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Developing assisted reproduction for reptiles, what's next?

Sean Perry
Mississippi Aquarium, Gulfport, MS

Abstract

Assisted reproductive technologies (ART) are often used as management tools to protect endangered vertebrate taxa; however, progress in developing ART for reptiles has been at a tortoise's pace! As proven useful for other species, the creation of functional and sustainable ART in reptiles can strengthen our conservation abilities by allowing zoos and aquariums to capture genetic material from select important individuals. This is a review of reptile research in ART dating back to the 1970's, highlighting important areas that still need investigation. Topics include artificial insemination, gamete collection, gamete storage, and genome resource banking. Additionally, potential application of in-vitro/ex-ovo fertilization, intracytoplasmic sperm injection (ICSI), cloning (somatic cell nuclear transfer), and genetic editing to reptiles, and the hurdles that need to be overcome, are included. For all reptile taxa, utilizing short-term gamete storage and genome resource banking, in conjunction with timed artificial insemination or ex-ovo incubation, could lead to profound advances in reptilian ART, with the hope of mitigating the loss of reptile biodiversity.

Keywords: Reptiles, assisted reproduction, conservation, gamete

Introduction

Over, 11,300 species of reptiles have evolved on every continent except Antarctica. At this moment in history, we are observing the biological annihilation of the natural world. Anthropogenic induced environmental alterations such as habitat loss and degradation, invasive species introduction, environmental pollution, infectious disease, unsustainable harvesting, illegal trade, and global climate changes are all substantial threats to current reptile populations worldwide. One in 5 reptile species is threatened with extinction. This is the harsh reality of our lifetime. Zoological institutions utilizing both in-situ and ex-situ conservation strategies have contributed to reptile conservation; however, assisted reproductive technologies may be 1 of the last hopes to preserve and conserve reptilian biodiversity for some species. We briefly review what has been completed to date in order to help develop assisted reproductive technologies (ART) for reptiles and discuss where ART is applicable to reptiles.

Gamete collection techniques

Males

Postmortem collection of samples can be used for endangered species that die in zoological settings and from animals subject to mass-mortality events to preserve genetic diversity.[1] During the reproductive season, epididymis and ductus deferens distend with sperm because these are the sites where sperm undergo in vivo maturation and storage prior to ejaculation. Therefore, postmortem sperm recovery should focus on sperm present in the epididymis and ductus deferens. Once the male reproductive tract is collected, sperm can be harvested by expressing the ductus deferens and/or macerating the epididymis under a dissection scope. Samples should not be collected directly from the testes, as testicular sperm have poor motility (1%); even after being exposed to a phosphodiesterase inhibitor (caffeine), motility only increased to 7%.[2] Successful AI has been performed using sperm recovered in crocodilians at postmortem.[3,4] Currently, postmortem collection is the best method to conduct experiments to develop ART for reptiles, as it provides large sample volumes from model reptile species that are necessary to further develop ART.

Antemortem semen collection was performed in a wide range of reptile species via various methodologies, including coelomic massage, digital phallic massage, a vibrational method, and electroejaculation. Coelomic massage was used successfully to collect semen from snakes and small lizards. In snakes, semen was collected by gently massaging the ventral caudal third of the coelomic cavity using light digital pressure in a caudoventral direction toward the cloaca.[5-13] Similarly, digital ventral coelomic massage was used in small geckos and skinks to collect semen samples.[14,15] In crocodilians, digital phallic massage was used to successfully collect semen under mild sedation and manual restraint. Success with this strategy was reported in American alligators (*Alligator mississipiensis*), broad-nosed caiman (*Caiman latirostris*), saltwater crocodiles (*Crocodylus porosus*), and Philippine crocodiles (*Crocodylus mindorensis*). To collect semen, the crocodilian is placed in ventral recumbency and straddled across 2 supporting structures; the phallus extruded from the proctodeum using a gloved finger; and the phallus base at the level of the urodeum massaged until a sample is produced within the sulcus spermaticus.[3,4,16-21] No reports exist of using this method in other species, although investigation may be warranted, especially in chelonians. One report described the use of a human vibrational device to collect semen from chelonians. Male turtles were held upright and an appropriately sized vibrator set to its highest frequency and placed against the carapace and moved along the carapace in linear and circular motions. If no erection was noted after carapacial stimulation, then the vibrator was placed on the plastron for 7 - 22 minutes. Semen collection success was species-specific, with good success in Blanding's turtles (*Emydoidea blandingii*) (12/12), but poor success in painted turtles (*Chrysemys picta*) (4/30) and wood turtles (*Glyptemys insculpta*) (2/17).[22]

