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Technical report

Semen and oocyte collection, sperm cryopreservation and IVF with the threatened North American giant salamander *Cryptobranchus alleganiensis*

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Abstract. Semen of high to moderate quality was collected following the hormonal induction of North American giant salamanders C*ryptobranchus alleganiensis*. Oocytes from one female yielded the first *C. alleganiensis* produced while maintained in aquaria under human care and the first externally fertilising salamander produced with cryopreserved spermatozoa and IVF. Further research is needed with North American giant salamanders to establish reliable techniques to produce large numbers of viable offspring, along with the application of cryopreserved spermatozoa.

Keywords: amphibian, breeding, conservation, hellbender, hormone, induction, propagation, reproduction.

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Introduction

Most populations of the North American giant salamander *Cryptobranchus alleganiensis* have experienced severe declines of up to 90% over the past 25 years, with a demographic shift to larger individuals, indicating a lack of recruitment (Wheeler *et al.* 2003; Burgmeier *et al.* 2011; Hopkins and DuRant 2011). Declines in populations have been linked to increased siltation, agrochemicals and decreased forest cover in affected watersheds (Hopkins and DuRant 2011; Jachowski and Hopkins 2018). Habitat-related threats will increase in the foreseeable future, and the sustainable management of *C. alleganiensis* will increasingly require cost-effective and reliable conservation breeding programs (CBPs) (Browne *et al.* 2018, 2019; Howell *et al.* 2020).

Population augmentation is already being practiced in many American states by the release of juveniles from wild-harvested eggs or *C. alleganiensis* bred in artificial streams (Civiello *et al.* 2019). Nashville Zoo at Grassmere in the US is contributing to the sustainable management of *C. alleganiensis* through the development of a conservation augmentation program for a highly imperilled, evolutionarily significant unit (ESU) located in the Lower Tennessee River. Zoo staff have been raising 170 juveniles from wild-harvested eggs from this population for a head-start program. In addition, staff have been developing reproductive technologies for the species that include the cryopreservation of spermatozoa and IVF with hormone-induced spermatozoa.

Financial constrains limit the number of brood stock housed in managed care (Howell *et al.* 2020) and will result in the loss of genetic variation and decreased fitness of offspring over time. This limitation on numbers is a particular problem in *C. alleganiensis* CBPs because of the large adult size and long generation times of these salamanders. To stimulate natural reproduction in *C. alleganiensis* while under human care (UHC), breeding has to date required complex and expensive stream systems. The use of reproduction technologies, including cryopreserved spermatozoa, can provide the most cost-efficient and reliable means to manage reproduction and genetic variation in *C. alleganiensis* CBPs (Browne *et al.* 2019; Civiello *et al.* 2019). Nashville Zoo has been developing techniques for sperm cryopreservation in tandem with the development of techniques for reproduction with IVF.

Sperm collection and cryopreservation techniques have been mostly developed for Anura, with approximately 30 species having spermatozoa cryopreserved and approximately 45 species having produced spermatozoa through hormone induction

(Browne *et al.* 2019). Only a few Caudata (8 spp.; Guy *et al.* 2020) have had spermatozoa collected or cryopreserved. However, approximately 50% of caudate species are threatened, compared with approximately 30% of anurans (https://www.iucn.org). From 2011 to 2020 we used hormone induction to collect *C. alleganiensis* semen and oocytes for sperm cryopreservation and for IVF. The metrics we assessed included semen volume, sperm concentration, sperm motility, post-thaw sperm motility, the number of oocytes, the percentage of abnormal oocytes, the number of developing eggs after IVF and the number hatched. We also collected *C. alleganiensis* semen from males in natural populations for comparison with semen from males UHC. This project has an approved Nashville Zoo internal animal care and use committee (IACUC) number #20.08. The aim of this study was to develop reproduction technologies that may help conserve this iconic salamander in the future.

