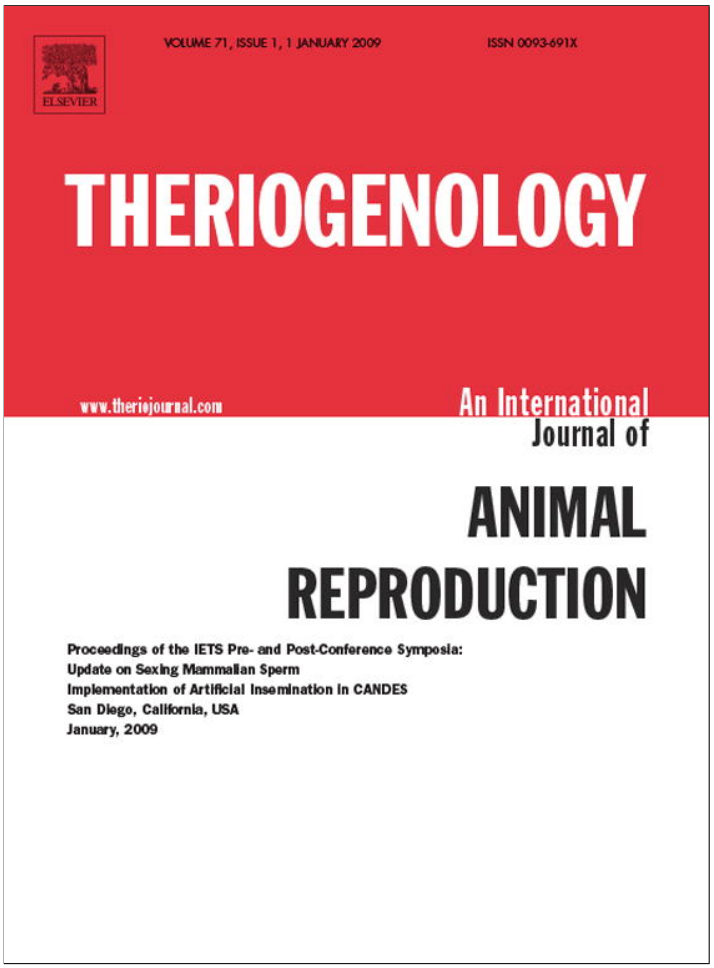

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Artificial fertilization for amphibian conservation: Current knowledge and future considerations

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Abstract

Amphibian populations in the wild are experiencing massive die-offs that have led to the extinction of an estimated 168 species in the last several decades. To address these declines, zoological institutions are playing an important role in establishing captive assurance colonies to protect species in imminent danger of extinction. Many of the threatened species recently placed into captivity are failing to reproduce before they expire, and maintaining founder populations is becoming a formidable challenge. Assisted reproductive technologies, such as hormone synchronization, gamete storage and artificial fertilization, are valuable tools for addressing reproductive failure of amphibians in captive facilities. Artificial fertilization has been commonly employed for over 60 years in several keystone laboratory species for basic studies in developmental biology and embryology. However, there are few instances of applied studies for the conservation of threatened or endangered amphibian species. In this review, we summarize valuable technological achievements in amphibian artificial fertilization, identify specific processes that need to be considered when developing artificial fertilization techniques for species conservation, and address future concerns that should be priorities for the next decade.

Keywords: Amphibian; Artificial fertilization; Egg; Hormones; Sperm

1. Introduction

Compared to mammals, amphibians display a wide range of reproductive strategies. Indeed, their reproductive strategies are so diverse that one would be challenged to make a general statement that reflects the entire taxa. Amphibians have evolved reproductive mechanisms often involving both an aquatic and terrestrial life stage that are successful based on their specific habitats. Most of the current literature on anurans (frogs and toads) is for temperate *Bufo* and *Rana* species; however, the great diversity of reproductive patterns in tropic anurans remains relatively unstudied. Caecilian reproductive mechanisms are even less well understood than those of tropical anurans. Fortunately, there is more known about the diversity of urodele (salamanders and newts) reproductive patterns because of their high density in the southeastern USA, where they are easily studied. The three living orders of Amphibia use both external and internal fertilization mechanisms reflecting a wide range of oviparous, ovoviviparous and viviparous strategies [1]. Typically, anurans are oviparous, salamanders and newts are ovoviviparous and caecilians are viviparous, although

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there are some exceptions to these categorizations, especially in anurans. Extensive reviews on amphibian reproduction can be found in Duellman and Trueb [1], Salthe and Mecham [2], and Whitaker [3].

The reproductive patterns of *Xenopus*, *Bufonids* and *Ranids* have been described in great detail for nearly a century. All three families of anurans share common traits that make them ideal model organisms, especially in the fields of developmental biology and embryology. These anurans have external fertilization, large eggs that are easily manipulated, developmental rates that proceed at a highly advanced pace compared to mammals, have no parental involvement, and have fecundity rates that can reach as high as 80,000 eggs per reproductive event [1]. It is therefore not surprising that 10% of all Nobel Prize recipients in physiology and medicine used frogs as a model (www.nobelprize.org), or that the first animal ever cloned was a frog [4].

Many of these early developmental biology studies required researchers to develop artificial fertilization (AF) techniques for greater control over their study designs. This dearth of knowledge on AF for several key laboratory species is now being applied to the conservation of endangered species within these same families (e.g. *Bufonidae* and *Ranidae*). In one context, this accumulation of knowledge on fertilization mechanisms, coupled with the ease of external fertilization in the lab, places assisted reproductive technologies (ARTs) for amphibians at a much more advanced stage than for any other companion animal or non-domestic species. A case in point is the release of over 2000 endangered Wyoming toad tadpoles produced by AF into the wild [5]. No mammalian conservation program can boast such numbers of released animals produced by ART. It is noteworthy that the terms AF and *in vitro* fertilization (IVF) are often used interchangeably by amphibian reproductive biologists to denote the artificial insemination (AI) of eggs in a Petri dish. However, AF is probably a more appropriate term for anurans that demonstrate primarily external fertilization compared to salamanders, newts and caecilians that exhibit internal fertilization. Whereas the majority of topics within this special issue of *Theriogenology* will focus on AI for companion animal and non-domestic mammalian species, a comparative paper on amphibian AF is warranted, considering their global extinction threat.

