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Applied reproductive technologies and genetic resource banking for amphibian conservation

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Abstract. As amphibian populations continue to decline, both government and non-government organisations are establishing captive assurance colonies to secure populations deemed at risk of extinction if left in the wild. For the most part, little is known about the nutritional ecology, reproductive biology or husbandry needs of the animals placed into captive breeding programs. Because of this lack of knowledge, conservation biologists are currently facing the difficult task of maintaining and reproducing these species. Academic and zoo scientists are beginning to examine different technologies for maintaining the genetic diversity of founder populations brought out of the wild before the animals become extinct from rapidly spreading epizootic diseases. One such technology is genetic resource banking and applied reproductive technologies for species that are difficult to reproduce reliably in captivity. Significant advances have been made in the last decade for amphibian assisted reproduction including the use of exogenous hormones for induction of spermiation and ovulation, *in vitro* fertilisation, short-term cold storage of gametes and long-term cryopreservation of spermatozoa. These scientific breakthroughs for a select few species will no doubt serve as models for future assisted breeding protocols and the increasing number of amphibians requiring conservation intervention. However, the development of specialised assisted breeding protocols that can be applied to many different families of amphibians will likely require species-specific modifications considering their wide range of reproductive modes. The purpose of this review is to summarise the current state of knowledge in the area of assisted reproduction technologies and gene banking for the conservation of amphibians.

Additional keywords: anuran, cryopreservation, frog, *in vitro* fertilisation, ovulation, reproduction, spermatozoa, toad.

Introduction

Amphibian conservation has received a great deal of attention in the last decade, owing to the large number of species that are presumed to have become extinct or are currently experiencing rapid population declines. It is estimated that nearly one-third (32%) of known amphibian species are threatened with extinction worldwide (IUCN, Conservation International, and NatureServe 2006). A comprehensive amphibian survey estimates that 34 species are likely to have become extinct; however, it is expected that the number more closely approaches 165 species, because many of the animals considered to be critically endangered have not been seen for years, and in some cases a decade (IUCN, Conservation International, and NatureServe 2006). Of greatest concern is the fact that nearly half of all amphibians are continuing to decline in population, suggesting that the number of threatened species can be expected to rise in the near future. In North America, 55 of 262 species are threatened, representing ~21% of all amphibian species on the continent (Young *et al*. 2004).

Although habitat loss still plays a major role in amphibian fatalities (Green 2005; IUCN, Conservation International, and NatureServe 2006), several causative agents that are not commonly attributed to the biodiversity crisis are associated with population declines. Since the early 1990s, a great deal of research has been focussed on increased ultraviolet radiation (Blaustein *et al*. 1997; Crump *et al*. 2001; Hakkinen *et al*. 2001; Blaustein and Belden 2005), climate change (Alexander and Eischeid 2001; Carey *et al*. 2001; Stallard 2001; Reaser and Blaustein 2005) and environmental stressors (e.g. pesticides and xenobiotics; Sparling *et al*. 2001; Hayes *et al*. 2002; Withgott 2002; Bridges and Semlitsch 2005*a*, 2005*b*) as potential culprits for decreasing populations. Because many amphibian life cycles are dependent upon a mix of aquatic and terrestrial habitats and the animals have highly porous skin, it is commonly believed that amphibians may be particularly susceptible to environmental contaminants, strange weather patterns and changing global temperatures, and as such, they are often viewed as 'indicator species' for environmental health. Although these global events are all likely to affect amphibian populations in some capacity, many of the reported population declines occur in pristine, unspoiled alpine forests. In fact, amphibian species occurring at high elevations, having restricted distributions or

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characterised by terrestrial life cycles (do not have aquatic tadpoles) are more likely to be threatened than are species with other characteristics (IUCN, Conservation International, and NatureServe 2006). Why do these traits cause certain species to be more susceptible to decline? For as-yet unknown reasons, these amphibians appear to be more susceptible to a rapidly spreading disease known as chytrid fungus (*Batrachochytrium dendrobatidis*) (Pessier *et al*. 1999). The catastrophic epidemics caused by the spread of this fungus are causing mass extinctions of amphibian populations worldwide (Rabb 1999; Bradley *et al*. 2002; Williams *et al*. 2002).

A great deal of time and many resources have been applied towards documenting amphibian declines, monitoring the status of their populations, and exploring causal factors (Rabb 1999; Alford *et al*. 2001; Kiesecker *et al*. 2001), yet, comparatively little effort is devoted to preserving those species that are suspected to be most at risk. In 2005, an amphibian conservation action summit was held in Washington DC, USA, with the goal of determining how best to respond to the global amphibian crisis. One of the action steps that emerged from this summit was the need to secure, in zoological facilities, assurance colonies of amphibians that are in imminent danger of extinction (Gascon *et al*. 2007). However, due to the limited space and resources available to zoos and aquariums, these institutions will be unable to preserve the growing number of amphibian species experiencing population declines worldwide.

The amphibian genetic resource bank

As the challenges, complexity and massive scale of such rescue efforts become more apparent, biologists are looking for additional means to secure some portion of the amphibian biodiversity at risk of being lost. The establishment of biomaterial resource collections to support captive breeding programs and research is one such tool that can be implemented and need not conflict or compete for resources with other *ex situ* or *in situ* conservation efforts. Soule (1991) proposed the creation of suspended *ex situ* programs, or genetic resource banks (GRBs), in which cryopreserved germplasm and seed banks maintained in a metabolically arrested state can contribute to biotic survival. The establishment of a GRB for amphibians is but one component of the toolbox needed to address the extinction crisis (Kouba *et al.* 2009) and strategies should be incorporated into species recovery plans to conserve as much diversity from founder populations at the initiation of captive assurance colonies. To provide the greatest variety of options for future species management or research, a collection of germplasm, embryos, tissues, DNA and cell cultures should be preserved (Ryder *et al*. 2000). Additionally, by creating biomaterial banks with a variety of different cell types and tissues, resources can be provided for other scientific disciplines such as pathogenesis of disease, phylogenetics, systematics and medical research.

For a GRB to be effective it is important to determine what the goal of its stored biomaterials should be. The initial goal of a GRB should be to help to maintain up to 90% of all existing gene diversity for an extended period (e.g. 100 years) by augmenting the effective population size long after the death of the gamete donors. Hence, GRBs are essentially the storehouses for amphibian genetic material, which can be deposited or withdrawn to support applied reproductive technologies (ART). Applied reproductive technologies are viewed as potentially important contributors to species conservation by helping to sustain the viability of extant populations through genetic management (Holt 2001). To establish such conservation measures, the development of GRBs is essential. These GRBs can then be used to store frozen spermatozoa, eggs and embryos from threatened populations, with the deliberate intention to use them in a breeding program in the future. Although GRBs have been established for various charismatic mega-vertebrates (Wildt 1992; Wildt *et al*. 1997), amphibians, reptiles and birds have been virtually ignored, placing them in greater danger of permanent widespread extinction than mammals. The benefits of establishing GRBs for mammalian conservation have been detailed extensively in several reviews (Wildt *et al*. 1997; Bainbridge and Jabbour 1998; Comizzoli *et al*. 2000; Pukazhenthi and Wildt 2004); however, similar concepts regarding the value of GRBs have not been presented for other vertebrate taxa, such as amphibians.

Several institutions initiated amphibian GRBs within the last decade including the Memphis Zoo, USA (Kouba *et al.* 2009), Cincinnati Zoo, USA (Roth and Obringer 2003), the University of Newcastle, Australia (Clulow *et al*. 1999) and the Institute of Cell Biophysics, Russia (Uteshev *et al*. 2002; Uteshev and Gakhova 2005), with each GRB containing various species of regional interest or conservation concern. While the primary goal of these research programs is to assist captive assurance colonies by decelerating the loss of genetic diversity, a by-product of their studies is a wealth of knowledge generated on the fundamental reproductive biology for rare species in population decline. The establishment of a GRB for amphibians is timely considering the precarious status of these animals in the wild and the enormous amount of information available on the biology of several key laboratory species. Since the early 1900s, amphibians have been widely used as model organisms for biological research, especially in the field of early experimental embryology. Because development in most anurans is external and their eggs are large, they can easily be manipulated *in vitro*. This wealth of knowledge on anuran fertilisation and embryo development will greatly assist in the production of ART for endangered amphibians and allow for the rapid use of gene-banked germplasm. Additional reasons for establishing GRBs for anurans include: (1) current accessibility to animal donors, (2) organised captive breeding and reintroduction programs exist for numerous species, (3) high fecundity with typically no parental involvement, (4) wild populations are at risk due to the rapid spread of disease and (5) several species are difficult to propagate in captivity. Moreover, a plethora of information is being collected on antibiotic peptides, anti-tumour agents, analgesics and adhesive compounds in frog skin (Tyler *et al*. 2007), which, if not preserved in GRBs, could be lost to science due to pandemic diseases such as chytrid fungus. Table 1 summarises several justifications for establishing a GRB for amphibians.