Materials and methods

We combined our field data with historical records to reveal seasonality and geographical variations in reproduction (Table 1). Spermatozoa and oocytes were collected through hormone induction in the Nashville Zoo's conservation breeding and research program from two *C. alleganiensis* ESUs, which are genetically distinct populations requiring individual management (Hime 2017). One population is from the Ohio River ESU and comprises three males, and one female (♀1) collected in 2007 (West Virginia DNR Scientific collection permit #2007.003). The Nashville Zoo also houses 36 offspring produced by IVF with gametes collected from the Ohio River specimens collected in 2007. Six of those IVF offspring produced semen in 2020. The other population, which is maintained separately, is from the highly threatened Lower Tennessee River ESU (Hime 2017). The individuals UHC at the Nashville Zoo from the Lower Tennessee River ESU consist of one large adult male brought to the zoo in 1999 by a fisherman, one adult female (♀2) collected in 2013 and one adult male collected in 2015 under Tennessee Wildlife Resources Agency (TWRA) Scientific Collecting Permits 1525 and 1529. In addition, zoo staff are head-starting 170 Lower Tennessee River *C. alleganiensis* collected as eggs between 2015 and 2018 (TWRA Scientific Collecting Permit 1781). Most of the offspring hatched from eggs harvested from nature are for release, with some being held back to increase genetic security and facilitate research to optimise cost-efficiency and the security of the Lower Tennessee River CBP. The first release of head-started individuals occurred in June 2021 and were from a cohort of offspring from the first egg collection in 2015.

The *C. alleganiensis* used in the reproductive studies were kept in aquaria with photoperiods ranging from 10.00 to 14.25 h daylight, achieved by changing timers monthly to simulate natural regimens. Water temperatures ranged from 4.4°C (winter low) to 28.0°C (summer high) by following seasonal temperatures in nature, and were controlled by aquarium refrigeration units. Water quality was tested weekly and maintained at a pH 7–8, <0.01 ppm ammonia NH_3^+ and nitrite NO_2^- and <10 ppm nitrates NO_3^-. Feed consisted of live golden shiners *Notemigonus crysoleucas* (Jonesfish; jonesfish.com), commercial food-grade shrimp (McRoberts Sales; mcrobertssales.com), common earthworms *Lumbricus terrestris* (various sources) and thawed frozen neonatal rats that are sold under the name 'rat pinkies' (Rodent Pro). From 2011 to 2020, the AMPHIPLEX method (Trudeau *et al.* 2013) was used to obtain gametes. The gonadotrophin-releasing hormone (GnRH) agonist (GnRH-A) [des-Gly^{10},D-Ala^6,Pro^9]-GnRH (0.4 μg g^{-1} bodyweight) and

Table 1. Reproduction seasonality of *Cryptobranchus alleganiensis* males as shown by semen collection in the field
NW, north-west; SW, south-west

Location	Dates	References
NW Pennsylvania	21 August–14 September	Smith (1907)
SW New York	21 August–14 September	Bishop (1941)
West Virginia	21 August–14 September	Green (1933)
Missouri	3 September	Dundee and Dundee (1965)
Missouri	13 September	Nickerson and Mays (2005)
Missouri	6 October	Nickerson and Mays (2005)
Arkansas, Spring River	12 August–12 October	Baker (1963)
Indiana	7 September–11 October	Kern (1986)
Indiana	20 August–11 September	Humphries (1999)
Indiana Blue River	22 September	Burgmeier *et al.* (2011)
Indiana Blue River	16–19 September	Burgmeier *et al.* (2011)
Gasconade and Big Pine rivers	18 September–25 October	Unger (2003)
North Fork of the White River	1 October–18 November	Unger (2003)
North Carolina. Davidson River	9–11 September	Unger (2003)
Georgia, Coopers Creek	12–15 September	Unger (2003)
Tennessee Hiwassee River	6 October	Present study
Tennessee Hiwassee River Drainage, Ocoee River tributary	5 September	Present study
Tennessee, Duck River tributary no. 1	13 September	Present study
Tennessee, Duck River tributary no. 1	17 September	Present study
Tennessee, Duck River tributary no. 2	13 September	Present study