The aim of this review paper is to first introduce the amphibian extinction crisis and then the global efforts to stem their loss. As a result of securing so many relatively undescribed species in biosecure facilities, a captive breeding crisis is now growing that will require rapid development of ART until more is known about how to induce natural breeding. The remainder of this paper will discuss the current state of knowledge regarding AF for amphibians and some of the related technologies or unique reproductive adaptations that impact gamete interactions during fertilization.

2. Amphibian extinction crisis

The global loss of amphibian biodiversity is a stark example of how increasing anthropogenic actions impact our global ecosystems. Currently, amphibian extinctions are 200 times higher than the mean extinction rate for all species over the last 350 million years [6], leading many paleontologists to describe our current global biodiversity deficit as parallel to the loss of the dinosaurs. One of the most comprehensive surveys for an entire class of vertebrates, the global amphibian assessment (http://globalamphibians.org), indicates that approximately 32% of the nearly 6000 amphibian species known to science are in imminent danger of extinction. This level of extinction debt is much greater than for mammals (22%) or birds (12%) [7]. In general, the public is more likely to identify with, and financially support, charismatic flagship species such as elephants, lions or giant pandas [8] than they are to espouse frog conservation. It is estimated that approximately 168 amphibian species have likely gone extinct since the early 1980s; even more alarming is that 43% of the total number of remaining species are continuing to decline [9]. Although habitat loss is the primary threat to amphibians in the wild [10], other factors such as disease, climate change and pollution are affecting amphibian species worldwide. The rapid spread of a global epizootic fungal disease known as chytridiomycosis [11] has decimated populations in protected areas with pristine habitat. Hence, finding the way forward for conserving amphibian biodiversity is much more challenging than for mammals, because threats to mammalian biodiversity are well-known and conservation efforts to alleviate these threats primarily address habitat loss, poaching and genetic bottlenecks. Stressors such as climate change and pollution are believed to be interacting with the spread of chytridiomycosis [12], which poses the question of how to confront these population collapses, especially in remote or protected areas where many of the declines are occurring.

In 2005, the IUCN species survival commission hosted an international summit in Washington DC to address the catastrophic loss of so many amphibians. Out of this summit, an amphibian conservation action

plan (ACAP) was developed to provide a way forward and help stem or reverse the current rate of species loss [13]. One component of the ACAP defines a plan to establish captive-assurance colonies within zoos and aquariums for species likely to go extinct within the near future [14]. Three years later (2008), zoos around the world answered the call for amphibian conservation action and announced a global campaign called 'Year of the Frog'. Facilities and holding spaces were created to assist state, government and non-governmental agencies tasked with the preservation of our remaining amphibian heritage. It is highly likely that these global triage efforts can save a substantial portion of our amphibian fauna; however a new crisis is mounting that threatens these initial short-term gains.

3. The captive breeding crisis

For the most part, little is known regarding the reproductive mechanisms of the amphibian species placed into captivity. Even more vague and challenging to their care-takers are the environmental stimuli that cue a natural reproductive event. In order to stimulate appropriate reproductive behavior in many anurans and urodeles, hibernation must be employed, which frequently causes the animals to become immune-compromised, leading to bacterial and fungal infections (unpublished). Even after hibernation, females frequently fail to produce eggs, retain mature eggs, or release the eggs in the absence of a male. Among veterinarians and zookeepers, dystocia is rapidly becoming recognized as a major cause of death in amphibians that fail to lay eggs. Although dystocia is a common problem and well described in reptiles [15], a search of Medline returned no citations on amphibians and dystocia. The authors are receiving an ever-increasing number of communications for hormone protocols that will induce egg laying for health purposes rather than for breeding, because so many amphibians were collected from the wild at the height of breeding season (since they are easy to find) before they have had a chance to eject their eggs. However, the problem does not reside with the female alone. Male anurans often fail to elicit correct breeding behavior, fail to produce sperm, or there is asynchrony in sperm and egg release. Many captive breeding facilities report pairs in amplexus (clasping of the female by the male) followed by egg-laying, yet no fertilization. The escalating crisis in breeding failures or reproductive disorders threaten the original established founder lines for several captive collections, making it difficult for many programs to meet their long-term sustainable, genetically diverse population goals. By incorporating assisted breeding techniques into captive management plans for endangered amphibians, several of these problems may be reduced or eliminated. Research is necessary to establish effective and appropriate protocols to accomplish these objectives and apply them to preserving endangered species. The first step in developing ART for any amphibian species is to characterize their seasonal hormone profiles and develop exogenous hormone administration techniques that induce spermiation and ovulation.

4. Hormone induction for spermiation and ovulation

Because anurans typically display external fertilization, developing AF techniques is rather simplistic. The rate-limiting step in the development of ART is related to the acquisition of gametes. Anyone who has ever attempted to breed amphibians using exogenous hormones probably encountered a suite of difficulties and frustrations. Our laboratory found that hormone stimulation protocols are genus-specific with poor transferability to other species. In order to develop a suitable protocol for hormone-induced ovulation and AF in female amphibians, it is critical to have a basic understanding of the amphibian ovarian cycle and how hibernation or circannual cycles affect hormone efficacy. In mammals, the role of pituitary gonadotropins is clearly differentiated with luteinizing hormone (LH) and follicle stimulating hormone (FSH) having clear functions with high specificity toward certain cell types and receptors [16,17]. However, in amphibians, there is still some question as to the specific role or action of these gonadotropins. This lack of knowledge is partly due to the limited availability of homologous gonadotropic hormones for amphibians. Thus, our understanding of gonadal control is largely based on experiments carried out with mammalian gonadotropins purified from pituitary glands [18]. Although some ambiguity still exists regarding specificity, it is clear that pituitary gonadotropins induce ovulation and spermiation in amphibians [18,19]. Several excellent review articles on follicular growth, vitellogenesis, steroidogenesis, and influences of hibernation on these processes can be found in Duellman and Trueb [1], Redshaw [20], and Jorgenson [21].