Advantages and opportunities of a GRB and associated ART for amphibian conservation

The potential impact of GRBs is dependent not simply upon successful methods for cryostorage of spermatozoa, but also on the

Table 1. Justification for establishing GRBs for amphibians (adapted from Holt *et al.* 1996 and Bennet 2001)

- *Reproductive failure*: incompatible breeding in toads and frogs could be overcome through the use of *in vitro* fertilisation
- *Increased security*: provides some protection against Chytridiomycosis outbreaks causing local extinctions to amphibian populations
- *Unlimited space*: due to space constraints for live animals, cryobanking offers a large amount of space to conserve diversity
- *Increased gene flow*: transportation of frozen gametes between zoos has advantages over moving live amphibians
- *Minimise introgression*: secures the integrity of a gene pool against the threat of hybridisation when introduced to new pond sites
- *Extend generation times*: the genetic lifespan of a toad or frog is extended thereby reducing loss of alleles (genetic drift)
- *Maximise genetic diversity*: storage of unrepresented founder amphibians, under-represented descendants and deceased animals
- *Minimise inbreeding*: restoring germplasm to unrelated or more distantly related amphibians from different breeding ponds
- *Manage effective population size* (N_e): equalise family size by manipulating age-specific fertility rates and sex ratios
- *Minimise selection*: detailed pedigree analysis combined with GRBs can reduce genetic drift and increase genetic diversity for small amphibian assurance colonies
- *Mutation*: extending generation lengths assists in decreasing the load of harmful mutations in small amphibian populations
- *Preservation of cell lines*: transgenic models in *Xenopus* spp. for human health studies
- *Future benefits*: possibilities of restoring lost genes; discovering medicinal compounds for curing illnesses; pathogenic studies for disease resistance; nuclear transfer or parthenogenesis (gynogenesis and androgenesis) experiments

related assisted technologies for manipulating reproductive processes and ovarian cycles to facilitate the utilisation of banked spermatozoa (Hodges 2001). Key among these technologies are those concerned with the timing of ovarian reproductive events, specifically, *in vivo* oocyte maturation, ovulation and oviposition. There is considerable variation in the length of amphibian ovarian cycles, both within and between species, and the prediction and timing of ovulation in natural cycles is not easy. Hence, there are several practical advantages of exerting external control over the timing of these events, particularly for improving the efficiency of *in vivo* or *in vitro* fertilisation (IVF). Because breeding in most amphibians occurs in a seasonal manner, the ability to induce maturation and ovulation in a quiescent (non-cyclic) ovary offers the potential for extending the breeding season and collecting gametes out of season for cryopreservation or IVF. Other advantages of manipulating the ovarian cycle and oviposition in amphibians include: (1) preventing retention of mature eggs (dystocia), (2) circumventing hibernation requirements for inducing reproductive behaviour, known to induce bacterial and fungal infections and (3) synchronising egg and sperm release to optimise fertilisation.

A common question often posed by reproductive biologists is how to actively incorporate gamete material (e.g. spermatozoa) stored in a GRB into a captive breeding program that necessitates the use of ART? This has been a difficult question to answer as challenges associated with animal management often complicate the application of assisted breeding technologies. While GRBs are useful to any species management program, the application of ART has not proven very effective in mammals and is being regularly implemented in only one species reintroduction program, that of the black-footed ferret (Howard *et al*. 2003; Howard and Wildt 2009). Invasive techniques like oestrous synchronisation, artificial insemination, IVF, and embryo transfer along with stress-related anaesthetics have all proven to be difficult to establish reliably in exotic mammalian species. By contrast, many aquatic-breeding amphibians exhibit external fertilisation (Whitaker 2001), eliminating the need for stressful invasive techniques and anaesthetics. Therefore, conserving amphibians through GRBs and ART is likely to be highly successful, especially considering the simplistic nature of amphibian external fertilisation and development in an undefined medium, such as water.

The low numbers of oocytes produced during a mammalian oestrous cycle and the requirement for internal fertilisation and placental development places mammals at a considerable disadvantage compared with amphibians when evaluating the long-term application and success of ART in population management. For instance, there are very few examples in the literature where mammals were produced by cryopreserved germplasm and subsequently released to the wild. In contrast, the potential success of such conservation technologies for amphibians is much greater when considering the high fecundity rate (up to 5000 offspring in a single reproductive event), producing enormous populations that can be reintroduced into suitable habitat. In fact, reintroductions have already taken place for many threatened species, such as the Wyoming toad, Puerto-Rican crested toad, Houston toad, midwife toad, boreal toad, green and golden bell frogs, Ramsey Canyon leopard frog, Romer's tree frog and Tarahumara frog from natural breeding (Griffiths and Pavajeau 2008; Kouba *et al.* 2009; B. Foster and D. Barber, pers. comm.) and it would take little additional effort to produce animals by ART for release into the wild. However, using banked genetic material for ART in amphibians will require further studies to improve upon present cryopreservation protocols.

GRBs and ART will also be useful in addressing several other pressing issues for captive breeding of amphibians. Due to disease concerns and quarantine issues related to the transfer of animals between institutions, many genetically valuable animals are not being moved between facilities in time to breed during the narrow seasonal window needed for release of tadpoles. Having frozen stored material on hand could serve as a back-up if males cannot be moved out of quarantine in time, or as an alternative to moving males between facilities. Furthermore, multiple paternities could be generated from one egg mass by using gene-banked spermatozoa from many different males and conducting IVF. Although the spermatozoa did not originate from

frozen–thawed samples, the use of multiple sires to fertilise eggs has already proven to be effective in the Wyoming toad (Browne *et al*. 2006*b*). As ART becomes more significant in reproducing amphibian captive stock, as is commonplace in the fish industry, it will be possible to fertilise a single female's eggs with spermatozoa from numerous males of genetic importance. Another advantage to gene banking amphibian spermatozoa is that for many institutions space is a limiting factor. This problem could be minimised by reducing the number of males held in captive facilities if sperm samples were available in cryostorage and ART protocols were available for the targeted species. Lastly, male amphibian sperm production is often seasonally dependent, especially for animals that hibernate. Low reproductive output or poor fertilisation rates from seasonal fluctuations in sperm production could be overcome by banking spermatozoa from males during the height of seasonal reproduction, insuring that sufficient numbers of spermatozoa are available for breeding any time of the calendar year. However, procurement of a suitable number of gametes for IVF and gene banking is sometimes quite challenging for species in which studies utilising exogenous hormone stimulation are limited.

Hormonal control of spermiation and ovulation

There are two commonly used hormone preparations for inducing spermiation and ovulation in amphibians, human chorionic gonadotropin (hCG) and luteinising hormone releasing hormone (LHRH) (reviewed in Whitaker 2001). Due to some inherent risk associated with administration of these hormones they should only be used when natural reproduction fails or for research studies designed to improve their use. In addition to LHRH and hCG for stimulating reproduction, frog pituitary extracts, containing a myriad of other hormones, can be purchased from commercial dealers or can be prepared by following the guidelines described by Rugh (1965). Typically, pituitary preparations are administered via the dorsal lymph sac to stimulate sperm letdown or ovulation into the cloacae (Cabada 1975; Subcommittee on Amphibian Standards 1996). However, it should be noted that pituitary extracts may harbor transmissible diseases and some caution should be used regarding the species and source of the extracts. In general, pituitary extracts are discouraged and rarely used considering the global loss of amphibians and the additional risks these extracts pose.

When first considering the use of exogenous hormones for assisted reproduction, careful consideration needs to be given to the route of administration. Injection sites that receive the greatest targeting in the literature are intra-peritoneal (i.p.) (Obringer *et al*. 2000; Kouba *et al*. 2003; Roth and Obringer 2003; Browne *et al*. 2006*a*, 2006*b*) or administration via the dorsal lymph sac (Wolf and Hedrick 1971). *Bufo americanus* produced greater amounts of spermatozoa and had higher circulating levels of plasma LH when receiving an i.p. injection compared with either subcutaneous or ventral/dorsal absorption (Obringer *et al*. 2000). Rowson *et al*. (2001) attempted to find a means whereby ventral absorption could be improved by testing various carrier or chaperone agents that would facilitate cutaneous transport across the integument. Of those compounds tested, dimethylsulfoxide (DMSO) provided the best results with 70% of the animals producing spermatozoa in response to treatment; however, sperm concentration was significantly lower than that obtained by i.p. injection. Moreover, the use of DMSO on the toads' dermis caused a swelling and reddening of the tissue in the immediate treatment area suggesting that some adverse health effects might have developed. Due to the selective transport nature of amphibian skin it is uncertain whether a topical hormone will ever provide as definitive results for sperm concentration as i.p. or dorsal lymph sac injections. However, research in this area is still warranted especially for amphibians the size of dart frogs (*Dendrobates* spp.) considering that these species are extremely small and it is very difficult to safely administer hormone injections even through 30-gauge needles. It may be that some frustrations in captive breeding programs using exogenous hormones are a result of inappropriate administration sites, such as in the leg muscle or under the skin, which may not provide the best route for circulation in amphibians.

Although the ability of exogenous hormones to stimulate spermiation in live adult anurans has been known for nearly 60 years, the majority of studies to date sacrifice the male to obtain sperm suspensions from testicular macerates (Wolf and Hedrick 1971; Hollinger and Corton 1980; Browne *et al*. 1998, 2002*a*, 2002*b*, 2002*c*, 2002*d*; Edwards *et al*. 2004; Fitzsimmons *et al*. 2007). Arguably, the advantage of using testis macerates is that a much higher sperm concentration can be obtained for fertilisation when compared with using hormonally stimulated live animals to obtain spermic urine. However, the highest fertilisation rates *in vitro* (Cabada 1975; Browne *et al*. 1998; Edwards *et al*. 2004) for ~100–200 toad eggs is achieved with sperm concentrations in the range of 10^5–10^6 spermatozoa mL^{-1}, which can be readily obtained from live animals using hormonal stimulation (Iimori *et al*. 2005; Browne *et al*. 2006*b*; Kouba *et al.* 2009). Waggener and Carroll (1998*a*) point out that, if sacrificed, the genetic contribution of the male cannot be studied in subsequent experiments, nor can *in vitro* studies on gamete function be examined accurately due to sperm preparations that contain both live and dead sperm cells as well as testicular accessory cells. Moreover, such sacrificial techniques are inappropriate for endangered animals where every male is genetically valuable. Non-invasive studies on induced spermiation in common model species (Waggener and Carroll 1998*a*; Obringer *et al*. 2000; Rowson *et al*. 2001; Kouba *et al*. 2003, 2009; Roth and Obringer 2003; Iimori *et al*. 2005) are more appropriate for application to endangered species when the goal is long-term preservation of the germ line and reproduction.