the dopamine D2 receptor antagonist metoclopramide (MET; 10 μg g ^{1} bodyweight) were dissolved together in saline and a single i.p. injection (1 μL g ^{1}) were given to both males and females with timing of injections based on a ultrasound assessment of the reproductive organs and time of year. Five wild-caught adult males, three from the Ohio River ESU and two from the Lower Tennessee River ESU, have been hormonally induced and all produced semen. Two wild-caught females, one from the Ohio ESU (♀1) and one from the Lower Tennessee ESU (♀2), were hormonally induced and produced eggs. In addition, six 7- to 8-year-old males produced by IVF at the Nashville Zoo were hormonally induced for the first time in 2020, all of which produced semen.

Ultrasound analysis has been used to document the reproductive status of *C. alleganiensis* for over a decade at the Nashville Zoo. It has been used every month of the year to document the size of reproductive organs and follicles. This long-term study is ongoing. Ultrasound analysis during the reproductive season has occurred as often as every day with some individuals to determine epididymal growth in males, follicle growth patterns in females and when final ovulation has occurred.

A General Electric Logiq e BT12 with a 12-L linear probe (5–13 MHz) for ultrasound analysis. The probe was inserted into a nitrile glove containing approximately 3–5 mL ultrasound gel. Then, a tightly wound rubber band was placed around the top of the nitrile glove and probe to contain the gel with the probe for underwater analysis. Transabdominal ultrasound examinations were conducted in both sexes while the individuals were held in a shallow container with just high water enough to cover their bodies (ultrasound chamber). To transport the salamander from the aquarium to the ultrasound chamber, specimens were gently guiding into a tall narrow plastic tub (transfer tub) while still in their aquarium. Individuals were then transported to the ultrasound chamber in the transfer tub filled with aquarium water. The transport tub was gently tilted so that the salamander and tank water were transferred to the ultrasound chamber. After ultrasound assessment, the *C. alleganiensis* were returned to their aquaria while in the ultrasound chamber still containing their original water to reduce handling stress.

Ultrasound analysis was used to determine when to inject males based on the size of the epididymal tubules located above the testes. Hormone injections were given when the hypoechoic (darker) epididymal tubules had grown to approximately 0.2–0.4 cm in diameter (Fig. 1*a*). Spermatozoa could be collected when the epididymal tubules become engorged with milt and filled the space above the testes. At this time, the epididymal tubules were between 0.6 and 1.0 cm in diameter (Fig. 1*b*).

Ultrasound analysis was also used to determine when to inject females based on the size of hypoechoic follicles (Fig. 1*c*). Finally, ultrasound analysis was used to determine when final ovulation had occurred, when oocytes could clearly be seen on ultrasound with the yolk sacs darker than, or hypoechoic to, the oocyte envelopes (Fig. 1*d*).

Semen was collected from males both UHC and in the wild by restraining the male with rubber shelf liners while held right side up in a horizontal position with the head slightly elevated. A 120-mL sterile specimen container was held under the cloaca. The semen was then stripped by applying pressure longitudinally along the sides from the anterior of the abdomen towards the cloaca. A small aliquot of the semen was then placed in a vial and diluted with simplified amphibian Ringer's (SAR; 1 L distilled water, 7.48 g NaCl, 0.23 g $CaCl_2$, 0.07 g KCl, 0.13 g $NaHCO_3$) to give suitable concentrations for sperm counts. Dilutions ranging from 1 : 1 to 1 : 10 (dilution factor) were required for accurate counts on the haemocytometer depending on the sperm density of a given sample. Then, 10 μL diluted sample was injected with a micropipette into the port on each side of the haemocytometer for counting; the sperm concentration (mL ^{1}) was calculated by multiplying the count in five squares by the dilution factor (e.g. 0.05 10^{6}). The total number of spermatozoa was calculated by multiplying the sperm concentration (mL ^{1}) by semen volume (mL). Sperm motility was assessed as the percentage of spermatozoa exhibiting movement graded 0–5, classified as follows: 0, no movement; 1, head movement with no velocity; 2, very low velocity; 3, low velocity; 4, moderate velocity; and 5, high velocity (from Schmitt and Webb 2002; Unger *et al.* 2013; note, we use the standard term 'velocity' instead of 'rate of forward progression').