4.1. Hormone use in female amphibians

A synthetic analog of luteinizing hormone releasing hormone (LHRH) [22] is used extensively by captive breeding programs in the USA to induce ovulation in a

diverse assemblage of female amphibians [3,23,24]. Concentrations of LHRH used in the captive breeding programs for endangered *Bufo baxteri*, *Bufo boreas* and *Bufo lemur* are typically in the range of 0.1 μg/g body mass; however, appropriate concentrations have rarely been tested empirically and are usually associated with trial and error breeding attempts. Whereas LHRH is used extensively to induce spermiation in male anurans [24–27], its use for stimulating ovulation is less well studied. It is crucial that simple concentration curves be developed for LHRH in reproduction programs that are employing this hormone. Michael et al. [23] tested varying concentrations of LHRH and other vertebrate hormones for their ability to stimulate ovulation in *Eleutherodactylus coqui*; they found that 20 μg of 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 *Bufo baxteri*, *Bufo boreas*, or *Bufo lemur*, suggesting that the inefficiency of this hormone in their program may be due to a lack of knowledge regarding its concentration potency. Whereas, LHRH is typically employed in the zoological industry, it is rarely used in the research industry, where it has been replaced by human chorionic gonadotropin (hCG).

Similar to LHRH, hCG is also used to stimulate ovulation in a wide range of species [3,5,18,28–31]. There are many arguments throughout the literature espousing that LHRH or hCG is better than the other hormone for certain species. Yet, none of these papers performed detailed dose–response curves for hormone efficacy. This reflects a large gap in the current state of knowledge regarding the effectiveness of specific hormones for inducing ovulation or spermiation. Species-specific differences in hormone use for ovulation have been known for some time. Hormone induction techniques and AF developed for *Rana pipiens* [32] are not applicable to *Xenopus laevis*, which required significant modifications [28]. Michael et al. [23] found that of the hormone concentrations they tested, LHRH was more effective at inducing ovulation in *Eleutherodactylus coqui* than the hormone hCG. In comparison, detailed dose–response curves conducted in our laboratory found that hCG was much more effective in stimulating ovulation for *Bufo americanus* and *Bufo fowleri* than LHRH.

Homologous frog pituitary extracts are also successful at initiating ovulation and spermiation in amphibians [20,30]; however, these preparations can contain transmissible diseases and caution should be taken when using extracts. In general, homozooic pituitary extracts are more potent than mammalian gonadotropins [20]. Yet, species-specific effectiveness of homologous pituitary extracts are also observed as *Bufo* ovulations can readily be induced by extracts from *Bufo*, less readily in *Leptodactylus* species, and not at all in *Xenopus* [20]. The use of pituitary extracts is not advisable for endangered species, in light of the massive amphibian extinction crisis; thus, hCG and LHRH are typically employed.

In mammals, it is not uncommon to synchronize estrus using GnRH or prostaglandins prior to AI to ensure appropriate timing with regard to ovulation and maximization of time and resources [33,34]. A similar, though slightly different, process is also common for amphibians that closely rely on hibernation for final oocyte development. Priming hormones are used successfully to prepare and initiate final maturation of oocytes in various species [30]. A priming hormone is typically given at 0.2–0.25 the normal ovulatory dose (e.g. 100 IU hCG instead of 500 IU hCG) and may precede a normal ovulation dose by 24–96 h. In our laboratory, a single hormone treatment of hCG suffices for stimulating ovulation in *Bufo americanus* [31] or *Bufo fowleri* [29]; however, *Bufo baxteri* are unresponsive to a single administration of hCG [5] when using the same protocol. Instead, priming hormones were found to be extremely effective at inducing ovulation for this species, with two priming hormone doses more effective than one priming hormone dose alone [5]. A novel aspect of this same study was the combined use of LHRH and hCG for priming as well as ovulatory treatments [5], and the two hormones given as a cocktail mixture appear to be more effective than either hormone alone (Kouba et al., unpublished).

4.2. Hormone use in male amphibians

Numerous studies have successfully used exogenous hormones to induce spermiation in a variety of frog and toad species [5,18,24,25,27,29,30,35–40]. The first experiments in anuran spermiation occurred during the 1940s and 1950s. A human pregnancy test was developed using male *Bufo arenarum*, wherein treatment with urine from a pregnant woman was found to induce spermiation [35]. The bioactive hormone in urine of pregnant women was identified as hCG which has activity similar to that of luteinizing hormone (LH) [41]. In anurans, LHRH, hCG and pituitary extracts effectively cause the release of sperm into urine. Goncharov et al. [42] found that the administration of LHRH promoted spawning in over 40 amphibian species and multiple studies used LHRH to induce spermiation in anurans [24,26,27,42,43]. Interestingly, our laboratory found that hCG is more effective

than LHRH in producing a greater concentration of high quality sperm in *Bufo fowleri* (Kouba et al., unpublished). Conversely, whereas hCG promoted higher quality sperm, we found that LHRH stimulates a stronger amplexus response (breeding behavior) from male toads than does hCG (Kouba et al., unpublished). This stronger effect of LHRH on breeding behaviors is likely due to its biochemical actions at the level of the pituitary rather than a direct action on the testes, as seen for hCG. Consequently, LHRH is the favored hormone in captive breeding programs that prefer natural reproduction versus AF.

Human chorionic gonadotropin is highly effective at initiating spermiation in anurans, although concentrations often exceed 2000× the recommended dose for a mammal on a weight basis, indicating reduced specificity. In many *Bufonids*, administration of 300 IU of hCG per 50 g toad typically produces a peak in sperm production 3–6 h after injection ([5], Kouba et al., unpublished). However, hormone efficacy appears to be species-specific and often transferring the same concentration to another species generates different sperm characteristics (e.g. concentration, motility and viability). For example, a single administrative dose of hCG (300 IU) to *Bufo fowleri* produces excellent quality sperm for AF; yet, *Rana pipiens* require a higher dose of hCG (500 IU) in combination with LHRH (10 μg) just to obtain spermic urine (Fig. 1). This cocktail injection of hCG + LHRH was more effective than either hormone alone, but still provided sperm of poorer quality (Fig. 1; Kouba and Willis, unpublished). These data emphasize the importance of conducting dose-dependent pharmacological trials for both males and females of individual species.

