Topical application of hormone gonadotropin-releasing hormone (GnRH-A) stimulates reproduction in the endangered Texas blind salamander (Eurycea rathbuni)

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Abstract

We present a landmark success of a pilot study in the noninvasive, topical hormonal stimulation of reproduction of salamanders using Texas blind salamanders (Eurycea rathbuni) as a model species. Improved reproduction is a critical milestone in the conservation of imperiled species. Captive reproduction of amphibians is often challenging due to specific and ambiguous environmental cues for each species. The Texas blind salamander is a federally listed troglobitic amphibian found only in the Edwards Aquifer beneath San Marcos, Texas. This species is long-lived, paedomorphic, and obligately aquatic. As with other cave-dwelling organisms, Texas blind salamanders exhibits delayed reproductive maturity and low reproductive output. The US Fish & Wildlife San Marcos Aquatic Resources Center maintains a captive assurance population of wild individuals to supplement natural populations in the case of a catastrophic impacts on the wild population. Despite housing this species since the 1980s, unassisted reproductive events remain infrequent and unpredictable. In 2020, we developed the noninvasive use of the topical application of GnRH-A to stimulate reproduction in 12 females combined with 12 males during a pilot study, that resulted in 11 clutches over a five-month period. These findings mark a significant increase from normally low production of 4.5 clutches annually (average from 2007 to 2019) and represent a landmark success for captive propagation of this species.

Keywords

amphibian, breeding, conservation biology, endangered, hormones, nonmodel organism, pheromones

1 | INTRODUCTION AND PURPOSE

Amphibians are suffering global population declines due to numerous factors, including disease, climate change, and habitat destruction (Gascon et al., 2007; Sodhi et al., 2008). Of the three taxonomic classes of amphibians, Caudata is suffering the highest percentage of extinction risk with assessments showing at least 57.6% of caudate species threatened with extinction (vulnerable, endangered, or...
critically endangered) (IUCN, 2021). Within Caudata, the family Plethodontidae has the highest representation of species, and International Union for Conservation of Nature (IUCN) assessment shows 59.3% of these species to be threatened with extinction. The genus *Eurycea*, within the family Plethodontidae, has 34 known species (Amphibiaweb, 2021), 26 of which have been assessed by IUCN as of 2021. IUCN assessments report 46.2% of this genus are threatened with extinction. The decline in amphibians has generated demand for development of reproductive techniques and captive breeding programs (Holt et al., 2010; Kouba et al., 2009). Amphibian reproductive mechanisms are widely variable and environmental cues for reproduction are unclear and often difficult to replicate in a captive setting (Browne & Zippel, 2007; Julien et al., 2019; Kouba et al., 2009).

Gonadotropin-releasing hormone (GnRH-A) is a natural hormone present in all known vertebrates (Gorbman & Sower, 2003; Roch et al., 2001). In amphibians, this hormone released from the hypothalamus to trigger release of luteinizing and follicle stimulating hormones from the pituitary, which then act on the male and female gonads to trigger gamete production and sex steroid hormone release (Moore et al., 2005; Vu & Trudeau, 2016). While the primary role of GnRH-A is reproductive, other hormones and organ systems are affected by its release (Muñoz-Cueto et al., 2020) and it acts as a pheromone in some amphibian species (Moore et al., 2005; Vu & Trudeau, 2016). GnRH-A can be injected, inserted intranasally, or topically applied for use in amphibian breeding (Browne & Zippel, 2007; Julien et al., 2019; Kouba et al., 2009; Silla et al., 2020; Vu & Trudeau, 2016). While GnRH-A is naturally occurring in amphibians, the response to exogenous application is somewhat species specific and generally requires testing to understand its impacts and efficacy in inducing reproductive behavior, ovulation, and sperm release (Kouba et al., 2009; Vu & Trudeau, 2016). Studies using GnRH-A have generally focused on anuran species (Browne & Zippel, 2007; Vu & Trudeau, 2016), though a few studies on salamander species have been published (Guy et al., 2020; Marcec, 2016).