Exogenous hormone use in male amphibians

In the late 1940s, Galli-Mainini of Argentina developed a human pregnancy test that involved injecting male *Bufo arenarum* with a small portion of urine from pregnant human female test subjects (Galli-Mainini 1947, 1948). After 3–4 h, spermic urine could be collected from the toad's cloacae by introduction of a small pipette, confirming the pregnancy. For nearly 30 years following this discovery, male amphibians were used as pregnancy test models and it was not until the discovery of monoclonal antibodies in the 1970s that the bioassay technique using live animals was replaced with immunoassays. The active hormone in a

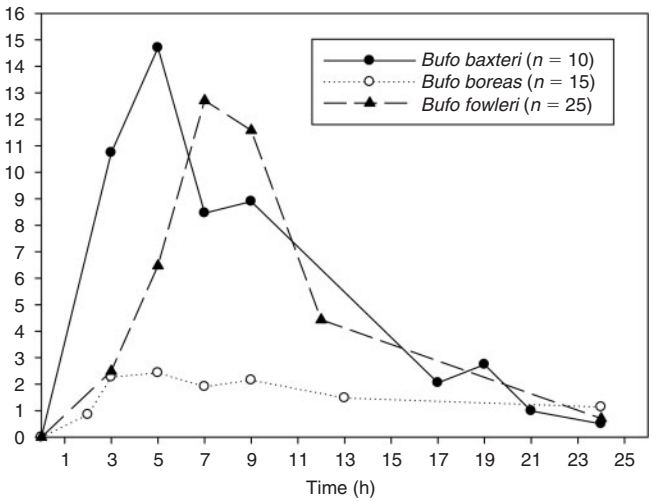

Fig. 1. Average sperm concentration in urine over time after a single intraperitoneal hormone injection of 300 IU hCG, for three different species of toads (*Bufonids*).

pregnant woman's urine that induces spermiation and ovulation in amphibians is hCG and is known to have LH-like bioactivity. Considerable sequential and structural overlap occurs between the β subunits of LH and hCG (Norris 2007), which likely allows for this cross-reactivity between human and amphibian hormones and their receptors. Genes for both LH and hCGβ subunits, which confer their unique biological activity, are located on chromosome 19, thus the βhCG gene may have arisen from an early duplication of the βLH gene sometime during the period when mammals evolved from reptiles (Norris 2007).

The hormone hCG induces spermiation in a wide variety of toads and frogs (McKinnell *et al*. 1976; Easley *et al*. 1979; Subcommittee on Amphibian Standards 1996; Clulow *et al*. 1999; Iimori *et al*. 2005; Browne *et al*. 2006*a*, 2006*b*; Pozzi *et al.* 2006) and is commonly used in the commercial breeding of *Xenopus laevis* (Schultz and Dawson 2003). In male amphibians, testosterone secretion increases in response to an hCG challenge for *Bufo marinus* (Iimori *et al*. 2005) and *Rana tigrina* (Kurian and Saidapur 1982) and testosterone concentrations peaked 2 h after administration of hCG for *Bufo marinus*. Peak sperm production typically occurs 3–6 h after administration of hCG with concentrations declining to near zero 12–24 h later for *Bufo baxteri* (Browne *et al*. 2006*a*), *Bufo fowleri* (Kouba *et al.* 2009) and *Bufo boreas boreas* (Fig. 1). Fig. 1 shows the concentration of spermatozoa produced over time in response to 300 IU hCG for three different species of male bufonids. The time to peak sperm production shown for the three bufonids in Fig. 1 supports the observations by Iimori *et al*. (2005) that sperm production is highest shortly after testosterone concentration is maximal. Species-specific differences can be observed in time to peak sperm production and concentration of spermatozoa, highlighting the observation that when adapting existing protocols to a new species, it will be important to understand how the animals respond to hormones in order to provide optimal chances for fertilisation. In contrast to bufonids, *Rana pipiens* sperm production, after a combined treatment of hCG (500 IU) + LHRH (15 μg), occurs rapidly with peak production in 30–60 min and is depleted in 2–3 h (Kouba *et al.* 2009).

While hCG is most commonly used for research employing IVF (Browne *et al*. 2006*a*, 2006*b*) and for commercial breeding of *Xenopus* species (Schultz and Dawson 2003), it is rarely used in zoological facilities or for captive breeding programs, where natural breeding is preferred over IVF. In zoos and aquariums the hormone LHRH has been used extensively for stimulating spermiation in toads and frogs (Easley *et al*. 1979; Goncharov *et al*. 1989; Waggener and Carroll 1998*a*; Rowson *et al*. 2001; Whitaker 2001; Kouba *et al*. 2003; Roth and Obringer 2003) as well as salamanders and newts (Vellano *et al*. 1974; Goncharov *et al*. 1989). Several different hormones and synthetic analogues of LHRH and LH have also been tried over the years for stimulating breeding in amphibians. The most effective synthetic analogue of LHRH to date has been D-Ala6, des-Gly10 ethylamide (Arimura *et al*. 1974), which has been modified for an extended half-life *in vivo*, thus increasing its effectiveness. Originally, this LHRH derivative was tested in goldfish (Lam *et al*. 1975) and subsequently gained popularity for assisted breeding in the commercial fish industry (Lam 1982), but has since proven effective in amphibians. However, in contrast to the efficient hormone application within the commercial fish industry, specific information regarding hormone concentrations or administrative procedures for amphibians has traditionally been passed along by word of mouth between institutions (Whitaker 2001), and the optimisation of LHRH derivatives for amphibian breeding remain largely unexplored.

The general lack of knowledge regarding appropriate LHRH hormone dosages for individual species has led to many frustrations that are currently encountered during assisted breeding in zoological institutions (e.g. poor fertilisation rates of what appear to be good quality eggs). Goncharov *et al*. (1989) found a wide range of sensitivity to LHRH after testing more than 40 amphibian species for induced spawning via exogenous hormone treatment. In some cases amphibians could be induced to spawn after one treatment of LHRH at $2\,\mu g\,kg^{-1}$, whilst in other instances repeated injections were needed at much higher concentrations ($8\,mg\,kg^{-1}$), representing a 4000-fold range in hormone potency. Because the LHRH dosage, number of injections, and intervals between injections differed so dramatically between species in these experiments (Vellano *et al*. 1974; Goncharov *et al*. 1989) it was noted early on that specific protocols would need to be developed for each. Thus, there is a great need for empirical studies on the efficacy of LHRH and its derivatives in inducing spermiation for anuran species being brought out of the wild to establish assurance colonies and that are difficult to breed in captivity.

In our laboratory, a direct comparison of the effectiveness of hCG (50–500 IU) and LHRH (0.1–10 μg) to induce spermiation in *Bufo fowleri* revealed that hCG provided significantly greater sperm concentration of better quality than LHRH (A. J. Kouba, C. Milam, M. Joyce, J. delBarco-Trillo, M. Carr, unpubl. data). However, LHRH induced more males to amplex females and display reproductive behaviours, suggesting that the hormone's actions have a stronger effect at the level of the brain, yet are not as effective in stimulating spermiation. Research is currently underway to see if a cocktail mixture of the two hormones, hCG

and LHRH, provides a better response than either hormone individually. Human CG has been used on a limited basis for induced natural breeding in the endangered *Bufo baxteri* (B. Foster, pers. comm.) and *Peltophrynes lemur* (D. Barber, pers. comm.) breeding programs with mixed results. The hormone hCG may be ineffective on its own for stimulating natural amplexus and mating in many species; however, its usefulness for IVF is much more relevant as higher concentrations of spermatozoa can be collected via cloacal lavage or urinary stimulation compared with LHRH.

Multiple use of hormones over time

For mammals, the efficacy of hCG over time wanes and is determined by an immunogenic response and production of gonadotropin-neutralising immunoglobulins due to administration of large foreign glycoproteins (Swanson 2006). At most, 3–4 injections may be effective in mammals before a sufficient antibody response is mounted to negate the hormone's effectiveness (Swanson *et al*. 1995, 1997). Amphibians, on the other hand, do not have as highly evolved antibody-based defence mechanisms and rely primarily on antimicrobial skin secretions or other granular substances for protection against pathogens (Duellman and Trueb 1986). Hence, amphibians can receive multiple injections throughout the year with no adverse immunological response. In our laboratory, male *Bufo fowleri* have been injected every other week for several years with no decrease in the number of responders or sperm concentration (A. J. Kouba and C. K. Vance, unpubl. data). The importance of this immunologic difference cannot be underscored enough and has been one of the primary reasons that ART in amphibians has advanced so rapidly and has a greater chance for success than in mammals. For example, repeated hormonal stimulations of endangered Wyoming toads (*Bufo baxteri*) are necessary each year and have resulted in over 100 000 tadpoles released into the wild (Dreitz 2006; B. Foster, pers. comm.). Wyoming toads have never bred in captivity without one sex having received hormone stimulation (A. Odum, pers. comm.) and the success of this program would be limited if not for the continued administration of hormones on a yearly basis to the same individual animals. However, Roth and Obringer (2003) found that *Bufo marinus* treated with LHRH twice weekly showed sperm depletion at a higher rate than those treated once a week. This response is most likely due to down-regulation of LHRH receptors and time needed to recycle these proteins back to the cell membrane, rather than to an immunologic response. Some evidence suggests that male amphibians procured from the wild respond poorly to exogenous hormones outside of their normal breeding season (Biesinger and Miller 1952); however, male anurans maintained in a laboratory setting under constant environmental conditions tend to respond better to exogenous hormones than those recently acquired from the wild during periods of aestivation.

Exogenous hormone use in female amphibians

The induction of ovulation in female amphibians by exogenous hormone administration has also been known for nearly 70 years. Similar to the pregnancy test described in the previous section using males, human urine containing hCG stimulates ovulation in female frogs (Bellerby 1934; Shapiro and Zwarenstein 1934). The worldwide distribution of amphibians for pregnancy testing was an enormous enterprise for more than 30 years and in 1962 more than 11 000 amphibians were imported into South Australia alone for pregnancy testing (Tyler *et al*. 2007). It has been surmised that this worldwide distribution of frogs, especially *Xenopus laevis* out of South Africa for pregnancy testing, may have initiated the global spread of the epizootic disease chytrid fungus (Weldon *et al*. 2004). While many studies involving sperm collection for ART utilised testis macerates from sacrificed animals, oocyte collection has predominantly been non-invasive or involved minimal stress. Detailed information on the use of hCG, LHRH or pituitary homogenates to induce ovulation is limited to only a few key laboratory anuran species (e.g. *Xenopus laevis* and *Rana pipiens*) and virtually nothing is known for urodeles or caecilians.