Semen was diluted 1 : 1 (v/v) with 90% SAR : 10% dimethylsulfoxide (v/v) to create cryosuspensions, then frozen in 1.5-mL Eppendorf tubes held approximately 5–10 cm over liquid nitrogen vapour inside the cryotank for 5 min and then slowly lowered into the liquid nitrogen. Samples were thawed over several minutes by rubbing the Eppendorf tube between cryogloves and then between bare hands until the frozen sample became liquid; the sample was then placed in an ice bath. Post-thaw samples were assessed for the percentage of activated spermatozoa. To activate spermatozoa, semen was mixed 1 : 1 with water, where the osmotic shift caused the spermatozoa to exhibit twitching, side to side movement of the sperm head, flagellar membrane motion and forward movement.

Spawned oocytes were collected for IVF when a female was observed spawning using remote video. The time between fertilising oocytes and the spawning of individual groups of oocytes varied due to various constraints, including travel time to the zoo after oocyte laying started, time to collect spermatozoa and, most importantly, intermittent oocyte deposition by the females. The number of oocytes, the percentage abnormal oocytes (defined as those with deformed or multiple yolk sacs), the number of developing eggs and the number of larvae hatched between 2014 and 2020 are provided in Table 2.

Dry fertilisation has been the most successful method for IVF. Our methodology for dry fertilisation has varied, but uses either freshly collected spermatozoa or spermatozoa held in an ice bath. With hormone-induced spermatozoa, we have seen no detectable difference in forward movement, flagellar membrane movement or abnormalities when held in an ice bath over several hours compared with fresh spermatozoa (unpubl. obs.). Spawned oocyte strands were collected from the female's tank for fertilisation. On several occasions, oocytes were collected as they were being laid. The spermatozoa were then applied, using a syringe, to fresh oocytes. Otherwise, oocytes were held in amphibian Ringer's solution, which was poured off and then the spermatozoa applied. Once spermatozoa had been applied, oocytes were allowed to sit on average for 1 min (range 1–10 min). Then aquarium water or reverse osmosis water was