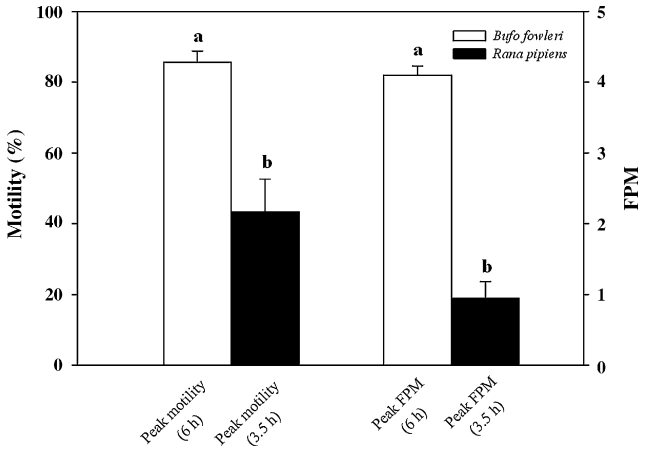

Fig. 1. Mean (±S.E.M.) comparison of peak sperm quality between *Bufo fowleri* at 6 h post-hCG and *Rana pipiens* at 3.5 h post-hCG + LHRH. *Bufo fowleri* ($n = 10$) were given a single treatment of 300 IU of hCG, whereas *Rana pipiens* ($n = 18$) were given 500 IU hCG + 10 μg LHRH. There were differences in peak sperm motility and forward progressive movement (FPM), as well as time to peak. *Rana pipiens* did not produce sperm after administration of 300 IU hCG and required a higher concentration of hCG combined with LHRH to elicit a spermiation response (Willis et al., unpublished).

5. Collection and storage of gametes

5.1. General collection of male and female gametes

Once an amphibian is stimulated with exogenous hormones, collecting the gametes can either be easy or quite challenging, depending on the gender or species. Male anurans deposit sperm into the urine which then passes out through the cloaca. Conversely, male urodeles produce spermatophores that are picked up by the female's cloaca and stored in a specialized organ called the spermatheca until needed for fertilization [1]. Obtaining sperm from male anurans will usually depend upon the animal's response to threatening occurrences. For instance, true toad species often have a defense mechanism such that when picked up or handled they urinate. In that regard, Fig. 2A shows a male *Bufo americanus* urinating into a Petri dish upon handling. The spermic urine is then processed and stored for either AF or cryopreservation. Once a male is given hormones for sperm collection, it should be placed into a small shallow reservoir of tap water so that it remains hydrated, allowing for urine production. Spermic urine can then be collected every hour over a 24-h period. This frequency of sperm collection in male anurans is much different than for mammals, where a refractory period is typically observed. Optimal peak sperm production can vary among species, but in most *Bufonids* tested in our lab (*Bufo americanus, fowleri*, *baxteri*, *boreas*, *lemur*, and *valliceps*) this peak occurs 3–5 h post-administration of hormone (Kouba et al., unpublished). Sperm collection from *Ranid* frogs is more challenging than collection from toads, because most species do not exhibit a similar urinating defense mechanism. Hence, sperm collection for *Ranids* (e.g. *Rana pipiens* and *Rana sevosa*) in our laboratory usually requires the gentle insertion of a small catheter into the cloaca, often referred to as cloacal lavage [24], which loosens the sphincter muscles of the bladder causing release of spermic urine. This sperm collection technique in *Ranids* is very similar to how eggs are expressed from females (Fig. 2B).

Amassing oocytes for AF is a relatively simple procedure, provided the female has already ovulated, meaning she has expelled the oocytes from the follicular tissue into the body cavity. Most females treated with