One of the rate-limiting steps in developing ART for amphibians is the acquisition of gametes, especially oocytes. Whereas males can be sacrificed and testis macerates collected easily for IVF, obtaining fertilisable oocytes can sometimes be difficult. Females receiving exogenous hormones have been shown to ovulate spontaneously (Browne *et al*. 2006*a*, 2006*b*) or oocytes can be stripped from the female after gentle pressure (Subcommittee on Amphibian Standards 1996). Injections of hCG or LHRH can result in ovulation of fertilisable eggs up to 7 h after administration (Brun 1975; C. K. Vance and A. J. Kouba, unpubl. data) due to contributions of oviducal secretions not present with eggs derived from the body cavity (Wolf and Hedrick 1971; Ishihara *et al*. 1984; Krapf *et al*. 2007). Body cavity oocytes are not capable of fertilisation unless co-incubated with steroids in culture. Progesterone can induce final maturation of amphibian oocytes *in vitro* within ~24 h (Schuetz 1971; Masui and Clarke 1979). It is preferable to develop protocols for spontaneous ovulation using non-invasive hormone techniques as egg jelly from oviducal secretions also plays an important role in preparing the spermatozoa for fertilisation (Ishihara *et al*. 1984) and especially in helping spermatozoa acquire the ability to undergo the acrosome reaction (Ueda *et al*. 2002).

The hormone LHRH (Arimura *et al*. 1974) has been used extensively for induction of ovulation by captive breeding programs in the USA (Whitaker 2001). Concentrations of LHRH used are typically in the range of 2–6 μg/50 g toad or frog; however, appropriate concentrations have rarely been tested empirically and are usually associated with trial-and-error breeding attempts, with more errors than successes. Whereas LHRH is used extensively to induce spermiation in male anurans (Waggener and Carroll 1998*a*; Roth and Obringer 2003), its use for stimulating ovulation is less studied. Currently, the captive breeding programs for the endangered *Peltophrynes lemur*, *Bufo boreas* and *Bufo baxteri* all recommend an LHRH concentration of 0.1 μg/g toad to induce ovulation. When animals are left unhibernated LHRH is not very efficient at stimulating ovulation, yet LHRH combined with periods of low temperatures before hormone stimulation provide a higher frequency of ovulation success (D. Barber and B. Foster, pers. comm.). Because extended hibernation can cause death to the animals from bacterial, viral or fungal infections, there is a rising interest in the use of exogenous hormones alone for induction of

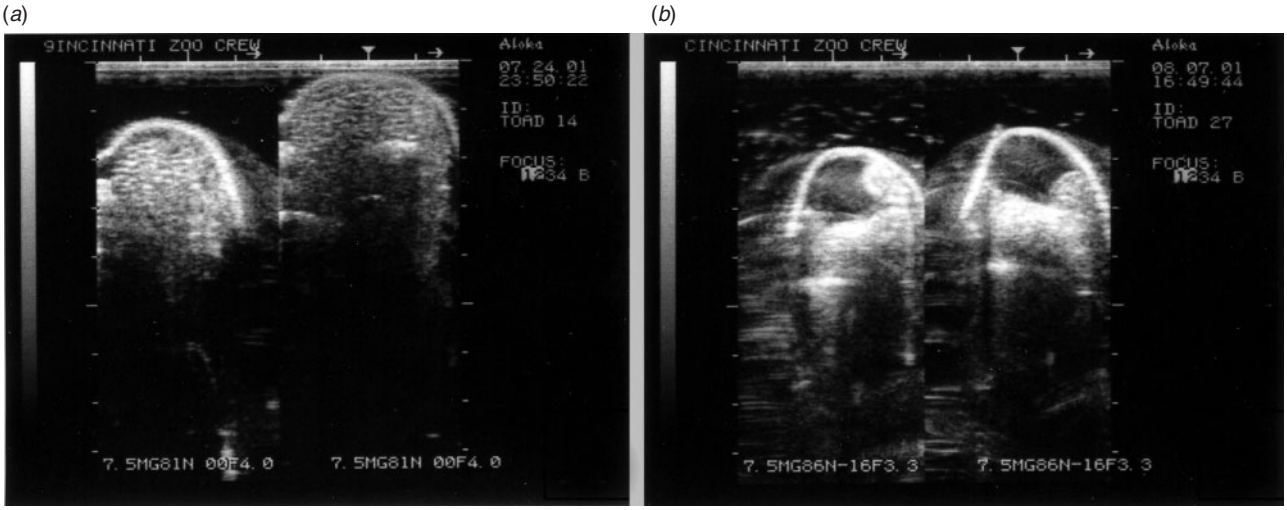

Fig. 2. Ultrasound images of an American toad (*Bufo americanus*), right and left side, (*a*) that did not ovulate compared with (*b*) a female that ovulated after hormone administration with hCG. The ovarian tissue is full of oocytes before hormone administration, yet after treatment ovarian tissue mass falls by an average of 40%.

ovulation. Therefore, it is crucial that simple dosage curves be developed for LHRH in programs that are employing this hormone. Michael and Jones (2004) tested varying concentrations of LHRH from different species (mammalian, avian, fish and a synthetic version), as well as hCG, for their ability to stimulate ovulation in *Eleutherodactylus coqui*. These investigators found that 20 μg of the synthetic LHRH was necessary to reliably stimulate ovulation in an average 9.0-g frog. On a per weight basis, this amount is more than 25 times higher than the amount given to *Peltophrynes lemur*, *Bufo baxteri* or *Bufo boreas*, suggesting that the inefficiency of this hormone in their program may be due to a lack of knowledge regarding its potency.

The hormone hCG has been used extensively by researchers to induce ovulation in female amphibians (Subcommittee on Amphibian Standards 1996; Browne *et al*. 2006*a*, 2006*b*; Kouba *et al.* 2009) and on a more limited basis within zoological institutions (Whitaker 2001). Concentrations of hCG typically used to induce ovulation in frogs and toads range from 500 to 1000 IU (Subcommittee on Amphibian Standards 1996) and are species-dependent. Unlike LHRH, concentrations of hCG used for ovulation are rarely expressed on a per weight basis and are usually articulated as a series of international unit (IU) concentrations for all females regardless of weight. While hCG has been used effectively to induce ovulation in a large number of amphibian species, hCG shows low cross-reactivity with amphibian receptors. Thus, the hormone is effective for stimulating ovulation only at extremely high concentrations, levels that would be considered excessive for any mammalian species. For example, a tiger (*Panthera tigris*) might receive a maximal dose of 1000 IU hCG to initiate ovulation (Graham *et al.* 2006), whereas *Xenopus laevis* regularly receive 500-IU injections for a breeding female (Subcommittee on Amphibian Standards 1996). This represents approximately a 2000-fold increase in the amount of hormone given a frog on a weight basis compared with a tiger (e.g. 200 kg for *P. tigris v.* 50 g for *X. laevis*). Furthermore, species-specific differences in hormone use and ovulation have been known for some time as artificial fertilisation techniques developed for *Rana pipiens* (Rugh 1965) are not applicable to *Xenopus laevis*, which required significant modifications (Wolf and Hedrick 1971). Michael and Jones (2004) found that LHRH was more effective at inducing ovulation in *Eleutherodactylus coqui* than the hormone hCG. In contrast, our laboratory found that 10 IU/g bodyweight hCG was more effective in stimulating ovulation for *Bufo americanus* and *Bufo fowleri* than 0.1 μg/g bodyweight LHRH as recommended by the captive husbandry manual for *Bufo baxteri* (Spencer 2002) or *Peltophrynes lemur* (Lentini 2002). Although we have not tested varying concentrations of LHRH on ovulation in *Bufo americanus* or *Bufo fowleri*, our model species, a single injection of 500 IU hCG is ~60% effective in stimulating spontaneous ovulation and increasing hCG concentrations above 500 IU (up to 1000 IU) failed to elicit additional ovulations. Ultrasound analysis of the body cavity found no statistical difference in *Bufo americanus* ovarian mass between animals that actually ovulate in response to hormones compared to those that do not (Johnson *et al*. 2002). It is unknown why a percentage of females fail to respond to hCG administration while others will ovulate. However, evaluation of serial ovarian mass ultrasound images can provide a useful tool for estimating the next sequence of hormone injections from previously ovulated females (Fig. 2).

Ovulation failure following single hormone injections in females with an ovarian mass similar to those that do ovulate may be due to immature follicular oocytes. To overcome this obstacle, priming hormones such as a reduced concentration of hCG or repeated injections of LHRH have been used successfully to prime and initiate final maturation of oocytes in various species (Vellano *et al*. 1974; Subcommittee on Amphibian Standards 1996). Crested newts are known to ovulate only after repeated LHRH injections ($n = 8$) every other day with 12 or 24 μg per

Table 2. The number of toads spawning, mean number of eggs produced and total eggs spawned during the fertile period (11.5–17.5 h post-induction) for *Bufo baxteri* having received one or two primings

Data are expressed as mean ± s.e.m. unless otherwise specified. There was a significant difference in the number of eggs spawned at each period, and the total mean number of eggs produced for one or two primings. Previously published in Browne *et al*. (2006*b*)