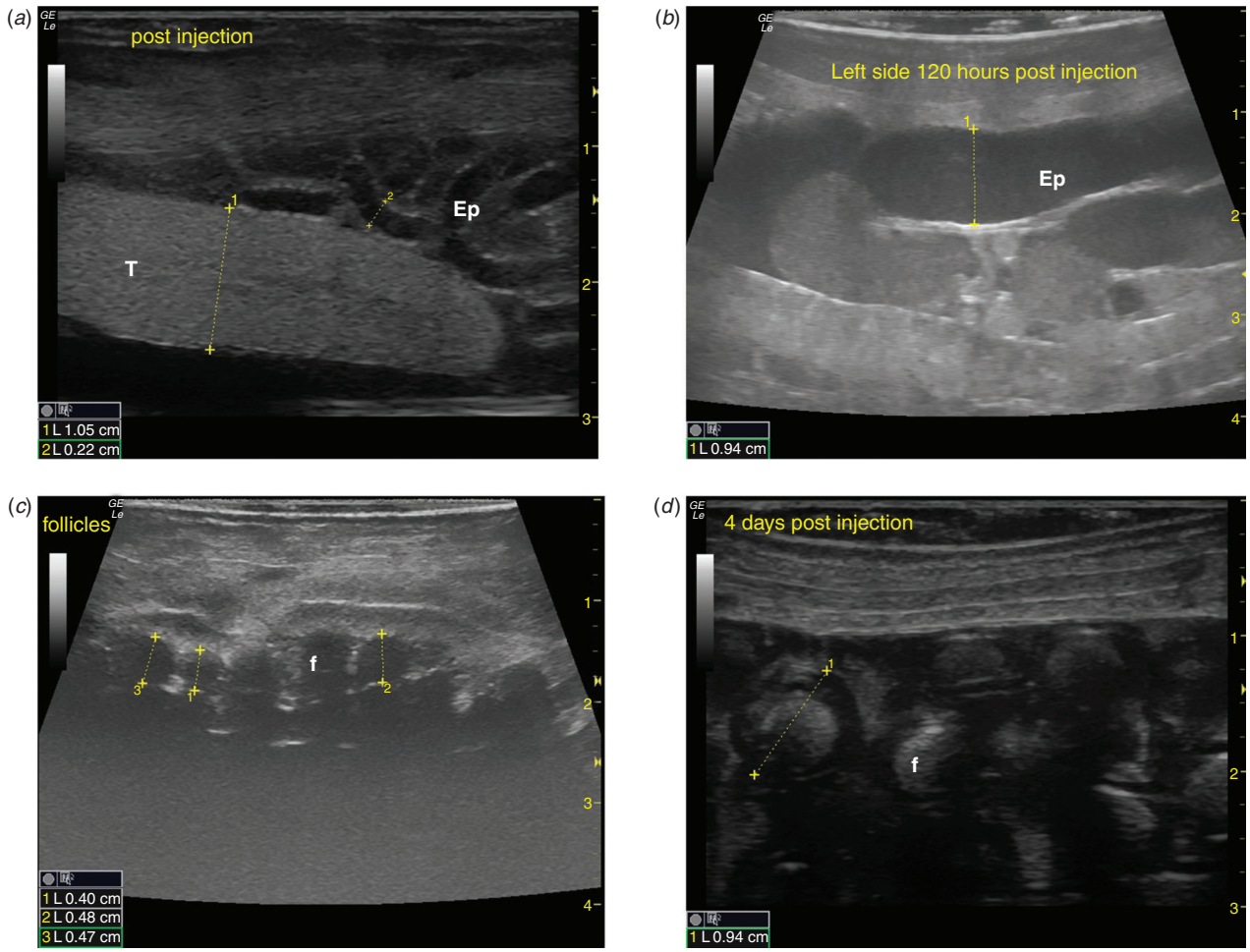

Fig. 1. Images of *Cryptobranchus alleganiensis* gonads captured directly using ultrasound. (*a*) Testes (T; 1.05 cm wide) with epididymal tubules (Ep; diameter 0.2 cm) are visible. (*b*) At 120 h after injection of the AMPHIPLEX mixture, the epididymal tubules are engorged and are increased approximately fivefold to 0.94 cm in diameter, and semen can be harvested. (*c*) Females are injected with the hormone mixture when the follicles (f) reach 0.4–0.5 cm in diameter. (*d*) Oocyte maturation, ovulation and the development of eggs can clearly be visualised at 4 days after injection.

Table 2. Number of oocytes spawned each year from 2014 and 2020, the percentage abnormal, the number of oocytes developing after IVF and the number of larvae hatched from two female salamanders (♀1 and ♀2)

In 2020, ♀1 was not hormonally induced and did not produce oocytes. In 2014, ♀2 was hormonally induced but did not produce oocytes. DNC, data not collected

	2014	2015	2016	2017	2018	2019	2020	Mean
♀1								
No. oocytes	460	397	567	489	517	857	0	548
% abnormal	13	8	23	4	25	66	0	20
No. developing	0	4	2	2	36	9	0	7.6
No. hatched	0	3	0	0	27	4	0	5.7
Weight (kg)	2.4	2.6	2.7	2.7	3.1	3.3	3.2	2.8
♀2								
No. oocytes	0	145	320	129	360	474	579	287
% abnormal	–	DNC	DNC	DNC	3	10	17	10
No. developing	0	0	0	0	0	0	12	1.7
No. hatched	0	0	0	0	0	0	0	0
Weight (kg)	0.6	1	1	1.2	1.2	1.4	1.5	1.1