Most commonly, GnRH-A has been provided as an injection in amphibian reproductive technologies. GnRH-A injection has proved helpful in inducing egg deposition of other salamander species (Guy et al., 2020; Marcec, 2016) and topical application has proven effective in two species of anurans (Silla et al., 2018, 2020). The following pilot trial documents the first attempt at topical application of GnRH-A to a salamander species, utilizing the Endangered Plethodontid species, *E. rathbuni*, as an opportunistic and practical model. This species represents a member of the largest family of salamanders, the Plethodontids, and it is hoped that techniques on this species may be transferrable to related species. Many Plethodontids are minute species, weighing less than 1 gram. Plethodontids have distinctly different physiology from other caudates primarily in that they are lungless salamanders that rely primarily on their skin to breathe (Whitford & Hutchison, 1965). This makes them particularly delicate, and traditional reproductive technologies may be too invasive for these species. Fortunately, most Plethodontids have multiple adaptations and organs developed specifically for releasing and absorbing pheromones (Sever & Staub, 2011). In Plethodontid species, the nasolabial folds area is most receptive to pheromones with additional pheromone receptors down the length of the body. Because of this they are potentially well suited for topical hormone application.

The Texas blind salamander (*Eurycea rathbuni*) is a blind, obligately aquatic, subterranean cave-dwelling species in the Edwards Aquifer underneath San Marcos, Texas. Fertilization occurs internally after transfer of a spermatophore packet during courtship (Bechler, 1988). While the IUCN has re-designated the species as Vulnerable, Texas blind salamanders remains classified as Endangered in the United States under the Endangered Species Act. Their habitat faces threats of drought, contamination, and overuse as the human population continues to increase (Zerrenner & Sommer, 2012). We have no population estimate, as captures are limited to nine historical surface accessible sites. The subterranean habitat has not been explored. Individuals are captured from wells, caves, and spring outflows to build captive assurance populations. Genetic analysis for relatedness of individuals between localities is pending. Texas blind salamanders have been held by the United States Fish and Wildlife Service at the San Marcos Aquatic Resources Center (SMARC) since the 1990s. Survival in the wild is greater than 10 years (Longley, 1978), though we estimate survival may extend upwards of 30 years based on other cave salamander life histories and the age of captive individuals. Growth rate drastically decreases with age and size, but is overall indeterminate (Vieira et al., 2020).

External sexual dimorphism is absent in this species (Sever, 1985) so sexing is dependent on the candling method, where eggs or testes are observed by shining light through the abdomen (Gillette & Peterson, 2001). We hypothesize that breeding is temporally unrestricted because larvae are found year-round in the wild. As in other cave dwelling organisms, sexual maturity is delayed.
and reproductive output is reduced (Culver & Pipan, 2019). Females begin to show small eggs at 1.5–2 years of age at the SMARC (Vieira et al., 2020). However, the presence of eggs does not necessarily result in the production of offspring. We have observed the period between reproductive events to be approximately 2 years, but conclusive records were not kept until 2017, thus more observations are needed to define a more precise range. In captivity, Bechler (1988) observed females initiate courtship, which is different from other species. We have also noted both females and males initiating courtship.

Many females in the SMARC colony exhibit varying stages of gravidity throughout the year. In spring 2020, 12 females simultaneously exhibited mid to late gravidity. This presented an opportunity for researchers to initiate a pilot reproduction trial with GnRH-A. This was the first opportunity we had where there were numerous adults in the population, extra tank space to hold the treated organisms, programmatic freedom, and staff available to conduct a trial all at once. Low collection rates of adult Texas blind salamanders inhibits a full experimental study with large number of salamanders for many years. Thus, the following pilot study was initiated using a smaller number of organisms to explore the efficacy of GnRH-A in the interim. Ideally, a large number of animals would be used to conduct a statistically sound full experimental design, however due to the rarity of collecting adult Texas blind salamanders and the number of years it would take to amass enough for a full factorial experiment, plus the promising results we saw from the pilot experiment, we felt that going forward with publishing a research note acknowledging this was a pilot experiment would serve the amphibian community more than waiting years to bring forth these results. Topical application of GnRH-A in this species could prove to be a safe and effective management method for captive breeding of this species, and could prove applicable to other Eurycea or Plethodontid species in the future.

2 METHODS

Wild Texas blind salamanders were collected under Texas Scientific Research Permit No. SPR-0616-153. Researchers adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidance of a veterinarian.