Hours post-induction	No. toads spawning and egg production with one priming			No. toads spawning and egg production with two primings		
	No. toads spawning	No. eggs (mean ± s.d.)	Total eggs spawned	No. toads spawning	No. eggs (mean ± s.d.)	Total eggs spawned
11.5	0	0	0	8	431 ± 184	3551
12.5	3	376 ± 176	1127	7	520 ± 152	3640
13.5	3	743 ± 242	2230	7	367 ± 68	3675
14.5	3	193 ± 17	1805	7	363 ± 44	2905
15.5	4	468 ± 175	1872	7	218 ± 36	3055
16.5	5	314 ± 70	1579	6	588 ± 156	3530
17.5	4	318 ± 94	1270	7	372 ± 89	2605
Total no. eggs	—	—	9883	—	—	22 961
Mean ± s.e.m.	—	—	1647 ± 167	—	—	3280 ± 159

animal; 6 μg of LHRH was ineffectual in stimulating ovulation (Vellano *et al*. 1974). A priming hormone is typically given at 1/5 to 1/4 the normal ovulatory dose (e.g. 100 IU hCG instead of 500 IU hCG) and may precede an ovulation injection by 24–96 h. In our laboratory, a single hormone injection of hCG can suffice for *Bufo americanus* (Johnson *et al*. 2002) or *Bufo fowleri* toads (Browne *et al*. 2006*a*) to induce ovulation; however, *Bufo baxteri* were unresponsive to a single injection of hCG (Browne *et al*. 2006*b*) when using the same protocol. Instead, priming hormones were found to be extremely effective in inducing ovulation for this species with two priming hormones more successful than one priming hormone alone (Browne *et al*. 2006*b*). Table 2 shows the difference in number of oocytes ovulated for females having received two *v.* one priming hormone. A novel aspect of this same study was the combined use of LHRH and hCG for priming as well as ovulatory injections (Browne *et al*. 2006*b*) and the two hormones given as a cocktail mixture may be more effective than either hormone alone, although to date this has not been tested. The steroid progesterone, which is often used to complete final oocyte maturation *in vitro* (Schuetz 1971), can also be given as a hormone injection and results from our laboratory suggest an increase in the number of ovulations and oocyte numbers for *Bufo fowleri* given this steroid in combination with hCG (Browne *et al*. 2006*a*). Progesterone alone did not stimulate any of the animals to ovulate and was ineffectual without hCG. Species-specific differences in the application of hormone protocols for ovulation are obvious in our laboratory between *Bufo americanus*, *Bufo fowleri* and *Bufo baxteri*. We speculate that final oocyte maturation in *Bufo baxteri* may be closely tied to their longer hibernation period and the lower temperatures they are exposed to compared with the other two temperate species. Thus, priming hormones are needed to trigger this maturation event in the absence of prolonged captive hibernation. The *Bufo baxteri* captive breeding program has found that hibernation in combination with hormone injections is much more successful at inducing ovulations than hormone injections alone (B. Foster, pers. comm.).

Artificial fertilisation for amphibians

The terms artificial fertilisation and *in vitro* fertilisation (IVF) are often used interchangeably within the literature for amphibian studies. The discrepancy occurs because most aquatic-breeding anurans display external fertilisation; hence, referring to this technique as *in vitro* does not make sense when there is no *in vivo* reference point. The term artificial fertilisation has gained a great deal of literature use because of this disparity. However, in urodeles (salamanders and newts) and caecilians internal fertilisation is predominant so the term *in vitro* fertilisation would be more appropriate. For descriptive purposes, this paper will refer to all studies where fertilisation is performed mechanically by a researcher in a Petri dish as IVF. *In vitro* fertilisation for amphibians has been performed for more than 50 years, predominantly for studies on early embryonic development or for commercial production of laboratory species such as *Xenopus laevis*. The development of ART for amphibians, especially IVF, is simplistic when compared with mammals because of the many advantages of external fertilisation in water. Complex cell culture media, incubation conditions, temperatures and micro-handling of oocytes (e.g. removal of cumulus cells from mammalian oocytes via enzymatic digestion or mechanical pipetting) are all necessary to perform IVF in mammals due to the fact that natural fertilisation is internal. None of these complex systems or protocols is needed for amphibian IVF, which makes the widespread development of ART for amphibians more applicable to the management of captive breeding programs than for mammals.

Once gametes are obtained from both sexes, IVF is a rather simple process for anurans. Because gamete production is often asynchronous when using ART, the greatest challenge is typically storing one gender's gametes until the eggs or spermatozoa have been obtained from the other sex. To date, no anuran spermatozoa have been observed to undergo cold-shock as seen in mammalian spermatozoa when rapidly exposed to low temperatures. Our laboratory found that spermic urine from *Bufo baxteri*, *Bufo fowleri*, *Bufo americanus* and *Bufo boreas* can be quickly

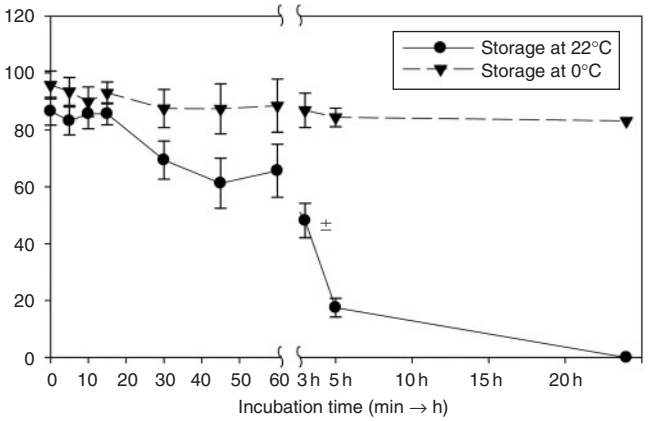

Fig. 3. *Bufo fowleri* sperm motility over time at two different storage temperatures, 22°C (●) and 0°C (▼). Sperm samples ($n = 10$) immediately plunged into ice slurry maintained motility over 24 h, while spermatozoa kept at room temperature ($n = 10$) quickly declined after 1 h and were completely immotile by 24 h. Data are expressed as the mean ± s.e.m. Previously published in Kouba *et al*. (2009).

immersed in an ice slurry at 0°C with no harmful side effects on motility or forward progression (Kouba *et al.* 2009). Fig. 3 compares percent motility over 24 h for *Bufo fowleri* spermic urine stored immediately in an ice slurry *v.* at room temperature. These results indicate that spermatozoa lost motility after sitting at room temperature for 24 h. Suspension in low temperatures is known to extend the lifespan of anuran spermatozoa and in some cases motility can still be observed up to 2 weeks after cold storage (Browne *et al*. 2001; A. J. Kouba and C. K. Vance, unpubl. data). However, sperm motility and forward progression decrease over time, thus the goal should be to use the spermatozoa as quickly as possible to ensure optimal fertilisation. Additionally, if spermic urine is to be stored between 0 and 4°C with cryodiluents it should also be noted that the process of inactivation and reactivation of sperm movement can significantly reduce the forward progressive motility, although the percent motility is rarely affected (Kouba *et al*. 2003). The ability to store spermatozoa easily for extended periods in the refrigerator or in an ice slurry means that although the exact time of ovulation may be unpredictable, spermatozoa for fertilisation can be readily available when eggs are procured. The simple nature of collecting toad spermatozoa, rapidly cooling the samples and short-term storage suggests that transport of spermatozoa between institutions for captive breeding and IVF should be an easy process. No such transports have occurred yet in American institutions and attempts for genetic exchange of gametes between holding facilities engaged in captive breeding programs should be a priority.

Anuran eggs, on the other hand, are much more sensitive to short-term storage than spermatozoa and research is currently underway to develop protocols for extending their fertilisation capacity. Once eggs are oviposited into water, the egg jelly quickly hydrates, resulting in structural changes that render it incapable of penetration by spermatozoa after a short period of 30 to 60 min (Hollinger and Corton 1980; Elinson 1986; Olson and Chandler 1999). During natural fertilisation, males amplex females and release spermic urine in short bursts as the female expels the eggs from her cloacae and thus sperm motility is not needed for extended periods of time due to the proximity of gametes upon release. Studies have shown that eggs can be ovulated into media with $\geq$200 mOsmol kg^{-1}, such as simplified amphibian Ringer's (SAR) solution, which extends fertilising capacity of the oocytes up to 8 h after ovulation in *Bufo marinus* (Browne *et al*. 2001) and more than 12 h for *Bufo fowleri* (R. K. Browne and A. J. Kouba, unpubl. data) and *Limnodynastes tasmaniensis* (Edwards *et al*. 2004). The time allowable for oocyte fertilisation capacity also appears to be affected by the temperature at which the eggs are stored. While Browne *et al*. (2001) found that storage of oocytes in SAR at 15°C for *Bufo marinus* and 10°C for *Bufo fowleri* was better than storage at room temperature, reduction of the storage temperature to 4–5°C was invariably detrimental to fertilisation (Wolf and Hedrick 1971; Browne *et al*. 2001). It is uncertain how the salts or ions in SAR temporarily delay the structural rearrangements of the egg jelly layer and block to fertilisation of oocytes laid in water or why temperature impacts SAR's effectiveness. Yet, the ability of SAR to extend fertilisation capacity for a short period is very useful considering most anurans that we studied spontaneously ovulate during the early morning hours (0100–0500 hours), similar to natural mating in the wild. Keeping healthy amphibians in SAR for extended periods (24 h) will cause excessive oedema and is only recommended when the animals cannot be monitored full-time (such as overnight).

In vitro fertilisation was widely used for amphibian gametes in the laboratory during the 1960s and 1970s in developmental and fertilisation studies. Wolf and Hedrick (1971) first described their techniques using the common laboratory species, *Xenopus laevis*. Since the first detailed description of IVF on *Xenopus* more than 35 years ago, few protocols have been developed for anurans outside of common laboratory models. The first IVF procedures using gametes procured from live animals of both sexes was accomplished by Waggener and Carroll (1998*a*). These researchers found that high rates (100%) of fertilisation could be obtained upon mixing freshly obtained eggs and spermatozoa from *Lepidobatrachus* species stimulated with the exogenous hormone LHRH. However, fertilisation decreased by 50% upon oocyte storage past 30 min at room temperature in SAR solution.

For IVF, eggs are removed from the water, placed into a dry Petri dish and mixed with spermatozoa for 5–10 min before flooding the dish with water (Kouba *et al.* 2009). If eggs were stored in SAR, they would need to be rinsed in water several times before IVF and subsequent placement into the dry Petri dishes. Incubating the gametes in any buffer solution with an osmolality higher than 50 mOsmol kg^{-1} will inhibit fertilisation (Edwards *et al*. 2004). It is likely that this inhibition to fertilisation is due to inactivation of sperm motility (Browne *et al*. 1998; Kouba *et al*. 2003; Edwards *et al*. 2004). Typically, fertilisation takes place with sperm concentrations ranging from 10^4 to 10^6 spermatozoa mL^{-1} (Wolf and Hedrick 1971; Browne *et al*. 1998; Edwards *et al*. 2004). Sperm concentrations for fertilisation will likely vary between species and investigations should be undertaken to determine the best sperm : egg ratio for any IVF experiments with new species.