The most widely used method to collect semen antemortem from reptiles is electroejaculation (EEJ), used to successfully and safely collect ejaculates from all reptile taxa other than the tuatara (Sphenodon punctatus). This is the preferred antemortem method to collect semen in larger lizards and chelonians because their anatomy limits the use of some of the other methodologies. Semen collection by EEJ in lizards and chelonians is typically performed under anesthesia or heavy sedation; however, some studies did not sedate animals for this procedure. Various electroejaculation units and protocols were reported to be successful for collecting semen from reptiles. Most had a variable voltage/amperage power source with a plastic or metallic probe. Much of the variation in EEJ techniques is due to the variation in the size of animals, unique species anatomy, and variable/limited availability of appropriately sized probes. In lizards, EEJ was first performed in green iguanas.[23] Since then, multiple studies were conducted to evaluate the safety and efficacy of EEJ in lizards, including veiled chameleons (*Chameleo calyptratus*), spiny lava lizards (*Tropidurus spinulosis*), spiny lizards (*Sceloporus torquatus*), and Grand Cayman blue iguana hybrids (*Cyclura lewisi x nubila*).[23-30] Safety of repeated EEJ in anesthetized veiled chameleons demonstrated that repeated anesthesia and EEJ can be performed safely and successfully at least once weekly in this species.[24] Overall animal health was assessed using physical examination, complete blood counts, biochemistry values, cloacal external morphology, and cloacal mucosal inflammation with endoscopy. Transient cloacal inflammation was observed after EEJ, but resolved spontaneously.[24] This study demonstrated that repeated semen collection can be done safely using EEJ in reptiles without impacting the long-term health of the reptiles. To date, EEJ effects on peripheral corticosterone (stress-related) concentrations in reptiles was not evaluated. In chelonians, EEJ is the most common antemortem method used to collect semen. EEJ was used to collect semen from leopard tortoises (*Stigmochelys pardalis*), green sea turtles, red-eared slider turtles, a Galapagos tortoise (*Chelonoidis nigra*), a ploughshare tortoise (Astrochelys yniphora), olive Ridley turtles, hawksbill turtles, and the black marsh turtle (*Siebenrockiella crassicollis*).[31-38] EEJ was performed with and without anesthesia in chelonians with no associated discomfort or trauma; however, no studies specifically evaluated those effects.[31,38] In reptiles anesthesia and sedation techniques were improved in the past 20 years. Sedation or anesthesia is recommended for EEJ to limit discomfort. An aversion to performing EEJ in chelonians exists because a single report of a death that occurred in a ploughshare tortoise 30 days after semen collection due to suspected renal disease.[31,33] However, no reports exist in the literature associating renal failure and EEJ in any species. In snakes, EEJ was successful in checkered garter snakes (*Thamnophis marcianus*) as an alternative to coelomic massage. EEJ in snakes pose a special problem because of the distance from the cloaca to the gonads. For the garter snakes, a special elongated EEJ probe was used due to the cranial position of the reproductive tract. In larger snakes, EEJ may be the only method that can be used to collect semen because coelomic massage can be challenging.[31,39] In crocodilians, EEJ was used successfully to collect semen from American alligators and saltwater crocodiles.[18,20]

Females

There are no reports of antemortem or postmortem collection with in vitro oocyte maintenance and maturation for reptiles. Oocyte recovery in live reptiles is complicated. Numerous factors contribute to this, including reproductive season/timing, ovary type/structure, ovulation timing, or deposition of albumen or egg shell. We do not have well established antemortem collection maintenance methods. Viviparous reptile species should be the first investigated to develop antemortem collection methods, as these species are reproductively more similar to mammals, and techniques are likely more transferable.

Semen handling and genome resource banking

A fully functional genome resource bank for reptiles is necessary for continued conservation efforts. A complete genome resource bank could help maximize our ability to store reproductive cells and tissues. The technology and techniques required to develop, maintain, and grow a genome resource bank include our ability to routinely collect sperm, oocytes, embryos, and somatic cells. Once these samples are collected, indefinite storage is required, which could be achieved using cryopreservation.