Twelve gravid females (43–56 mm SVL, 1.89–4.55 g, 2.6—more than 5 years old) and 12 males (45–57 mm SVL, 2.24–4.40 g, 2.6—more than 5 years old), previously held in mixed-sex systems of mature adults, received topical GnRH-A before combination into one new holding system. Like previous systems, the new system was one large trough, with no subdivisions (partial recirculation, 325-L fiberglass tank 2.6 m length × 0.5 m width × 0.25 m water height). Animals were held at a density of 0.07 salamanders per liter and offered varied habitat, including plastic aquarium structures, artificial vegetation, rocks, hard and soft meshes, and PVC structures for egg deposition.

Texas blind salamanders of similar size classes are held in mixed-sex group tanks to encourage courtship opportunities; while we try to keep the sex ration at 1F:1M, there are more males in the population than females so some tanks have 1F:1.5M. There are a few tanks that have only males, and there are tanks of only immature individuals whose sex cannot yet be identified. The density of Texas blind salamanders varies depending on their size, ranging from 0.5 to 0.8 salamanders per liter. Water pumped from the Edwards Aquifer via two different wells, is not filtered, goes through a station chiller so that when it reaches the systems it is approximately 21°C. Systems have partial recirculation of water to mimic flow and a fresh water input that turns over the volume of a tank/system every 24 h. Salamanders are fed two to three times weekly a variety of food including live amphipods, live black worms, live daphnia, frozen Mysis shrimp, frozen copepods, live blind cave shrimp (Palaeonetes antrorum), and live troglobitic crustaceans (Stygobromus sp.).

\[\text{[des-Gly}^{10}, \text{D-Ala}^6]\text{-LH-RH ethylamine powder (GnRH-A) (5 mg, Sigma-Aldrich, Inc, catalog #L4513)}\]

was reconstituted with deionized water at a concentration of 2 mg GnRH-A/1 ml water. Topical dosage was of 25 µg GnRH-A per 1 g of salamander, or 12.5 µl solution/g. Starting with females, each individual was weighed and solution dose calculated. Animals were not anesthetized as the potential stress and risk without sedation was deemed lower than stress with the addition of a lengthy anesthesia procedure and possible reactions in this species between anesthetic M-222, bicarbonate buffer, and GnRH-A. The salamanders remained docile during the application process, did not exhibit startle responses or show aversion to the solution. Each salamander was held in a damp dipnet while GnRH-A was applied topically with a micropipette. Pipette tips were changed between individuals. GnRH-A was applied to nasolabial folds and excess volume was dripped along the dorsal side of the body. To increase absorption of GnRH-A, the individual was held in the net for 10 s after application before being transferred to a shallow dish of water, where the dorsal side, where the GnRH-A solution was applied, was kept above the water line for an additional 5 min. All 12 females were then added to the reproduction tank.
These processes were repeated with 12 male salamanders, who were then added to the tank all at once approximately 45 min after the females had been added to the tank.

Fresh water input was reduced during the 48 h following GnRH-A application to help retain and recirculate pheromones (both applied and naturally produced) within the system. After 48 h, fresh water input was adjusted to replenish the volume of the tank every 12–16 h.

Each batch of eggs were counted and moved to a separate glass aquarium within 72 h of first deposition of a clutch. Each clutch was maintained in its own independent aquarium with flow through water pumped from the Edwards Aquifer that had been chilled by the station chiller. As eggs developed, those that were not fertilized or became infected with water mold/fungus were removed. Hatchlings were counted (hatching occurred ~30–40 days after oviposition). Larvae and juveniles were maintained by clutch in independent aquariums with flow through water and fed artemia. Five months after LHRH application, males were removed from the system and females were checked for levels of gravidity.

Due to the low numbers of sexually mature wild Texas blind salamanders in captivity, a control group was not used for this pilot study. However, the remaining adults in the captive assurance population that were not treated with GnRH-A are used to draw some comparisons. There were 25 adult females and 38 adult males in the non-experimental portion of the population; with 5 of the males and 1 female incorporated into the non-experimental population (after finishing quarantine) 1 week after the pilot study started. The nontreated salamanders were in similar systems with the same types of habitat enrichment items, water flow and fresh water exchange, in the same building and room, and fed on the same schedule and same types of food as the pilot treatment group. The number of females in mid- to late-stage gravidity in the nontreated population fluctuated during the same time period as the pilot trial (from five to 14 gravid at any given time). Females in the nontreated population were held in tanks with 1F:1M to 1F:1.5M ratios.