Recently, *Bufo baxteri* tadpoles were produced by IVF in our laboratory, representing the first time an endangered amphibian has ever been produced by ART (Browne *et al*. 2006*b*). Subsequently, more than 2000 ART-produced tadpoles were released into the wild as part of a species reintroduction program monitored by the USA Fish and Wildlife Service. In 2008, ~5300 *Bufo boreas* tadpoles were produced by IVF at Colorado's Native Aquatic Species Restoration Facility (NASRF) after a collaborative partnership for training, technology transfer and capacity building was established between the Memphis Zoo and NASRF. These *Bufo boreas* tadpoles were subsequently released into the wild by the Colorado Department of Wildlife and Fisheries. Such technologies represent a conservation milestone for the preservation of endangered amphibians. Unfortunately, only ~16% of the total eggs laid (32 844) during the IVF experiments for *Bufo baxteri* were fertilised, indicating that there is significant room for improvement (Table 2). Toro and Michael (2004) performed a series of experiments to evaluate artificial activation and auto-activation of *Eleutherodactylus coqui* eggs during IVF, and they found that ~33% of all unfertilised eggs auto-activate after hormone treatment and ovulation. It is also likely that manipulation of the oocytes during IVF artificially activates the eggs. Once this activation takes place, the block to polyspermy occurs in as little as 15 min for *Eleutherodactylus coqui* preventing sperm penetration (Toro and Michael 2004). It may be that the poor fertilisation seen in our IVF studies with *Bufo baxteri* was due to a large percentage of auto- and artificially-activated eggs. Thus, future studies for captive breeding protocols should take great care in the movement and transport of eggs before fertilisation. The research done by Toro and Michael (2004) represents the first IVF techniques ever attempted in a tropical direct-developing species (no aquatic tadpole stage). Although IVF has been performed in a limited capacity for urodele studies, such as *Cynops pyrrhogaster*, most research has incorporated the sacrifice of one or both sexes for obtaining gametes (Watanabe *et al*. 2003).

Cryopreservation

Post-thaw survival of spermatozoa

When considering the wide range of taxa and species whose spermatozoa have been cryopreserved since the discovery of glycerol as a cryoprotective agent (CPA) in 1949 (Polge *et al*. 1949), there is a surprisingly limited number of publications dealing with freezing of amphibian spermatozoa. The fact that so little is known about amphibian sperm cryopreservation is even more surprising considering that seminal experiments on sperm freezing were first conducted with frogs in 1938 (Luyet and Hodapp 1938). Yet, a thorough literature search revealed only 14 journal articles describing cryopreservation technologies applied to amphibians. Almost all of these articles focussed on aquatic-breeding anurans and to the authors' knowledge no studies looked at viability or motility of urodele or caecilian spermatozoa following cryopreservation. Advanced technologies for cryopreservation of spermatozoa are typically observed in species with commercial value, such as domestic livestock (Curry 2000), poultry (Fulton 2006; Long 2006; Blesbois *et al*. 2007), fish (Lahnsteiner *et al.* 2000; Tiersch *et al*. 2007; Yang *et al*. 2007) and oysters (Dong *et al*. 2005; Kawamoto *et al*. 2007). Because there are currently no large-scale economic interests associated with amphibian species, the driving force behind these limited studies is centred on conservation or genetic management of captive species rather than commercial enterprise. Another reason for such gene banking studies was proposed by Sargent and Mohun (2005), in which transgenic lines for *Xenopus laevis* can be efficiently cryopreserved for future genetic screens, reducing the enormous amount of holding space that would otherwise be needed to maintain such a research colony.

Cryopreservation of toad spermatozoa from endangered amphibian species faces many challenges. Although cryopreservation has been accomplished in some common species such as *Xenopus* spp. (Buchholz *et al*. 2004; Sargent and Mohun 2005), *Bufo americanus* (Barton and Guttman 1972; Beesley *et al*. 1998), *Bufo marinus* (Browne *et al*. 1998, 2002*d*) and *Eleutherodactylus coqui* (Michael and Jones 2004), all of these investigators used minced testis from killed animals. These investigations provided a wealth of information on the practicality of anuran sperm freezing and different cryodiluents or cryoprotectants that afford survival of spermatozoa at low temperatures. In our laboratory, however, these cryopreservation protocols applied to spermatozoa collected from four *Bufonid* species (*B. fowleri*, *B. americanus*, *B. baxteri* and *B. boreas*) using live animals via exogenous hormone administration did not show the same post-thaw motility or viability as those collected from macerated testis of sacrificed animals (A. J. Kouba, C. K. Vance and E. Willis, unpubl. data). It is unclear what constituents found within testis macerates provide this additional protection. It may be that the testicular fluid provides additional protection or the higher sperm concentration found in testis macerates *v.* ejaculated spermatozoa allows a greater percentage of spermatozoa to survive cryopreservation. There are currently no published reports on the cryopreservation of spermatozoa from live male anurans treated with exogenous hormones. In our laboratory, using various extenders, cryoprotective agents and freezing rates, experiments have resulted in poor post-thaw sperm motility, ranging from 0 to 20%, with a great amount of variability within and between animals, and with experiments often not being reproducible. The authors noted occasioninal post-thaw motilities near 20% upon reactivation when using TEST egg yolk citrate buffer (Irvine Scientific Co., Santa Ana, CA, USA) with 6% glycerol. However, once the post-thaw samples are diluted with water to create a hypo-osmotic environment to reactivate the spermatozoa, something from the TEST extender precipitates out of solution and adheres to the membranes of the sperm cells, nearly eliminating any forward progression. Repeated washing and centrifugation can minimise some of this precipitation; however, stress from the centrifugation reduces the motility that would normally be gained from such a procedure. We are uncertain what effect, if any, the precipitate from the TEST extender would have on fertilisation. Sargent and Mohun (2005) found that *Xenopus laevis* and *Xenopus tropicalis* testis macerates cryopreserved in 0.2 M sucrose and egg yolk provided a relatively high degree of fertilisation, suggesting that the egg yolk is not the origin of the precipitate we are observing in the TEST buffer when the osmolality is reduced, or if so, that it does not adversely affect fertilisation in *Xenopus* species.

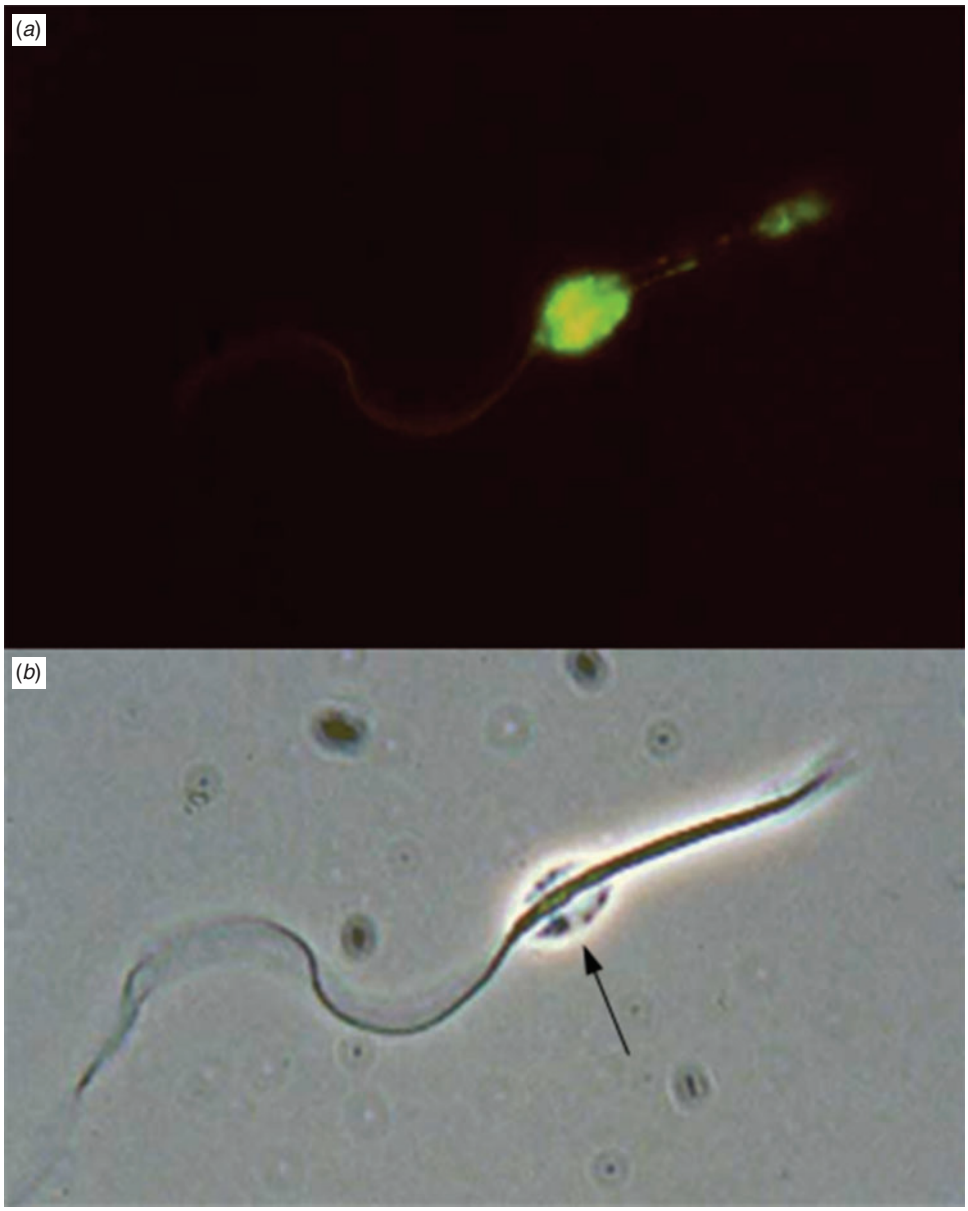

Fig. 4. (*a*) *Bufo americanus* spermatozoa stained with Mitotracker Green (100 nM) to display the mitochondrial vesicle; (*b*) a phase contrast image of the same spermatozoa with an arrow pointing to the vesicle. Previously published in Kouba *et al*. (2003).