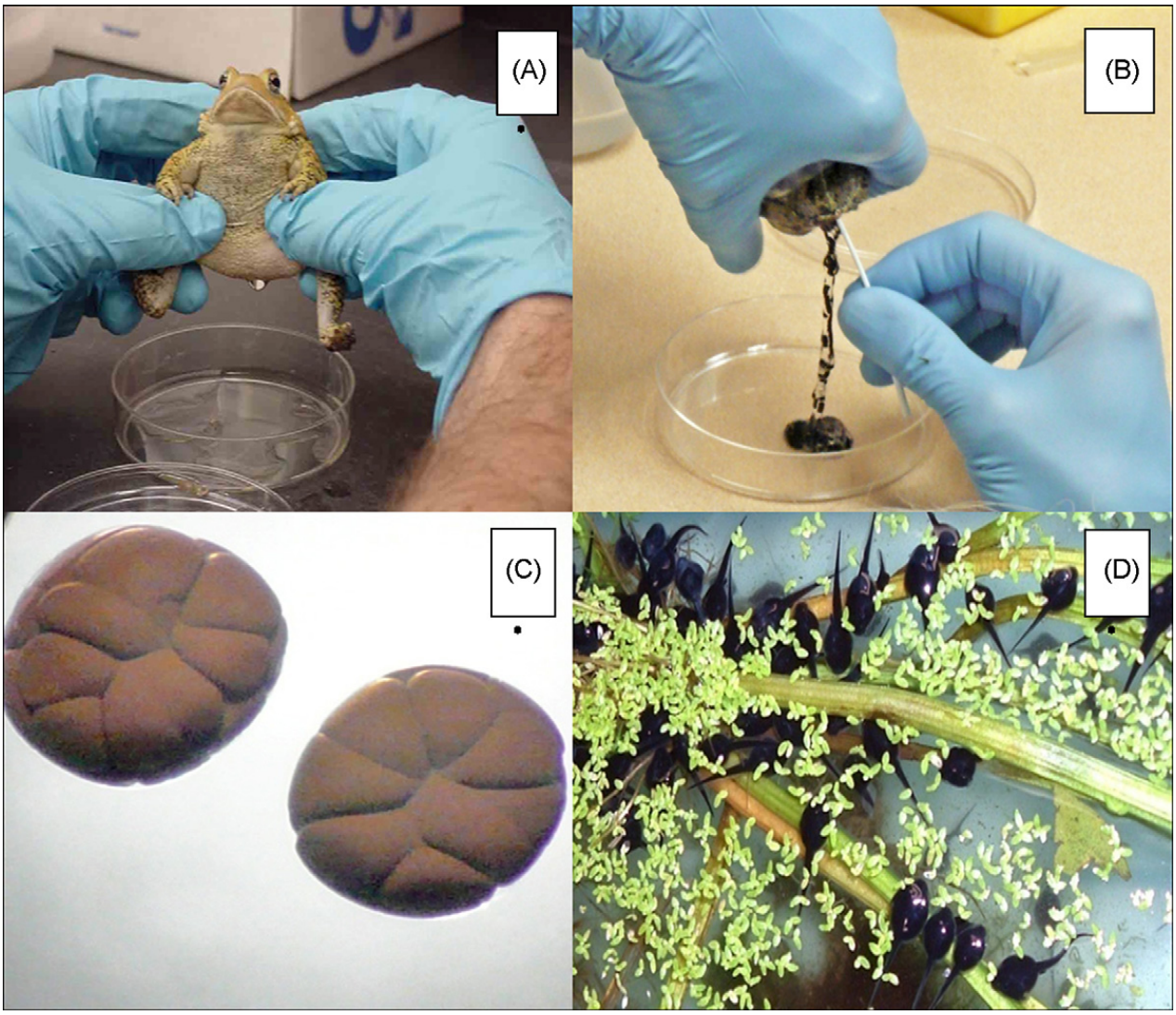

Fig. 2. Series of photographs representing technologies and achievements in amphibian artificial fertilization. Panel A: standard method for spermic urine collection after hormone administration in toad species that urinate upon handling. Panel B: oocyte expression from the endangered *Bufo boreas*, using gentle pressure of a catheter into the cloaca. Panel C: early developing embryos of the critically endangered frog, *Rana sevosa*, produced by AF in our laboratory. Panel D: tadpoles of the critically endangered *Bufo baxteri* produced by AF that were eventually released as part of a reintroduction program.

hormones will oviposit spontaneously if placed in water, provided they have received a sufficient quantity and repetition of exogenous hormones. If repetitious administration of hormones fails to elicit oviposition, it should be assumed that the female has probably not ovulated. Instead, a new recruitment of maturing follicles could have been initiated with hormone treatment, thus attempts to ovulate the female should be revisited 1–2 months later. It is also possible to check if a female has ovulated and is preparing to oviposit by gently inserting a small catheter into the cloaca to see if any eggs are expelled when the female urinates (Fig. 2B). If a few eggs are present the female can be gently squeezed by applying pressure to the abdominal area while using the catheter to release the pressure of the cloaca and free the oocytes from the oviduct (Fig. 2B). The extraction of eggs in such a manner is called oocyte expression; this technique allows for greater management over AF experiments, because the researcher controls how many eggs they desire to express from the female, whereas the remainder of the eggs in the oviduct linger in an optimal environment. An entire series of AF trials can then be established over the course of 8–10 h, depending on your quantity of stored sperm.

5.2. Storage of gametes

In captivity, asynchronous release of gametes is common and it is necessary to store gametes for short

intervals. However, incubation temperature can impact viability of the eggs and sperm in the storage medium. For example, *Bufo fowleri* eggs oviposited into an isotonic medium last longer if stored at 10 °C (Browne and Kouba, unpublished), whereas *Bufo marinus* eggs survive better at 15 °C [44]. If AF cannot be performed immediately upon oviposition, it is imperative that an isotonic solution like Simplified Amphibian Ringers (SAR) be used, because eggs laid in water will lose fertilization potential in 10–30 min [28,45]. However, oocytes can only be stored for 4–6 h, even if ovulated into an isotonic medium ([28,44,45], Browne and Kouba, unpublished). Hence, there is a very narrow window for AF to occur.

Since toad oocytes have such a short lifespan *in vitro* compared to sperm ([44]; Kouba et al., unpublished), cold sperm storage in anurans is much more efficient than oocyte storage and provides the greatest amount of flexibility when conducting AF. Browne et al. [44,46,47] found that testes macerates from the anuran families *Bufonidae*, *Hylidae* and *Myobatrachidae* survive for several days when held at 0 °C and can even be used for fertilization 10 d following cold storage in *Bufo marinus* [44]. These investigators did not test storage capacity of sperm collected from live animals and their results may not be applicable to ejaculated sperm from endangered species where sacrificing the animal for testes collection is not an option. There are significant differences between ejaculated sperm from live animals and testes macerates from euthanized animals, especially in their ability to survive cryopreservation storage. Anuran sperm cryopreserved from testes macerates has been performed in several species [48–52]; however there are no published reports to date of sperm cryopreservation occurring for spermic urine from live males' post-hormone administration. We found that cryopreservation of ejaculated sperm provides a highly variable post-thaw motility typically not exceeding 10%, although live offspring have been produced by frozen sperm in our lab. Protocols are currently being developed to improve this post-thaw motility so that frozen sperm samples can be transported between facilities for AF. Indeed, chilled sperm transport is currently being considered for amphibian AF in the U.S. by several zoological institutions working with the authors. Our laboratory observed that spermic urine from live males of several toad species (*Bufo americanus*, *baxteri, fowleri*, and *boreas*) can be placed into the refrigerator at 4 °C and kept for up to 10 d, as evaluated by sperm motility or fertilization, with some species differences. Unlike mammalian sperm, amphibian sperm do not display cold-shock, and immediately upon collection should be placed into the refrigerator at 4 °C or plunged into an ice-bath at 0 °C. Toad spermic urine kept at room temperature quickly lose motility compared to those in cold storage (90% motile versus 20% motile at 5 h; Fig. 3) and spermic urine diluted in water will lose motility within 10–15 min. Thus, anuran sperm for AF should never be stored in solutions less than 100 mos-