Table 3. Volume of semen collected from wild-sourced males under human care over 3–7 days after hormone injections

No. exp., total number of males expressing semen; % Exp., percentage of males expressing semen; Day 3 to Day 7 = Volume in mL of each male's semen collected; mL/exp. male = average volume of sperm (mL) per expressing male

Year	No. males induced	No. exp.	% Exp.	Volume of semen collected from individual males (mL)					Total volume collected (mL)	Mean semen volume (mL) per expressing male
				Day 3	Day 4	Day 5	Day 6	Day 7		
2020	2	2	100	0	0	0	4, 14.5	0	18.5	9.3
2019	4	4	100	0	1.6, 7	0	1, 4.5	1.7, 8.6	24.4	6.1
2018	4	4	100	2, 2.5, 3.6, 4	0	0	7, 7.8	0	26.9	6.7
2017	4	4	100	0	0	0	3, 6.5	2, 4, 5	20.5	5.1
2016	5	4	80	0	7, 15	0	3, 4, 6	2, 3	40	10
2015	4	3	75	0	6	8	0	2, 6, 9	31	10.3
2014	3	2	66	7	0	0	6.5, 7	0	20.5	10.3
2013	3	2	66	0	1.7, 5	0	0	0	6.7	3.4
2012	2	2	100	0	1.75, 3	0	0	0	4.75	2.4
2011	1	1	100	0	0	0	3	0	3	3
No. individuals expressing semen				5	9	1	13	10		
Total volume semen expressed for day (mL)				19.1	48	8	77.8	43.3		
Mean volume of expressing males (mL)				3.82	5.3	8	6	4.33		6.2

added to cover the oocytes, mixed gently and then allowed to sit for 5–20 min before the oocytes were transferred to incubation chambers. The incubation chambers consisted of 38 L aquaria in a flow-through system with small submersible pumps (various brands used) added to each tank to create gentle movement of the eggs.

Results and discussion

Wild populations of *C. alleganiensis* east of the Mississippi River reproduced during a 5-week period between late August and early October, whereas those west of the Mississippi River reproduced from late September to mid-October. Semen was collected from early to mid-September from most locations in Tennessee. The main stem of the Hiwassee River in Tennessee, downstream from a large dam, is the only site we know of west of the Mississippi where *C. alleganiensis* have been shown to breed in October (Table 1).

The percentage of wild-sourced males UHC induced to express semen (comprising two to five individuals in a given year, 2011–20) ranged from 65% to 100%. The mean semen volume of males UHC that expressed semen was 6.2 mL (range 2.4–14.5 mL; Table 3), which was greater than that of the younger zoo-bred males (mean 4.0 mL; range 2.5–7.0 mL; Table 4) and similar to that of males collected in the field (mean 5.8 mL; 0.1–10.2 mL; Table 5).

The six mature males that were produced with IVF (four 7 year olds, and two 8 year olds) were hormonally induced for the first time in 2020 by injection with AMPHIPLEX; semen was then collected from these individuals 6–8 days later (Table 4). The beginning and end of sampling of these individuals was based on ultrasound analysis of the size of the epididymides. In contrast, the older wild-sourced males UHC expressed semen earlier and over a longer period after induction (Table 3). Semen was not collected daily from individuals, but only as needed to artificially fertilise oocytes. Sperm concentrations for samples from *C. alleganiensis* collected from nature

Table 4. Age of hormone-induced captive-born *Cryptobranchus alleganiensis* and the volume (mL) of semen collected in September 2020 at the Nashville Zoo

Spermatozoa were collected on Days 6–8 after injection from six *C. alleganiensis* (two males produced in 2012 and four males produced in 2013). These males had just reached sexual maturity and were the first captive-born North American giant salamanders to be hormonally induced for sperm collection

Age (years)	Day 6	Day 7	Day 8
8	7		
8	3.6		5.2
7		4.3	
7	3		
7	2.8		
7	2.5		3.2

averaged 35 10^6 mL ^{1} ($n = 47$), and were higher than the mean for hormonally induced males at the Nashville Zoo in 2020, which included both recently mature and older males (4.1 10^6 mL ^{1}; $n = 8$; Table 5).