Semen handling and extension

Semen handling and extension methods were investigated in at least 1 species from each reptile taxon, except the tuatara. Standard semen handling methods should be adhered to when handling reptile sperm. A general trend is that reptile sperm can be successfully stored at low temperatures for shorter durations. Species evaluated include the American alligator (*Alligator mississippiensis*), Saltwater crocodile (*Crocodylus porosus*), Broad snouted caiman (*Caiman latirostris*), Green iguana (*Iguana iguana*), Green anole (*Anolis carolinensis*), McCann's skink (*Oligosoma maccanni*), Hybrid Grand Cayman Blue Iguanas (*Cyclura lewisi x nubila*), Eastern water skink (*Eulamprus quoyii*), Corn snake (*Pantherophis guttatus*), Banded watersnake (*Nerodia fasciata*), Red Eared Slider (*Trachemys scripta elegans*), Leopard Tortoise (*Stigmochelys pardalis*), Olive Ridley(*Lepidochelys olivacea*), and Hawskbill sea turtle (*Eretmochelys imbricata*).[10,15,19,20,21,23,30,34,35,38,40-42] Semen extenders are summarized (Table 1).

Semen cryopreservation/vitrification

In reptiles, only a few reports exist describing experiments evaluating sperm cryopreservation and none exist exploring vitrification. Species that were studied to date include the saltwater crocodile, American alligator, Argentine black and white tegu (*Tupinambis (Salvator) merianae*), eastern water skink (*Eulamprus quoyii*), Argus monitor (*Varanus panoptes*), red diamond rattlesnake (*Crotalus ruber*), Burmese python (*Python bivittatus*), and the red eared slider turtle.[3,4,16,17,25,41-49] The most extensive work regarding reptilian cryopreservation

Table 1. Semen extenders that have been evaluated for short-term storage of reptile sperm

Taxon	Species	Media	Temperature (°C)	Total motility (%)	Duration
Crocodilians	American Alligator (*Alligator mississippiensis*)	BEST with egg yolk and/or milk	5	25 - 50	2 - 5 days
	Saltwater crocodile (*Crocodylus porosus*)	PBS	30	< 30	120 minutes
	Broad snouted caiman (*Caiman latirostris*)	BEST with egg yolk	5	> 60	5 days
Lacertilians	Green iguana (*Iguana iguana*)	Modified Ham's F-10 + Test Yolk buffer	5 (Equitainer)	60	24 hours
	Green anole (*Anolis carolinensis*)	Ham's F-10 INRA 96 Sperm Wash Media	4	25	12 hours
	McCann's skink (*Oligosoma maccanni*)	Ham-F 10	4	70	5 days
	Hybrid Grand Cayman Blue Iguanas (*Cyclura lewisi x nubila*)	INRA 96 Test Yolk Buffer	4	4 - 41	24 hours
	Eastern water skink (*Eulamprus quoyii*)	PBS TLHepes Ham's F-10	20	70	16 hours
Serpentes	Corn snake (*Pantherophis guttatus*)	Modified Ham's F-10 + Test Yolk buffer	5 (Equitainer)	> 50	48 hours
	Banded watersnake (*Nerodia fasciata*)	INRA 96 Test Yolk Buffer Sperm Wash Media	4	> 60	72 hours
Chelonians	Red Eared Slider (*Trachemys scripta elegans*)	Same as *N. fasciata*	4	> 60	96 hours
	Leopard Tortoise (*Stigmochelys pardalis*)	Modified Ham's F-10 + Test Yolk buffer	4	0	24 hours
	Olive Ridley Sea Turtle (*Lepidochelys olivacea*)	test yolk buffer Tyrode medium with albumin, lactate, pyruvate Beltsville poultry semen extender 3% sodium citrate buffer phosphate buffered saline EEL 1% bovine serum albumin Ham F-10	4	0 - 5	6 hours
	Hawskbill (*Eretmochelys imbricata*)	Same as for *L. olivacea*	4	< 5	6 hours

was performed in saltwater crocodiles (Table 2).