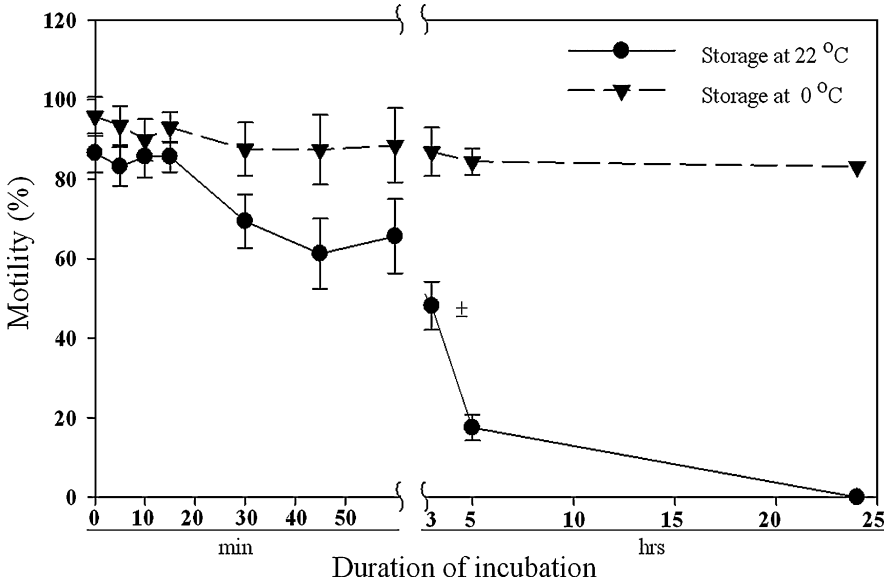

Fig. 3. Mean (±S.E.M.) motility of *Bufo fowleri* sperm over time at two storage temperatures, 22 °C (●) or 0 °C (▼). Sperm samples ($n = 10$) immediately plunged into ice slurry maintained motility over 24 h, whereas sperm kept at room temperature ($n = 10$) quickly declined after 1 h and were completely immotile by 24 h (Kouba et al., unpublished).

mol/kg. For additional information on mediums used for sperm dilution, egg incubation and AF see the review by Whitaker [3].

5.3. Impacts of osmotic stress on sperm motility and AF

Anuran and urodele sperm *in vivo* are inactive due to the isotonic environment of the testes (~280 mosmol/kg). In newts, the spermatheca maintains a high osmolality until sperm are activated by surface contact with the egg [53,54]; however, in anurans sperm motility is activated by deposition into a hypotonic (<50 mosmol/kg) aqueous environment, where sperm experience an extreme and rapid decrease in osmotic pressure [3,27]. Extreme hypoosmotic stress results in immediate cell death of mammalian sperm [55], but anuran sperm endure hypotonic stress for ~10 min, long enough to fertilize eggs in the external environment [25,26,56–58]. Sperm maintained between 100 and 200 mosmol/kg remain active, as evidenced by motility, yet do not experience the same cellular damage and loss of motility once the osmolality reaches <50 mosmol/kg.

Anuran spermatozoa have a unique ability to briefly resist hypoosmotic damage from water and actually require this drop in osmotic pressure to initiate motility prior to cell death. This cell death and eventual loss of motility can partially be attributed to lipid peroxidation as bovine serum albumin (BSA) can extend the lifespan of anuran sperm motility when diluted in water ([59], Vance and Willis, unpublished) and provides similar protective mechanisms to mammalian sperm purposefully exposed to lipid peroxidative damage [60]. Immediately upon ejaculation, sperm are moved from the testes into the urinary bladder where they are activated and experience a threefold drop in osmolality (Fig. 4). For AF, sperm are stored as described above and upon insemination experience another fivefold drop in osmolality, <20 mosmol/kg, from dilution into water (Fig. 4). When sperm are used from testes macerates for AF [28,44,47,61,62] the intermediate step of spermic urine storage is skipped.

Using cryopreserved sperm from live hormonally stimulated males for AF creates even more challenges for sperm survival due to the dramatic changes in the osmotic environment (Fig. 4). For instance, sperm go through a four-step process of being inactive–active–inactive–active (Fig. 4), due to the extenders used for freezing compared to sperm obtained from testes macerates, which only have a two-step process of activation. While sperm cryopreservation followed by

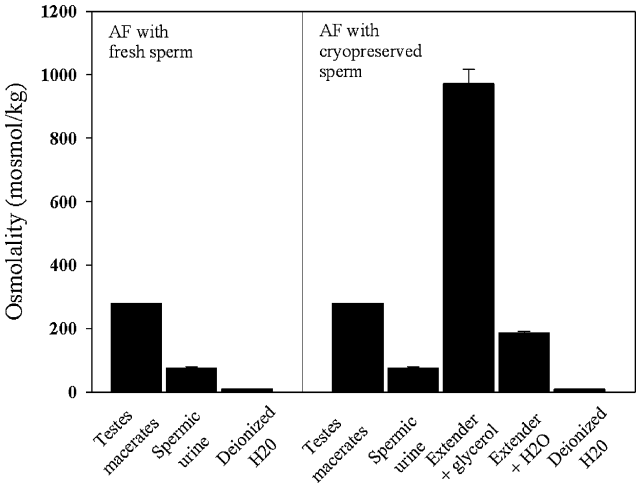

Fig. 4. Ionic changes experienced by anuran sperm during AF using freshly collected samples or following cryopreservation. Sperm released from the testes (~300 mosmol/kg) and used for fresh AF experience two 3-fold decreases in osmolality prior to gamete fusion (spermic urine ~100 mosmol/kg, followed by ~30 mosmol/kg in pond water during fertilization). During cryopreserved AF, sperm experience a second intermediate step of inactivation, with a 13-fold increase in osmolality over spermic urine, after exposure to extender + cryoprotectant. Cryopreserved samples are then reactivated for sperm quality assessment by diluting in water (lowering the osmolality to ~175 mosmol/kg) prior to fertilization. Finally, during fertilization, diluted cryopreserved sperm + oocytes are flooded with water, where sperm experience another decrease in osmolality (adapted from Willis et al., unpublished).