In 2020, the mean percentage motility of hormone-induced *C. alleganiensis* spermatozoa ($n = 8$), as shown by flagella movement, was 60% (range 14–96%), with a mean velocity of 3.45 (range 3–4.5). Moderate to high motilities averaging 85% are generally found in *C. alleganiensis* spermatozoa collected from nature (Unger *et al.* 2013). Refrigerated spermatozoa from wild *C. alleganiensis* retained 50–70% motility for up to 3 days (Unger *et al.* 2013). However, refrigerated hormone-induced spermatozoa retained no velocity and virtually no motility after 1 day, although motility and velocity were maintained for up to 2 days when induced semen was held in an ice bath at 0°C (Table 6).

The post-thaw recovery of cryopreserved spermatozoa collected from hormone-induced males after activation was

Table 5. Comparison of sperm concentrations and semen volumes collected from hormonally induced *Cryptobranchus alleganiensis* under human care (UHC) or from wild males

Hormone induction UHC consists of 7- and 8-year-old males produced by IVF with gametes from the Ohio River ESU wild-collected adults and two wild-collected adults from the Lower Tennessee River ESU with 8 and 22 years UHC. One sample from a wild-collected Ohio ESU specimen that was UHC for 14 years was contaminated with faecal material, so no calculations were made. Wild-sourced sperm 'natural' samples were collected from wild *C. alleganiensis* in the field

Sample	Date	Concentration (10^6 mL ^{1})	Volume (mL)	No. spermatozoa (10^8)	Reference
Hormone-induced, UHC					
7- and 8-year-old males ($n = 6$)	September 2020	3.5	4.0	0.14	Present study
Long-term UHC					
8 years ($n = 1$)	September 2020	10.6	4.0	0.40	Present study
14 years ($n = 1$)	September 2020	–	3.0	–	Present study
22 years ($n = 1$)	September 2020	1.0	14.5	0.14	Present study
Wild-sourced spermatozoa					
Natural ($n = 1$)	17 September 2010		2.5		Present study
Natural ($n = 1$)	17 September 2010	6.0	0.53	0.032	Present study
Natural ($n = 1$)	14 September 2010	5.0	2.0	0.10	Present study
Natural ($n = 1$)	2 September 2015		0.60		Present study
Natural ($n = 43$)	2002	36	0.10–10	1.5–2.7	Unger (2003)

Table 6. Velocity, number of motile spermatozoa/number spermatozoa counted and the percentage of motile spermatozoa from five *Cryptobranchus alleganiensis* hormonally induced in 2020 from collection up to 2 days in storage in an ice bath

Day 0 is is the date semen was collected

Individual	Velocity	No. motile/no. counted	% Motility
Male 1			
Day 0	3–4	30/221	14
Day 1	4	194/212	92
Day 2	1–2	5/291	2
Day 3	0	0	0
Male 2			
Day 0	4	155/219	71
Day 1	3	14/228	6
Day 2	1	5/205	2
Day 3	0	0	0
Male 3			
Day 0	4	239/250	96
Day 1	3	95/200	50
Day 2	0	0	0
Male 4			
Day 0	3–4	30/205	15
Day 1	3–4	18/225	8
Day 2	0	0	0
Male 5			
Day 0	3	177/236	75
Day 1	3	138/200	69
Day 2	2–3	168/228	74
Day 3	0	0	0