Amphibian spermatozoa contain a unique structure called a mitochondrial vesicle that is integral to movement (Kouba *et al*. 2003; Roth and Obringer 2003; George *et al*. 2005). The mitochondrial vesicle, located on mature ejaculated spermatozoa, was observed by other investigators and has been referred to as an accessory cell (Waggener and Carroll 1998*b*) or mitochondrial collar (Braz *et al*. 2004). It is structurally very different from a cytoplasmic droplet and is an integral component of mature spermatozoa. Fig. 4 shows a *Bufo americanus* spermatozoon stained with the fluorescent probe, Mitotracker Green. While the authors observed this large structure associated with spermatozoa from six different *Bufonid* species, its presence is not easily distinguishable via microscopy (400×) in two *Ranid* species (*Rana sevosa* and *R. pipiens*). Hence, species-specific or family-specific differences exist relative to its size or appearance. Previous observations found that once this structure is lost or ruptured, sperm motility immediately stops (Waggener and Carroll 1998*b*; Kouba *et al*. 2003; Roth and Obringer 2003). Observations by the authors suggest that the anuran sperm cell's mitochondrial vesicle (Kouba *et al*. 2003) is particularly susceptible to cryodamage. Following cryopreservation of spermatozoa from live *Bufonids*, we have noted that

~90% of post-thawed spermatozoa are missing the mitochondrial vesicle, a necessity for sperm motility. The second most common visual structural change following cryopreservation is the rupture of the biflagellar tail and splitting of the axoneme.

Cryoprotectants and buffers that maintain membrane integrity of this vesicle are crucial to future success of this technology. While several studies described viability of spermatozoa following cryopreservation as analysed by membrane exclusion dyes, such as SYBE-14 (Michael and Jones 2004), none reported post-thaw motility equal to or comparable with pre-freeze levels. While viable spermatozoa can be used for such procedures as intracytoplasmic sperm injection (ICSI), low motility following thaws will provide poor results for IVF. Because most amphibians have external fertilisation, IVF can be accomplished by most zoos and aquariums and requires no special equipment, whereas ICSI requires specialised instrumentation and expertise few zoological institutions have access to. Thus, if zoos are to utilise frozen spermatozoa in an IVF procedure it is imperative to develop cryopreservation protocols that provide a reliable post-thaw sperm motility greater than 20% in order to fertilise more than a small portion of the ovulated oocytes.

Table 3 provides a brief summation of relevant cryopreservation studies to date, including some unpublished data from the authors. As discussed above, most of the published studies utilised minced testis from killed animals. Such procedures are not applicable to endangered or threatened species where every animal is genetically valuable. Because spermatozoa can be obtained easily and safely from live animals treated with exogenous hormones, future studies on cryopreservation should employ less invasive techniques when the goal is to develop technologies for the conservation of endangered species. The studies outlined in Table 3 show a wide array of different extenders and permeating cryoprotective agents (CPAs). Although most of these reports tested different extenders and CPAs, only the optimal freezing solution that provided the best post-thaw motility, viability or both under their experimental conditions is shown for each study. Studies conducted by the authors and Browne *et al*. (1998, 2002*a*, 2002*b*, 2002*c*, 2002*d*) utilised straws (0.25 cc) for storing spermatozoa and maintained samples in liquid nitrogen at −196°C. The remainder of the investigations shown in Table 3 used eppendorf tubes or cryovials for storage and kept samples frozen at −80°C, thus potentially limiting the samples' overall life expectancy. Moreover, sperm freezing rates within a sample are better controlled in straws than eppendorf tubes or cryovials and may account for the variability seen in post-thaw viability and motility. Most of the studies outlined in Table 3 did not test the ability of frozen–thawed spermatozoa to fertilise eggs and none of the studies examined motility, viability and fertilisation potential, but rather focussed on one, and in some cases two, of these parameters. For greater details on the various extenders or CPAs each investigator attempted, the authors recommend the references shown in the last column.

Post-thaw survival of oocytes and embryos

To date, very little research has been done, or at least published, on the cryopreservation of amphibian oocytes or embryos. Mammalian oocytes freeze poorly due to the size of the cell and high cytoplasmic content; when considering amphibian oocytes are nearly 25 times the size of mammalian (human) oocytes (2–2.5 mm *v.* 100 μm) it is expected that the challenges to oocyte freezing will be magnified. The fact that amphibian oocytes are so much larger is likely to complicate adapting any current methods for oocyte cryopreservation in mammals to amphibians; hence, new technologies will need to be developed that address water/cryoprotectant transport across the cell membrane. In comparison, fish oocytes, which are similar in size to amphibian oocytes, freeze poorly because of low membrane permeability to cryoprotectants, inadequate removal of water, and large volume of yolk that compartmentalises the cytoplasm, which also maintains high water activity.

In 2006, two studies on *Xenopus laevis* oocyte cryopreservation were published as companion articles (Guenther *et al*. 2006; Kleinhans *et al*. 2006). The primary emphasis behind these two studies was to understand intracellular ice formation in oocytes at different stages and to develop models for expressing and characterising aquaporin channels in the plasma membrane. As more studies like this progress, it may be that technologies become available to preserve female germplasm for amphibian conservation. Although freezing amphibian oocytes or embryos anytime soon seems unlikely, it may be that cryopreservation of isolated totipotent embryonic cells could be a way to preserve a portion of the maternal genome. Isolated embryonic cells from *Bufo bufo* showed a high survival rate post-thaw (87.1 ± 10%) when placed into a medium containing 10% sucrose and 10% DMSO (Uteshev *et al*. 2002). The cryoprotectants ethylene glycol and formamide were less effective than DMSO in maintaining membrane integrity after cryopreservation. More important was the finding that homotransplantations of post-thawed embryonic cells into enucleated eggs undergo early development up to the blastula stage (Uteshev *et al*. 2002). Such findings are encouraging and indicate that steps should be taken to isolate a few embryos from endangered species reproducing in captivity, dissociate the cells and cryopreserve them in a frozen tissue bank for conservation of the genome.

A great deal of research has gone into developing means to freeze zebra fish oocytes and embryos as this species is an important research model; however, there are still significant challenges associated with current protocols (Hagedorn *et al*. 2004). As technologies advance for cryopreservation of oocytes or embryos for fish, it may be that there will be applications to amphibians due to similar external fertilisation mechanisms, size of eggs and the presence of yolk. Regardless, studies on developing cryotechnology for female amphibian germplasm are warranted immediately and should occur concurrently with fish as there may be subtle differences in cryosurvival.

Future research opportunities

Nuclear transplantation and parthenogenesis

Some of the first nuclear transfer experiments were conducted using *Rana pipiens* and *Xenopus laevis*. Embryonic nuclei at most developmental stages, up to and including early tadpoles, can be placed into enucleated eggs and give rise to viable tadpoles and in some cases fertile adults (Kikyo and Wolffe 2002; Di Berardino 2006). Transfer of adult somatic cells in culture could

Table 3. Summary of cryopreservation results for amphibian spermatozoa

AF, actual fertilisation; AM, actual motility; EH, exogenous hormones; EM, euthanised male; LM, live male; NR, not recorded; RM, relative motility (post-thaw motility/starting motility × 100); RF, relative fertilisation; TM, testis macerates

Genus/species	Source/origin	Optimal extender and cryoprotectant	Storage temperature and container	Post-thaw motility (%)	Post-thaw viability (%)	Post-thaw fertilisation (%)	References
Xenopus laevis	EM, TM	0.2 M sucrose with egg yolk (no permeating cryoprotectant)	−80°C, 500-μL eppendorf tubes	10–50 RM	NR	23–81 RF	Sargent and Mohun 2005
Xenopus tropicalis	EM, TM	0.2 M sucrose with egg yolk (no permeating cryoprotectant)	−80°C, 500-μL eppendorf tubes	10–50 RM	NR	30–53 RF	Sargent and Mohun 2005
Eleutherodactylus coqui	EM, TM	Fetal bovine serum (FBS) + 1 M glycerol	−80°C, not specified	NR	53.9	NR	Michael and Jones 2004
Bufo americanus	EM, TM	75% FBS + 25% Alsevers medium + 15–20% ethylene glycol	−80°C, 1-mL aliquots in stoppered tubes	90–98 AM, 0 AM after 4 days storage	NR	NR	Barton and Guttman 1972
Rana sylvatica	EM, TM	Suspension buffer + 50% FBS + 0.5 M DMSO	−80°C, 500-μL eppendorf tubes	NR	81	NR	Beesley *et al*. 1998
Bufo americanus	EM, TM	Suspension buffer + 50% FBS + 0.5 M DMSO	−80°C, 500-μL eppendorf tubes	NR	47.8	NR	Beesley *et al*. 1998
Rana pipiens	EM, TM	Suspension buffer + 50% FBS + 0.5 M DMSO	−80°C, 500-μL eppendorf tubes	NR	59	NR	Beesley *et al*. 1998
Rana sylvatica	EM, TM	50% suspension buffer + 150 mM glucose	−8°C, 250-μL container (not specified)	10–15 RM	55–65	NR	Costanzo *et al*. 1998
Rana pipiens	EM, TM	50% suspension buffer + 150 mM glucose	−8°C, 250-μL container (not specified)	10–15 RM	55–65	NR	Costanzo *et al*. 1998
Xenopus laevis	EM, TM	Suspension buffer + 50% FBS + 0.5 M DMSO	−80°C, eppendorf tubes and straws	50–100 RM	NR	0	Buchholtz *et al*. 2004
Rana sylvatica	EM, TM	Suspension buffer + 3.0 M glycerol	−80°C, not specified	NR	17	NR	Mugnano *et al*. 1998
Rana temporaria	EM, TM	SAR + 15% DMSO + 10% saccharose + 1% BSA	−196°C, testis plunged into LN2	NR	NR	30	Kaurova *et al*. 1997; Uteshev and Gakhova 2005
Bufo marinus	EM, TM	10% sucrose + 15–20% DMSO or glycerol	−196°C, 125-μL straws	60–70 RM	NR	60.5 AF	Browne *et al*. 1998, 2002*a*, 2002*b*, 2002*c*, 2002*d*
Bufo fowleri	LM, EH	TEST egg yolk buffer + 6% glycerol	−196°C, 125-μL straws	10–15 AM	NR	NR	A. J. Kouba and C. K. Vance, unpubl. data
Bufo americanus, *Bufo fowleri*	LM, EH	10% sucrose + 10% DMSO	−196°C, 125-μL straws	5–15 AM	NR	NR	A. J. Kouba and C. K. Vance, unpubl. data
Bufo fowleri	LM, EH	AndroMed Extender + 3.5% glycerol	−196°C, 1.0-mL eppendorf tubes, Mr Frosty	12 AM	NR	NR	E. Willis and A. J. Kouba, unpubl. data