AF successfully produced a few tadpoles in our lab, significant modifications to our current protocols will need to be investigated to overcome the poor survivability of frozen–thawed sperm. We hypothesize that skipping the intermediate activation–inactivation step is one reason why cryopreservation of testes macerates followed by AF is more successful at producing live-offspring [48,51,52] than cryopreserved ejaculated sperm collected from live animals. However, for endangered species, sacrificial protocols for gamete retrieval are not practical and more recent studies are focusing on the development of reproductive technologies utilizing ejaculated sperm in urine obtained from live animals after hormone treatment [5,25,27].

5.4. Impacts of egg jelly layers on sperm function during AF

The vitelline envelope of amphibian eggs is surrounded by several structurally and biochemically discrete jelly layers which are synthesized and deposited on the egg as it passes through specific regions of the oviduct [63]. In anurans, the number of investment layers of jelly varies among species from three in the African clawed frog *Xenopus laevis*, to four

in the toad *Bufo bufo* and as many as six in the leopard frog *Rana pipiens*. Eggs from urodeles are best characterized in the newts *Notophthalmus viridescens* and *Cynops pyrrhogaster* which have five and six jelly layers, respectively [53,64]. In *Bufo bufo* [65] and *Rana pipiens* [66] addition of succeeding investment layers past the innermost jelly layer increases the frequency of fertilization. By contrast, coelomic (body cavity) or artificially dejellied eggs are not effectively fertilizable [67,68]. The oviductal egg jelly is crucial to AF success of oocytes and is implicated in oocyte maturation, sperm–oocyte recognition and binding, induction of the sperm acrosome reaction and the structural block to polyspermy [63–65,68–77].

The fibrous glycoconjugate network of egg jelly layers loosely hold together small diffusible protein and ionic components including a reservoir of Ca^{2+} and Mg^{2+} needed for sperm activity, capacitation and fertilization [66,69,78,79]. Allurin, a diffusible protein that exhibits sperm chemoattractant activity and stimulates sperm movement, is released from the jelly layer in *Xenopus laevis* eggs [80,81]. In the newt *Cynops pyrrhogaster*, sperm motility is pH dependent and induced by contact with the surface of the outermost egg jelly layer [53,82] rather than by changes in osmolality as described for anurans. In anurans, efficient sperm penetration and passage through the jelly layers requires a low electrolyte concentration and sperm motility within the jelly layer is dependent on the carbohydrate composition of the glycoconjugates present in the jelly and stages of agglutination of the jelly [83]. Prior to hydration, egg jelly cation concentrations rapidly decrease upon deposition into aqueous solution [84]. Thus, the egg jelly layer may have a role in maintenance of pH and of specific cation concentrations as monovalent cations Na^+ and K^+ are lost faster than divalent cations Ca^{2+} and Mg^{2+} [84,85]. Such management of specific contributions to ionic strength would allow the sperm to move through a hypotonic solution but maintain the necessary divalent cations required for sperm activity and gamete fusion [84]. In anurans, calcium ions contribute to sperm binding via the plasma membrane or through the inner acrosomal membrane to the vitelline envelope of the oocyte, as seen in *Xenopus laevis* [68,70,77].

In addition to amphibian jelly layer contributions to egg maturation and induction or maintenance of sperm motility, egg jelly has also been determined to play an important role in the sperm acrosome reaction [70]. Dejellied eggs from various species can be fertilized by reintroducing the diffusible components of egg jelly in the form of egg-water into the insemination media to facilitate sperm activity [66,67,78,86]. The sperm acrosome reaction occurs in the outer jelly layers for the frog *Discoglossus pictus* [87], or the newts *Pleurodeles waltl* and *Cynop phyrrhogaster* [53,54,88]. However, toad sperm undergo capacitation as they interact with the vitelline envelope, rather than during passage through the jelly coat [84]. Although sperm motility in anurans requires low osmolality, acrosomal integrity is rapidly lost under conditions of hypotonic stress and maintaining an intact acrosome through the jelly layer is critical for many species requiring an intact acrosome for interaction at the vitelline coat [89]. Once toad sperm reach the vitelline envelope, calcium ions are required for the acrosome reaction, such that the sperm can release the protein lysin to penetrate the vitelline membrane [69–71,84,85]. These diffusible components of the jelly layer initiate the sperm acrosome reaction through a signal transduction pathway, which requires cholesterol efflux for membrane conformational changes and promotes phosphorylation cascades [78]. Understanding the concepts related to the involvement of egg jelly during fertilization is critical to developing AF for amphibians, especially since there is a great deal of interest in fertilizing eggs from recently deceased females in captive breeding programs. If AF is to be undertaken on coelomic oocytes, it may be necessary to complete final maturation of the eggs using progesterone [21] and re-introduce egg jelly water for appropriate gamete interactions.

6. Artificial fertilization

The process of AF for amphibians has been detailed extensively for nearly 50 years and a meticulous literature search will reveal thousands of articles employing this technique. However, there are very few instances where AF has been applied toward threatened or endangered amphibians. The challenges to AF are relegated to the processes described above, in particular, developing appropriate hormone protocols for collection of high quality gametes, short- and long-term storage of gametes, and lastly the physiological effects of temperature, ionic strength, and egg jelly on fertilization potential. Although AF using sperm from live males was first documented in 1976 by McKinnell et al. [36], more than 95% of the AF studies to date have utilized testes macerates from sacrificed males. These studies have provided substantial information which can be applied to AF for conservation of threatened species and several detailed articles summarize this collective information [3,30]. Table 1 reviews some of

Table 1
Summary of anuran artificial fertilization (AF) using gametes collected from live animals.