Fig. 2. (*a*) An adult male *Cryptobranchus alleganiensis* that was hormonally induced to produce cryopreserved spermatozoa. (*b*) First *C. alleganiensis* produced through IVF with cryopreserved spermatozoa. Photograph taken December 2015. (*c*) The same *C. alleganiensis* produced with cryopreserved spermatozoa approximately 5 years later in January 2021.

high, with approximately 40% exhibiting twitching, approximately 80% exhibiting flagella motion and limited forward movement, and only <10% of spermatozoa were abnormally shaped (data not shown). Nevertheless, *C. alleganiensis* had a higher post-thaw recovery of sperm motility than Chinese *Andrias* sp. (Peng *et al.* 2011). The ambystomid *Ambystoma tigrinum* (Marcec 2016) and three Salamandridae species (Guy *et al.* 2020) also had lower post-thaw motility, but these internally fertilising species have lower motility to begin with (Guy *et al.* 2020). In 2015, we produced one male using post-thaw cryopreserved spermatozoa and IVF that is thriving at the Nashville Zoo (Fig. 2).

Table 7. Total number of oocytes produced over the days after induction from the years 2014 through 2020 by wild-collected ♀1 and ♀2
Velocity scale as referenced in Material and methods section of this paper

	Days after induction								
	3–5	5–7	7–9	9–11	11–13	13–15	15–17	17–19	19–21
♀1	1634	1193	460						
♀2		1197	271	328	45	55	36	60	15

Starting in 2011, hormone induction produced oocytes from ♀1 every year until 2019. In 2018 and 2019, ♀1 produced an increasing percentage of abnormal oocytes and was therefore not induced in 2020 (Table 2). Over the years, ♀1 (collected in 2007) spawned most oocytes 3–5 days after induction and the rest by 7 days. In contrast, ♀2 (collected in 2013) began spawning 5 days after induction and continued to spawn oocytes until Day 11, with scattered low numbers of oocytes produced until Day 20 (Table 7). The number of oocytes spawned per year increased significantly between 2014 and 2020 for ♀2, from 0 to 579 ($r^2 = 0.83$, $P < 0.01$), and stochastically (2011–19) for ♀1, from 460 to 857 ($r^2 = 0.56$, $P < 0.1$; Table 2).

Conclusion

Our CBP and zoo-based research has made contributions to the sustainable management of North American giant salamanders. Semen from wild *C. alleganiensis* is easily collected in large volumes during the reproductive season. Successful cryopreservation of semen may be the best way to conserve gene diversity from declining populations. Hormone induction produced large semen volumes and oocyte numbers, with some IVF oocytes producing adults. In 2012, we produced the first *C. alleganiensis* larvae with IVF, and, in 2015, the first larva using cryopreserved spermatozoa (Virata 2016). To date, very few *C. alleganiensis* individuals have been tested for the hormone induction of gamete release, and little is actually known about the neuroendocrine control of reproduction in Caudata (Vu and Trudeau 2016; Kikuyama *et al.* 2019). Further research is needed to optimise the collection of viable spermatozoa and oocytes, IVF and early egg incubation techniques.

In the future, we may consider the use of oxytocin injections in the hope of stimulating females to deposit oocytes sooner after final ovulation. In addition, having the females lay oocytes over a shorter period of time would allow researchers to collect fresh semen to fertilise more oocytes in a shorter period of time, which has the potential to improve fertility.

There is still much to learn before *C. alleganiensis* reproductive technologies can be used to reliably produce large numbers of individuals for CBPs; we hope others will become involved in this research. Unfortunately, some *C. alleganiensis* ESUs may soon be lost without conserving the gene diversity that is left in senescent and declining populations. The availability of genomic and transcriptomic resources for an increasing number of salamanders (Hime *et al.* 2019, 2021; Hu *et al.* 2019; Nowoshilow and Tanaka 2020) will also aid in phylogenetic targeting of new management techniques for *C. alleganiensis* CBPs.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of funding

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Data availability statement

The data supporting this study will be shared upon reasonable request to the corresponding author.

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