### TABLE 1 Clutch tracking data

| Clutch # | Date deposited | Eggs deposited | Number hatched | Hatching success (%) |
|----------|----------------|----------------|----------------|----------------------|
| C1       | April 2, 2020  | 35             | 0              | 0                    |
| C2       | May 25, 2020   | 36             | 17             | 47.2                 |
| C3       | June 28, 2020  | 32             | 7              | 21.9                 |
| C4       | July 11, 2020  | 43             | 23             | 53.5                 |
| C5       | July 14, 2020  | 24             | 5              | 20.8                 |
| C6       | July 20, 2020  | 28             | 2              | 7.1                  |
| C7       | July 26, 2020  | 23             | 10             | 43.5                 |
| C8       | July 28, 2020  | 28             | 12             | 42.9                 |
| C9       | August 15, 2020| 17             | 11             | 64.7                 |
| C10      | August 24, 2020| 26             | 22             | 78.6                 |
| C11      | September 7, 2020| 21             | 13             | 61.9                 |
| Totals   | September 7, 2020| 313            | 122            | 39.0                 |

**Note:** Clutches C1–C10 occurred while males were in the system. C11 was produced just after males were removed from the system, 5-months posthormone application. Only one female that received GnRH-A did not deposit eggs. Within 72 h of deposition eggs were removed and counted. Hatching success was calculated as the number of larvae divided by the number of eggs.
For further comparison and historical context, we gathered paper and electronic records on the captive assurance colony at SMARC for population numbers and clutch deposition. Total population numbers are known for 2007–2016, but sex information was not recorded. We averaged the sex ratios from 2017 to 2020 (45.3% female) to estimate the females in these years. During 2007–2016, only adult Texas blind salamanders were collected. A new type of collection net was put into use starting in June of 2017 that allowed for the collection of very small juvenile salamanders; but in the discussion of captive population numbers, sexually immature salamanders are not listed in the population totals of this paper. Texas blind salamanders have been held using similar methods through the years, but the care givers have changed and the programmatic directions have been different. Initially, SMARC only held a few Texas blind salamanders and the program directive was to be very hands-off in the interaction with those salamanders, over the years the goals of the captive population program have changed in terms of collection and research. Currently, the captive population is part of the Edwards Aquifer Refugia Program that includes individually tagging and tracking life history events and conducting research that aids in captive holding, collection, and conservation. Salamanders have been kept in mixed sex groups, water temperature set at 21°C, partial recirculation, well water source the same, and feeding schedule and types the same. The main differences were salamanders were not tagged prior to 2017 and were kept in a partially enclosed building until late fall of 2018 when they moved into a new fully enclosed building. A portion of the Texas blind salamander population was in a tag retention and readability study in 2019–2020 (Moon et al., 2021); they were kept in their normal conditions as described above, with monthly tag checks and notations as to if they were gravid.

3 | RESULTS

No negative reactions to GnRH-A were observed. Eleven clutches of eggs were produced in the 5 months following the application of GnRH-A (Table 1). Four hundred and four eggs were deposited, and 157 larvae hatched (Table 1, Figure 1). In the nonexperimental population, three clutches were produced in the month prior to the pilot

| Year   | Clutches produced | Adults | Females | Males | Estimated females (45.8%) | Clutches per female |
|--------|-------------------|--------|---------|-------|----------------------------|---------------------|
| 2007   | 0                 | 65     |         |       |                           | 0.00                |
| 2008   | 5                 | 81     |         |       |                           | 0.13                |
| 2009   | 5                 | 71     |         |       |                           | 0.15                |
| 2010   | 8                 | 52     |         |       |                           | 0.34                |
| 2011   | 2                 | 57     |         |       |                           | 0.08                |
| 2012   | 3                 | 51     |         |       |                           | 0.13                |
| 2013   | 2                 | 80     |         |       |                           | 0.05                |
| 2014   | 11                | 115    |         |       |                           | 0.21                |
| 2015   | 9                 | 135    |         |       |                           | 0.15                |
| 2016   | 7                 | 118b   |         |       |                           | 0.13                |
| 2017   | 2                 | 24     | 12      | 12    |                           | 0.17                |
| 2018   | 3                 | 48     | 23      | 25    |                           | 0.13                |
| 2019   | 1                 | 70     | 30      | 40    |                           | 0.03                |
| Mean (SD) | 4.46 (3.38)     |        |         |       |                           | 0.13 (0.08)        |
| 2020 Whole population | 16 | 87     | 37      | 50    |                           | 0.43                |
| 2020 Nonexperiment | 5  | 63     | 25      | 38    |                           | 0.20                |
| 2020 GnRH-A experiment | 11 | 24     | 12      | 12    |                           | 0.92                |