not generate tadpoles or adults, although some early development did occur. This led to the conclusion that early amphibian embryonic cells are more pluripotent or totipotent (depending on the cell stage) than are adult somatic cells. Nevertheless, recent advances in mammalian somatic cell cloning improved upon methodologies that could be applied to amphibians and should continue to be investigated. Amphibians present excellent models for advancing fundamental cellular and developmental problems due to several factors including the ability to stimulate egg production year round, the large number of eggs per spawning and the external development of the embryos. Holt *et al*. (2004) suggested that somatic cell nuclear transfer experiments for endangered species recovery may have the greatest potential for success in amphibians and fish rather than in mammals. Nuclear transplantation was first accomplished in amphibians during the 1950s (Briggs and King 1952) long before any successes were reported for domestic mammalian species in the late 1980s (Prather *et al*. 1987; Tsunoda *et al*. 1987). Cloning methodologies first reported for the leopard frog (King 1966), *Xenopus laevis* (Elsdale *et al*. 1960) and *axolotl* (urodele) (Signoret *et al*. 1962) could possibly be applied and tested for less common species that are now of conservation concern.

To the authors' knowledge, no developmental or molecular biology laboratories are pursuing collaborations to clone an endangered amphibian, despite the relative ease of cloning for amphibians. Investigations should be undertaken to insert somatic cell nuclei from an endangered amphibian into the enucleated egg of a more common species. For example, *Bufo baxteri* somatic cells could be inserted into *Bufo hemiophrys* or *Bufo fowleri* enucleated eggs and development of the resulting offspring followed. Furthermore, stem cell or germ cell nuclei transfer into enucleated eggs may also prove useful. For such technologies to have any possibility for species conservation, it is vital that genetic resources be quickly established, via gene banks, for a significant portion of the genetic diversity from both maternal and paternal amphibian genomes. Currently, there is a great deal of interest in establishing genomic resources for *Xenopus laevis* and *tropicalis* because of their importance as a research model for transgenic and gene knockout studies (Hirsch *et al*. 2002), nevertheless, molecular biologists continue to struggle with having to maintain large genomic breeding colonies due to the lack of knowledge on how to maintain viable stored germplasm.

Another technology that could be used to reproduce declining amphibians takes advantage of the fact that they can be reproduced by parthenogenesis. It is well known that the nuclear content of fish and amphibian spermatozoa or eggs can be destroyed by UV or gamma radiation followed by fertilisation with an untreated gamete to form a haploid embryo (Nace *et al*. 1970; Subcommittee on Amphibian Standards 1996; Komen and Thorgaard 2007). Destruction of the sperm nuclear content is called gynogenesis while destruction of the egg nuclear content is call androgenesis. By inhibition of either the second meiotic division or first cell division through heat shock, the haploid embryo can subsequently be made diploid (Komen and Thorgaard 2007). Hence, diploid animals carry a duplicate set of maternal chromosomes from the untreated gamete and are by definition homozygous.

Some asexual gynogenetic metazoans depend on spermatozoa of sexual males to trigger embryogenesis, although the genetic information of males will not be used (de Meeus *et al*. 2007). Such mating systems that combine both asexual and sexual reproduction are a major puzzle to evolutionary biology. Induced gynogenesis can also be triggered by 'pricking' the amphibian egg with a sharp needle or object, resulting in activation of the eggs (Shaver 1953). Similarly, heat shock or intense pressure would be necessary to retain the second polar body to create a diploid animal that is viable and of maternal origin. This technique of artificially activating the eggs is not as efficient as using spermatozoa for activation. Moore (1955) found that inactivated *Rana clamitans* spermatozoa could retain motility and fertilise *Rana pipien* eggs; such hybridisation would normally create lethal hybrids. The Memphis Zoo is working with the critically endangered *Rana sevosa* and most of the animals in captivity (of reproductive age) are female. As we begin to face a crisis situation with this species, it may be that we will need to consider the production of gynogenetic diploid animals using inactivated UV-treated spermatozoa from a closely related *Rana* species to perpetuate the genetic lines of these females. Our laboratory has been able to stimulate female *Rana sevosa* to spontaneously ovulate when given an intra-peritoneal injection of 500 IU hCG plus 15–20 μg LHRH preceded 48 h earlier by a priming dose at 1/5 the ovulatory dose, and has recently produced tadpoles through IVF.

Gender determination for ART in monomorphic species

One challenge that has restricted many natural and assisted breeding efforts for amphibians in captivity is the inability to accurately differentiate male from female animals. Many species of amphibians if raised or kept in captivity for an extended period of time do not display secondary sexual characteristics that define their gender, even though in the wild they would be sexually dimorphic. Two examples of amphibian species that are difficult to sex include the giant Chinese and Japanese salamanders (*Andrias davidianus* and *A. japonicas*, respectively) and *Rana sevosa*. In our experience with *Rana sevosa*, males raised in captivity rarely display dark nuptial thumb pads, vocal sacs or call when picked up. Nor is it possible to gauge sex by size in captivity because of the consistent diet the gopher frogs are fed. Many of the smallest *Rana sevosa* at the Memphis Zoo were females even though we had classified them as males based on their size. There are several methods for invasively identifying sex, such as endoscopic evaluation, which are traumatic and dangerous for use on endangered species unless absolutely necessary. However, recent work by Szymanski *et al*. (2006) showed that gender identification by non-invasive faecal steroid analysis might be useful to separate males from females. However, the same hormone assay did not function equivocally for two different species tested, *Bufo americanus* and *Bufo boreas*. Testosterone metabolites were more definitive for sex determination in *Bufo americanus* faeces, while oestrogen metabolites were more appropriate for sex determination in *Bufo boreas* (Szymanski *et al*. 2006). Results from their study support a recurring theme in this paper; species-specific differences frequently exist between amphibian species and protocols will need to be

modified and tested for application to even closely related animals. Our laboratory found that an injection of hCG and LHRH promoted thumb pad development and calling upon handling of male *Rana sevosa* and similar developments have occurred for *Rana tigrina* (Kurian and Saidapur 1982). It may be that hormone injections will help display male sexual characteristics in those animals not exposed to environmental cues because of captive upbringing. Continued research in this area is warranted considering the number of institutions that are unable to breed their amphibians because of unknown gender or potential aggression between similar sex animals if placed into the same enclosure (e.g. giant salamanders).

Conclusion

An idealistic genetic management system for endangered amphibians placed into captivity should include a breeding program according to the founders' recorded pedigree and a cryopreserved GRB. The interaction between the breeding program and GRB should be dynamic and interactive, maximising each strategy's potential. Amphibian breeding programs that suffer from reproductive failure should use ART as a tool to ensure that neither program fails nor is genetic diversity lost, which could result in the crash of an assurance colony. Amphibian ART for endangered species has benefited enormously from the vast amount of knowledge that has been accumulated on select key laboratory species. Although this review is not an exhaustive summary of everything known regarding amphibian assisted breeding, the reader will note that most of the information presented here is for anurans, indicating a general lack of knowledge regarding urodele and caecilian reproductive biology, which places these orders of amphibian in greater threat of extinction than anurans.

Significant achievements and advances have been made with regard to ART for amphibians, especially in our ability to collect anuran spermatozoa, store the spermatozoa for short periods of time, use it for IVF and cryopreserve macerated testis that can then be used to generate tadpoles. However, we still do not have an efficient way to cryopreserve spermatozoa from live males, nor has any significant research been done to cryopreserve amphibian embryos or oocytes. Furthermore, long-term financial resources need to be applied towards developing better hormone regimens for stimulating reproductive behaviours (amplexus) and ovulation that are safe and reliable. Such techniques will be a boon to institutions that lose a significant number of animals from unsuccessful hibernations intended to stimulate breeding. For ART to be successful in any amphibian species, significant groundwork must be done to determine which exogenous gonadotropins and concentrations are most effective at inducing spermiation or ovulation. Individuals using exogenous hormones for amphibian breeding should take great care when handling these substances and the animals receiving the hormones as they can affect the natural reproductive cycles of people in the laboratory or related facilities. Studies on cryopreservation or IVF cannot move forward efficiently without developing reliable protocols for the non-invasive collection of gametes. Therefore, it is crucial to have a basic understanding of the endocrinology, spermatogenesis and steroidogenesis of the species targeted for study.

The application of reproductive technologies to assisted breeding for amphibians is a rapidly evolving field with a growing number of scientists worldwide utilising such tools for conservation management. Nevertheless, there is an urgent need for more academic reproductive physiologists to collaborate with zoos that are challenged with maintaining amphibian brood stock due to a simple lack of knowledge regarding the animal's reproductive biology. Each amphibian species is unique and may display reproductive modes that are alien to classically trained mammalian reproductive physiologists. Mechanistic questions related to why one species or another is not reproducing should drive the selection of ART and associated research designs for an endangered amphibian species at risk of extinction. It is important to note that reproductive failure can also be associated with poor husbandry, incorrect environmental parameters or insufficient nutrition; thus, ART may not always be the most efficient method for resolving lack of breeding. The massive number of amphibian extinctions has created a triage situation for the limited number of biologists in the field who are trying to find a 'quick fix' before everything is lost. The most urgent need is to recruit additional reproductive physiologists that can lend additional resources and support staff to basic studies on reproductive biology for relatively unstudied amphibian species.

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