As with other taxa, methods to cryopreserve reptile sperm were not consistent across species, although, major themes emerged when reptile sperm are cryopreserved. First, although the effects of cold shock on sperm was only evaluated in saltwater crocodiles and Argus monitors, both appeared to be tolerant of cold shock.[45,48] Second, cryoprotectant type and concentration affected prefreeze motility. Cryoprotectant cytotoxicity and the protective effects against cryodamage is a complex multifactor interaction in reptiles.[50] Third, DMSO and glycerol were used across species and have promise. Finally, freeze periods vary

Table 2. Published semen cryopreservation methods that have been applied to reptile species (please directly refer to the source for methodologies)

Taxon	Species	Media	Cryoprotectant	Concentration	Storage container	Dreeze rate (°C/Minute)
Crocodilians	Saltwater crocodile Johnston, et al: 2014b, Johnston, et al: 2017	Dulbecco's PBS w/o Ca2+ and Mg2+	DMSO Dimethylacetamide (DMA) Glycerol	0.68 M [5%] 1.35 M [10%] 2.7 M [20%]	250 µl French straws	- 6 - 21
		Dulbecco's PBS w/o Ca2+ and Mg2+	trehalose raffinose sucrose	0.3 M	250 µl French straws	- 6 - 21
		Dulbecco's PBS w/o Ca2+ and Mg2+	Glycerol +sucrose	0.68 M [5%] + 0.2 M sucrose 0.68 M [5%] + 0.3 M sucrose 1.35 M [10%] + 0.2 M sucrose 1.35 M [10%] + 0.3 M sucrose	250 µl French straws	- 6 - 21
	American alligator		DMSO Glycerol	5% 10% 20% 30%		-
Lacertilians	Argentine black and white tegu (*Tubinambis merianae*) Young, et al: 2017	Test-Yolk- M199 with HEPES	DMSO Glycerol	8% 12% 16%	Cryovials	- 0.3 - 1 - 6.3
	Eastern water skink (*Eulamprus quoyii*) (Hobbs, et al: 2018)	PBS w/o Ca2+ and Mg2+ Tris-yolk buffer Beltsville poultry semen extender	DMSO	1.35 M	200 µl French straws	- 6
	Argus monitor (*Varanus panoptes*)	PBS w/o Ca2+ and Mg2+	DMSO Glycerol	5% 10% 15%	250 µl French straws	- 32.1
Serpentes	Red diamond rattlesnake (*Crotalus ruber*) (Zacariotti, et al: 2011)	Lake's extender TEST-yolk buffer	DMSO Glycerol	2% 4% 4% 8% 10%	Cryovials	See abstract for protocols
	Burmese python *(Python bivittatus)* (Young, et al: 2017)	TEST-yolk buffer	DMSO Glycerol DMSO + Glycerol	8 % 12% 16% 4:4% 6:6% 8:8%	Cryovials	- 0.3

among studies but it appears that faster freezes may preserve a higher proportion of cells, although this remains species specific. Sperm integrity may be more important than motility when measuring success, but this remains to be determined. Studies using vitrification as a cryopreservation method have not been performed in reptiles.

Artificial insemination

Artificial insemination (AI) with successful fertilization, oviposition/parturition, and production of offspring has only been reported in 3 snake species and 1 crocodilian. AI was successful with freshly collected or short-term extended semen. Currently, no guidelines exist for recommendations regarding insemination volume, sperm concentration, and semen deposition location. Use of cryopreserved semen needs to be investigated further. American alligator was the first reptile to be successfully artificially inseminated with fresh sperm and produce live offspring.[16,18,19] Currently, AI has limited value for the American alligator industry but should be pursued for threatened and endangered crocodilians.

Successful AI was reported in 3 species of snakes: corn snakes on 2 separate occasions, a garter snake (*Thamnophis marcianus*), and an Amazon tree boa (*Corallus hortulanus*).[12,39,51] Corn snakes were the first snake species demonstrating offspring could be produced using fresh and cooled semen following posthibernation insemination. In this case, no follicular monitoring was performed, and all animals were inseminated at the same time. Sperm was deposited into the oviduct using a metallic ball tip syringe. Insemination was performed in 10 females, but only 3 females produced eggs. Two of these clutches produced offspring with 33% (5/15) and 38% (5/13) hatch rates. Genetic testing was performed on the offspring from both clutches to confirm paternity with microsatellites.[12] Other reports of successful AI in snakes include 3 clinical cases, 2 corn snakes and an Amazon tree boa where the female snakes were monitored for follicular progression prior to insemination. AI was performed using endoscopic assisted oviductal catheterization and a transcervical insemination catheter. The corn snakes used in this study had successfully produced eggs in prior years from natural breeding, whereas the Amazon tree boa was a wild-caught adult with an unknown reproductive history. Paternity was never confirmed for either species. In the corn snakes, it was reported that from the 2 clutches all eggs were fertile and hatched. The authors reported that the Amazon tree boa underwent parturition 4 months after insemination and successfully produced 2/7 live young. Paternity testing was not performed in these cases. In lizards, a single report of AI exists. A micropipettor was used to inseminate a McCann's skink at the lateral side of the cloaca. No offspring were produced from the procedure, but environmental issues and a disease outbreak may have contributed to this outcome.[15]