aRecords on the number of females and males were recorded starting in 2017 with a shift in programing. We estimated the number of females for 2007–2016 based on the average percentage of females from 2017 to 2020.

bThe large drop in number of adults between 2016 and 2017 is due to a theft of salamanders from the facility; this is still an on-going investigation. There were 118 adult Texas blind salamanders at the facility before the theft in November 2016, but only 12 remained after the theft.
experiment and two clutches were produced after the end of the pilot experiment. Historical and current records of clutch deposition data for the Texas blind salamander captive assurance colony are presented in Table 2.

When the females in the experimental group were checked for gravidity levels at the end of 5 months some females were generating new eggs at very early stages of maturity (Figure 2). One female (visible implant elastomer tagged Yellow–Pink–Pink, YPP) was fully gravid at the check, with noted swelling around the cloaca, which is likely a sign of having been inseminated (Figure 2e). No other females were fully gravid at the check. A clutch was produced 6 days after the males were removed from the system. The individual tagged YPP was examined for eggs 3 days after the clutch was laid to confirm that she was the mother; no eggs were seen in her body cavity. One female died of unknown causes during the study with no mechanical damage noted, nor signs of disease. This was one of the older females of unknown age estimated to be greater than 5 years of age. She had no eggs in her abdomen at death.

4 | DISCUSSION

With 11 egg clutches produced by 12 females in 5 months, we are confident that the treatment of GnRH-A increased the number of clutches produced when compared to clutches historically produced by Texas blind salamanders in captivity. During the same year, only five clutches were produced by the sexually mature adults in the untreated remainder of our captive population (25 females, 38 males). During a tagging study the previous year (February 2019–February 2020), 83 Texas blind salamanders were monitored: 23 of those were mature females, 13 of those females had eggs, but only one clutch was produced (23 females, 30 males, 29 immature salamanders). We believe each clutch in the pilot study was produced by a single female because even if this species undergoes bimodal or continuous reproduction, it is unlikely there would have been enough time for a female to deposit multiple clutches during the 172 days of the study. With this assumption, GnRH-A treated salamander production exceeded the average number of 4.46 (SD 3.38) clutches produced a year from all wild stock females (no treatments) during 2007–2019 at the SMARC (Table 2). For the whole 2020 captive assurance population (experimental and non-experimental) 0.43 clutches per female is triple that of the 13-year estimated average of 0.13 (SD 0.08) per female. The 2020 nonexperimental population clutch per female number (0.20) is consistent with the 13-year average, with the 2020 experimental group clutch per female number (0.92) seven times higher than the 13-year average. In perspective, an estimated 53 females in 2015 took an entire year to produce the number of clutches that 12 GnRH-A-treated female salamanders produced in 5 months. If clutches were not laid by individual females, this pilot trial at least shows that application of topical GnRH-A increases fecundity of females in this species, and future studies will determine individual effects.

It is unclear what role GnRH-A could have on egg production in females postoviposition. Without GnRH-A, significant gaps between gravidity have been observed, with females being nongravid for 1–2 years between events; however, some of the GnRH-A-dosed females in this trial began producing new eggs within 6 months after oviposition. We recommend that the SMARC continue to follow and document this group, especially to compare if they reach peak gravidity at a different time than other nontreated females in the captive assurance population.

This pilot study documents the likely efficacy of topical application of GnRH-A to stimulate reproductive behavior in Texas blind salamanders and the first successful topical application of GnRH-A in any salamander
species. Reliably reproducing Texas blind salamanders is a crucial step in creating a functional assurance colony, and in order to determine if this is a reliable method the study will need to be repeated. We acknowledge that the results presented here must be viewed in the light of not having a full factorial experiment with a true control. However, since acquiring enough adult Texas blind salamanders to do a large full-scale experiment would take many years and the results of this pilot study were very promising with implications to conservation of this species and others, we wanted to report the information in a prompt time frame.

