collection in the Panamanian Golden Frog (*Atelopus zeteki*). A. Intraperitoneal injection of GnRHa; B. Spermic urine collection by abdominal massage; and C. Spermic urine collection by gentle insertion of a catheter in the cloacae. *Image Gina Della Togna*.

Fig. 4. A. Collecting semen from a cryptobranchid, *Cryptobranchus alleganiensis*, through abdominal massage at Nashville Zoo, USA. *Image Robert Browne*; and B) hormonally induced tiger salamander, *Amystoma tigrinium*, semen from the cloaca at National Amphibian Conservation Center Detroit Zoological Society, Detroit, USA. *Image Ruth Marcec*.

Fig. 5. A flow diagram of the different stages in the collection, preparation of cryosuspensions, acclimation, and freezing of amphibian sperm as reported across various amphibian studies (Supplementary Table 2,3). Temperatures in °C. *Neurergus kaiseri*. *Image Richard Bartlett* http://news.mongabay.com/2010/0208-hance_luristannewt.html

Fig. 6. The phylogenetic relationship between four anuran families, with the Pipidae diverging form the others ~210 mya, the Ranidae from the Hylidae and Bufonidae ~170 mya, and the Hylidae and Bufonidae diverging ~70 mya (Adapted from Brelsford et al. [119]).

Fig. 7. A. *Ambystoma mexicanum* sperm stained with eosin nigrosin, 40× magnification. B. *Ambystoma* spermatophore stained with Trypan Blue. Glycoproteins on the spermatophore highly stain but the sperm package is intact to the vital stain and the enclosed sperm do not stain, and C. Hoesch staining is used to test acrosomal integrity. *Image Manuel Gonzalez*.

Fig. 8. Flow chart of suggested Proof of Concept study to complete the life cycle of a threatened species using cryopreserved sperm in a CBP. Warm temperatures in brown, and cool and colder temperatures in blue. *Image Robert Browne.*

Fig. 9. Global diversity of amphibian species, the distribution of threatened amphibians, average income, and the locations of research for the collection and storage of amphibian sperm for the sustainable management of amphibian biodiversity. Anurans (black circles) and Caudata (Yellow circles). Both Anura and Caudata are found in Australia, but only Anura are native. The size of circles roughly approximates research on sperm storage.

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