Reports of AI in chelonians are limited. Based on the literature, AI was only attempted in the ploughshare tortoise and the Yangtze giant softshell turtle (*Rafetus swinhoei*). In 1982, AI was attempted in a ploughshare tortoise following semen sample collection via EEJ. The female laid a clutch of 7 eggs 12 days after the AI procedure. A single dead embryo was reported in this clutch. It was concluded that this clutch was not secondary to the AI due to quick oviposition. Additional clutches were laid with dead embryos and one live hatching in December 1982 and February 1983. These latter events could have been related to the AI or sperm storage from natural copulation in 1982. This was never further investigated with paternity testing.[33] In the case of the Yangtze giant softshell turtle, AI was performed thrice since 2015 in the last known female of the species, and she successfully laid eggs following insemination, but none have been fertile.

When performing AI in reptiles confirmation of paternity is recommended. Multiple paternity was documented in all major groups of reptiles and is suggestive of high levels of female promiscuity.[52] Multiple paternity arises in reptiles via 2 routes: 1) either mating occurs with more than 1 male during the same reproductive cycle, or 2) mating occurs with 1 or more males during each reproductive cycle and sperm storage occurs.[52] Tests for multiple paternity in animals with sperm storage is needed because of this reproductive strategy. Additionally, alternative mating strategies such as parthenogenesis occur in reptiles complicating paternity confirmation. In order to confirm success with AI, testing is needed for each offspring to confirm paternal lineage and rule out parthenogenesis.

In-vitro/ex-ovo fertilization/intracytoplasmic sperm injection

In-vitro/ex-ovo fertilization and ICSI have not been described in any reptile to date. This likely stems from our incomplete knowledge of ovulation timing. Additionally, a major hurdle to overcome is developing a method to harvest the ovum following ovulation but before fertilization in a minimally invasive fashion. Currently, successful ovum collection would require surgical or terminal procedures following ovulation due to their large size, as is performed in birds.[53] Reptiles, like birds, undergo polyspermic fertilization; thus in-vitro/ex-ovo fertilization would likely be the preferred technique to allow for successful fertilization and developmental progression. If ICSI is attempted in reptiles, coinjection of sperm extract would likely enhance development. In avian species this is required, as injection with a single sperm is not successful.[54] Coinjection is needed for ICSI-generated zygotes for avian species.[53] We should look at birds as the model to enhance these technologies in reptiles.

Cloning/somatic cell nuclear transfer

No reports exist in the literature using cloning or somatic cell nuclear transfer in reptiles.

Genetic editing in reptiles

Genetic editing in reptiles is in its infancy. Transgenic leopard geckos and ball pythons (*Python regius*) have been successfully developed inserting genes with lentiviral vectors to induce fluorescent proteins.[55,56] The first successful report of genetic editing using the CRISPR/Cas9 system was reported in the brown anole (*Anolis sagrei*). Surgical ovarian microinjection introduced a Cas9 protein coupled to a mixture of 3 distinct synthetic tyr guide RNAs into immature oocytes to terminate the tyrosinase gene that resulted in phenotypic albino brown anoles.[57]

Conclusion

Since the1970s, great advances that laid the groundwork to apply ART in reptiles. So, what is next? Where should our limited resources be focused in developing ART for reptiles? Currently, biodiversity loss is outpacing our ability to innovate new ART technologies for reptiles. Additionally, we are limited in our ability to successfully reproduce some of these threatened and endangered species in captivity. Efforts should be focused, and we should preserve what species we have that involves developing a robust genome resource bank exclusively for reptiles and preserving the extant genetic biodiversity. Success with artificial insemination will progress with further refinement of techniques as we better understand the female reptile reproductive anatomy and physiology. These are technologies that are successful and being utilized in other species. With time, research and success, more individuals will realize ART's role in reptile conservation.

Conflict of interest

There are no conflicts of interest to declare.

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