Topical hormone application offers a safe and minimally invasive method for *Eurycea rathbuni* that might be applicable to related Plethodontids, which are often small and delicate. Many species of Plethodontids weigh less than 1 g, and all of the salamanders in this family are lungless and sensitive to handling. Topical hormone application not only offers a minimally invasive technique, but a technique that works with the physiology of the animals and their receptive skin. Plethodontidae represents the largest family of salamanders, and with 59.3% of the species in this family assessed as vulnerable, endangered, or critically endangered by IUCN they are in need of techniques for reproductive management. The success of topical hormone application in a salamander, particularly a Plethodontid, is an important step for captive assurance of these species worldwide.

ACKNOWLEDGMENTS
This work was supported by Edwards Aquifer Authority and the San Marcos Aquatic Resources Center USFWS, who financially supported this work but were not involved in the research or writing. Thank you to L. Moon, J. Crow, J. Barnett, T. Funk, and B. West for assisting with organizational care and support and D. Britton for text review. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the US Fish and Wildlife Service.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTIONS
Conceptualization, Methodology, Formal analysis, Investigation, Writing—Review & Editing, Visualization, Supervision, Project administration, Funding acquisition: Lindsay Glass Campbell. Investigation, Writing—Original draft: Kelsey A. Anderson. Conceptualization, Methodology, Writing—Review & Editing, Supervision: Ruth Marcec-Greaves.

DATA AVAILABILITY STATEMENT
Data can be requested from the corresponding author.

ETHICS STATEMENT
All the authors have contributed to the manuscript and agree with the findings and conclusions. This manuscript is an original work and has not been previously published.

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REFERENCES
AmphibiaWeb. (2021). AmphibiaWeb: Information on amphibian biology and conservation [Web application]. Retrieved from https://amphibiaweb.org/
Bechler, D. L. (1988). Courtship behavior and Spermatophore deposition by the subterranean salamander, *Typhlomolge rathbuni* (Caudata, Plethodontidae). *The Southwestern Naturalist*, 33, 124–126.
Browne, R. K., & Zippel, K. (2007). Reproduction and larval rearing of amphibians. *ILAR Journal*, 48, 214–234.
Culver, D. C., & Pipan, T. (2019). *The biology of caves and other subterranean habitats* (2nd Ed.). Oxford University Press.
Gascon, C., Collins, J. P., Moore, R. D., Church, D. R., McKay, J. E., & Mendelson, J. R., III (Eds.). (2007). *Amphibian conservation action plan: Proceedings IUCN/SSC amphibian conservation summit 2005* (p. 65). Gland, Switzerland: The World Conservation Union (IUCN).
Gillette, J. R., & Peterson, M. G. (2001). The benefits of transparency: Candling as a simple method for determining sex in red-backed salamanders (Plethodon cinereus). *Herpetological Review*, 32, 233–235.
Gorbman, A., & Sower, S. (2003). Evolution of the role of GnRH in animal (metazoan) biology. *General and Comparative Endocrinology*, 134, 207–213.
Guy, E. L., Gillis, A. B., Kouba, A. J., Barber, D., Poole, V., Marcec-Greaves, R. M., & Kouba, C. K. (2020). Sperm collection and cryopreservation for threatened newt species. *Cryobiology*, 94, 80–88.
Holt, W. V., Abaigar, T., Watson, P. F., & Wildt, D. E. (2010). Genetic resource banks for species conservation. In W. V. Holt, A. R. Pickard, J. C. Rodger, & D. E. Wildt (Eds.), *Reproductive Science and Integrated Conservation* (pp. 267–280). Cambridge, UK: Cambridge University Press.
IUCN (2021). The IUCN red list of threatened species. Version 2021-2. Retrieved from https://www.iucnredlist.org.
Julien, A. R., Kouba, A. J., Kabelik, D., Feugang, J. M., Willard, S. T., & Kouba, C. K. (2019). Nasal administration of gonadotropin releasing hormone (GnRH) elicits sperm production in Fowler’s toads (Anaxyrus fowleri). *BMC Zoology*, 1(1). https://doi.org/10.1186/s40850-019-0040-2
Kouba, A. J., Vance, C. K., & Willis, E. L. (2009). Artificial fertilization for amphibian conservation: Current knowledge and future considerations. *Theriogenology*, 71(1), 214–227. https://doi.org/10.1016/j.theriogenology.2008.09.055
Longley, G. (1978). Status of *typhlomolge* (= *Eurycea* rathbuni), the Texas Blind Salamander. *Endangered Species Report* 2 (Vol. 2, pp.1-45). Albuquerque, NM: U.S. Fish and Wildlife Service.
Marcec, R. (2016). Development of assisted reproductive technologies for endangered North American amphibians.
Moon, L. M., Butler, M. J., & Glass, C. L. (2021). Evaluation of tagging methods for unique identification of individuals in three aquatic Eurycean salamander species. *Ichthyology & Herpetology* Forthcoming.