Species	Hormones		Sperm concentration ($\times 10^3$ sperm/mL)	Fertilization rate (%)	Reference
	Male	Female			
Lepidobatrachus laevis	LHRH 0.403 μg/g	LHRH 0.1–0.575 μg/g	51–774	100	Waggener and Carroll [24]
Lepidobatrachus llanensis	LHRH 0.612 μg/g	LHRH 0.1–0.575 μg/g	214–422	100	Waggener and Carroll [24]
Bufo baxteri[a]	hCG 6 IU/g	hCG + LHRH 2 IU/g + 0.016 μg/g priming dose 10 IU/g + 0.08 μg/g ovulatory dose	1300	12.7	Browne et al. [5]
Bufo fowleri	hCG 6 IU/g	hCG + LHRH 10 IU/g + 0.2 μg/g	1000	90	Kouba et al. (unpublished)
Rana sevosa[a]	hCG + LHRH 10 IU/g + 0.3 μg/g	hCG + LHRH 1 IU/g + 0.03 μg/g priming dose 10 IU/g + 0.3 μg/g ovulatory dose	71.3	67	Kouba et al. (unpublished)

Hormone concentrations are expressed on a per weight basis.
[a] Priming hormones were required in two of the species (adapted from [5,24], Kouba et al., unpublished).

the recent published articles and unpublished data that describe AF using gametes obtained from both live male and female amphibians that may be directly applicable to conservation. Waggener and Carroll [24] describe AF procedures for two *Lepidobatrachus* species using LHRH to stimulate spermiation and ovulation with 100% fertilization under their experimental conditions. The remainder of work was done in our lab using either hCG alone (e.g. *Bufo fowleri*) or a novel combination of both LHRH and hCG in a cocktail mixture (e.g. *Bufo baxteri* and *Rana sevosa*) (Table 1).

When first developing AF for an amphibian species, it is vital to understand the ratio of sperm to eggs that provide the optimal fertilization rate. Several investigators reported optimal sperm concentrations for AF in *Bufo marinus* [48], *Limnodynastes tasmaniensis* [61], *Xenopus laevis* [28] and *Bufo arenarum* [90]. Optimal sperm concentrations for anuran AF typically range from 5×10^5 to 1×10^6 [45,48]. Sperm concentrations and fertilization curves have yet to be tested in our lab, primarily because our experiments are working with endangered species and our goal is to use all the sperm available for fertilization in order to produce tadpoles. However, the respective sperm concentrations and fertilization rates of our experiments for *Bufo baxteri* and *Rana sevosa* can be seen in Table 1 and are similar to published reports for other species.

Recently, Toro and Michael [91] conducted one of the first AF for a direct-developing frog, *Eleutherodactylus coqui*. Almost all studies to date performed AF on aquatic breeders that fertilize their eggs in water. This research is an important step forward for the conservation of tropical amphibians, even though testes macerates were used for the experiments. Interestingly, these investigators found that eggs obtained from hormone stimulation were auto-activated, possibly by the manipulation of the gametes, which reduced their overall fertilization rate [91]; thus, auto-activation of oocytes need to be considered during handling and manipulation of gametes. These results may explain why there is substantial variability in fertilization rates described in the literature.

To conduct AF in anurans, a procedure called 'dry fertilization' is conducted by mixing sperm and jellied eggs together in a Petri dish for 5 min, without the presence of water. After 5 min, the eggs are slowly flooded with tap water and set aside for evaluation of cleavage rate within 4–5 h (Fig. 2C). Cleavage rate is very easy to identify in amphibian embryos and is easily visible using a stereomicroscope. If oocytes are ovulated into SAR or a similar medium, it will be necessary to wash them at least three times before AF as the higher osmolality in these solutions inhibits sperm motility and thus, fertilization [28,61]. There have been limited studies on AF in endangered or threatened species yet the current technologies, which do not require sophisticated laboratory equipment or skills, could be easily adapted for zoological institutions attempting to breed animals placed into assurance colonies. An excellent example of how ART can be used to conserve a species is the production of over 2000 endangered *Bufo baxteri* in our laboratory by AF with the subsequent release of the tadpoles into the wild (Fig. 2D; [5]).

7. Future considerations

A great deal of literature is published on anuran AF mechanisms, yet very little effort has been applied to developing ART for salamander, newt or caecilians. In North America, urodeles are experiencing the greatest rate of declines [9], more so than anurans, so future studies should focus resources on applying the technologies described herein to conserving the greatest threat to our amphibian biodiversity. The reason studies on urodeles have lagged behind anurans is that internal fertilization mechanisms in salamanders represent a greater challenge for development of ART than in anurans. However, Osikowski [92] showed that artificial insemination can be performed in salamanders using sperm obtained from live males that resulted in offspring, illustrating the fact that such technologies should be pursued for endangered or threatened salamander species.

Furthermore, there is an urgent need to develop cryopreservation technologies for amphibian spermatozoa, embryos and if possible oocytes or follicular tissue. It is imperative that genetic resource banks be established for DNA, gametes, embryos, blood, cell cultures and tissues for preserving the greatest amount of genetic diversity in captive programs and facilitating studies on phylogenetics, systematics or for medical research. More advanced ART such as intracytoplasmic sperm injection (ICSI) and cloning should also be developed for conserving threatened species and is likely to be more easily implemented into captive programs considering the simplistic nature of working with such large oocytes, high fecundity, and external fertilization.

Due to the longevity of spermic urine held at 0–4 °C, institutions should be able to begin exchanging genetic material in the form of spermatozoa between facilities, since the stress of transporting animals into and out of quarantine often impacts the ability of zoos to breed animals during the narrow window available to release tadpoles. Moreover, oocytes from one female can be fertilized by several males through AF, thus improving the genetic diversity of resulting offspring [5]. Genetic improvement of the captive populations could be achieved by multiple paternities.

Lastly, breeding failures in captive facilities cannot all be attributed to a lack of knowledge on reproductive mechanisms or environmental stimuli that initiate a reproductive event. There are also issues associated with captive husbandry, nutrition and health that impact captive amphibian breeding behavior or physiology. More research needs to be focused on how these three additional fields of study affect the natural reproductive processes of amphibians in captivity.

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