Moore, F. L., Boyd, S. K., & Kelley, D. B. (2005). Historical perspective: Hormonal regulation of behaviors in amphibians. *Hormones and Behavior*, 48, 373–383.

Muñoz-Cueto, J. A., Zmora, N., Paullada-Salmerón, J. A., Marvel, M., Mañanos, E., & Zohar, Y. (2020). The gonadotropin-releasing hormones: Lessons from fish. Academic Press.

Roch, G. J., Busby, E. R., & Sherwood, N. M. (2011). Evolution of GnRH: Diving deeper. *General and Comparative Endocrinology, 171*(1), 1–16. https://doi.org/10.1016/j.ygcen.2010.12.014

Sever, D. M. (1985). Sexually dimorphic glands of Eurycea nana, Eurycea neotenes and *Typhlomolg e rathbuni* (Amphibia: Plethodontidae). *Herpetologica, 41*(1), 71–84. http://www.jstor.org/stable/3892131

Sever, D. M., & Staub, N. L. (2011). Hormones, sex accessory structures, and secondary sexual characteristics in amphibians. In *Hormones and reproduction of vertebrates* (pp. 83–98). Elsevier. Retrieved from. https://linkinghub.elsevier.com/retrieve/pii/B9780123749314100057

Silla, A. J., McFadden, M., & Byrne, P. G. (2018). Hormone-induced spawning of the critically endangered northern corroboree frog Pseudophryne pengilleyi. *Reproduction, Fertility and Development, 30*, 1352–1358.

Silla, A. J., Roberts, J. D., & Byrne, P. G. (2020). The effect of injection and topical application of hCG and GnRH agonist to induce sperm-release in the roseate frog, *Geocrinia rosea*. *Conservation Physiology, 8*(1), 1–10. https://doi.org/10.1093/conphys/coaa104

Sodhi, N. S., Bickford, D., Diesmos, A. C., Lee, T. M., Koh, L. P., Brook, B. W., Sekercioglu, C. H., & Bradshaw, C. J. (2008). Measuring the meltdown: Drivers of global amphibian extinction and decline. *PLoS One, 3*(2), e1636.

Vieira, W. A., Anderson, K., Glass Campbell, L., & McCusker, C. D. (2020). Characterizing the regenerative capacity and growth patterns of the Texas blind salamander (*Eurycea rathbuni*). *Developmental Dynamics, 250*(6), 880–895. https://doi.org/10.1002/dvdy.245

Vu, M., & Trudeau, V. L. (2016). Neuroendocrine control of spawning in amphibians and its practical applications. *General and Comparative Endocrinology, 234*, 28–39. https://doi.org/10.1016/j.ygcen.2016.03.024

Whitford, W. G., & Hutchison, V. H. (1965). Gas exchange in salamanders. *Physiological Zoology, 38*(3), 228–242.

Zerrenner, A., & Sommer, T. (2012). Final environmental impact statement: Edwards aquifer recovery implementation program habitat conservation plan.

**How to cite this article**: Glass Campbell, L., Anderson, K. A., & Marcec-Greaves, R. (2022). Topical application of hormone gonadotropin-releasing hormone (GnRH-A) stimulates reproduction in the endangered Texas blind salamander (*Eurycea rathbuni*). *Conservation Science and Practice, 4*(3), e609. [https://doi.org/10.1111/csp2.609](https://doi.org/10.1111/csp2.609)