City of Austin, Watershed Protection Dept.
2024 Annual Report
U.S. Fish and Wildlife Service Scientific Permit (TE-833851)

Reporting period: January 1, 2024, through December 31, 2024.

This report documents activities involving Barton Springs, Austin Blind, and Jollyville Plateau salamanders (*Eurycea sosorum*, *E. waterlooensis*, and *E. tonkawae*, respectively) and karst invertebrates.

Authors: Nathan F. Bendik, Dee Ann Chamberlain, Sarah Donelson, Matthew Westbrook.

General Annual Reporting Requirements for Barton Springs and Austin Blind salamanders

1) Precise locations of previously undocumented surveyed areas
None.

2) Dates of surveys conducted
Please see # 4, below.

3) Survey methods
Barton Springs and Austin Blind salamander surveys were performed quarterly throughout the year at Parthenia, Eliza, Old Mill (Sunken Gardens) and Upper Barton springs. For each survey, the date, weather, type of flow (base flow or storm flow), and aquifer discharge were recorded by the U.S. Geological Survey station at Parthenia Spring. Additionally, discharge and water quality measurements were measured at each of the other springs. Photographs of substrate were taken to measure relative sediment cover and embeddedness. We approximated fish abundance (by species) using a very cursory count within or near the salamander habitat. Each site was searched using a drive survey method where all non-embedded substrate is searched, except for at Old Mill Spring, where a timed survey is used due to the low abundance of salamanders commonly encountered at that site. Every individual salamander found was identified to species and categorized by an estimate of total length (<25 mm, 25–50 mm, >50 mm) or measured from photographs. Photographic capture-recapture surveys were performed at all sites except Parthenia Spring. Salamanders were captured using small handheld dip nets or basters, photographed, and released as soon as possible, within 3 hours. The total number of salamanders of each species and size class found were recorded, although we only present the totals below.

4) Survey results
Salamander counts from the reporting period are presented in Table 1 below. Tallies include the number of individuals captured plus the estimated number of those missed. Surveys in Parthenia were performed by visual count only, without capturing salamanders. Environmental data are presented in Table 2. Fish observation data are presented in Figure 2.

Table 1. Barton Springs and Austin Blind salamander counts from 2024 surveys. Tallies include individuals captured and photographed (sites at Eliza, Old Mill, Upper Barton) plus individuals observed but not captured (all sites).

Site	**Date, Time**	***E. sosorum***	***E. waterlooensis***
Barton Spring	2/23/2024 12:40	24	0
Barton Spring	5/23/2024 12:25	64	0
Barton Spring	8/29/2024 10:45	25	0
Barton Spring	11/21/2024 11:05	11	0
Eliza Spring	2/6/2024 9:30	609	1
Eliza Spring	2/8/2024 8:47	534	0
Eliza Spring	2/12/2024 9:45	473	2
Eliza Spring	5/7/2024 9:30	874	1
Eliza Spring	5/9/2024 9:30	582	0
Eliza Spring	5/14/2024 9:30	509	0
Eliza Spring	8/20/2024 17:20	772	0
Eliza Spring	8/22/2024 8:55	550	0
Eliza Spring	8/26/2024 9:15	516	1
Eliza Spring	11/1/2024 9:30	423	0
Eliza Spring	11/5/2024 9:05	343	1
Eliza Spring	11/6/2024 10:45	270	0
Eliza Stream (outflow from Eliza Spring)	2/5/2024 9:15	202	0
Eliza Stream (outflow from Eliza Spring)	2/7/2024 10:15	160	0
Eliza Stream (outflow from Eliza Spring)	2/9/2024 10:25	150	0
Eliza Stream (outflow from Eliza Spring)	5/6/2024 10:30	346	0
Eliza Stream (outflow from Eliza Spring)	5/8/2024 9:30	228	0
Eliza Stream (outflow from Eliza Spring)	5/10/2024 9:55	216	0
Eliza Stream (outflow from Eliza Spring)	8/19/2024 9:30	379	0
Eliza Stream (outflow from Eliza Spring)	8/21/2024 9:30	256	1
Eliza Stream (outflow from Eliza Spring)	8/23/2024 9:30	223	0
Eliza Stream (outflow from Eliza Spring)	10/31/2024 16:10	340	0
Eliza Stream (outflow from Eliza Spring)	11/4/2024 9:30	288	0
Eliza Stream (outflow from Eliza Spring)	11/6/2024 8:00	169	0
Old Mill (Sunken Gardens) Spring	2/23/2024 13:21	1	1
Old Mill (Sunken Gardens) Spring	5/22/2024 11:40	1	0
Old Mill (Sunken Gardens) Spring	9/4/2024 10:30	1	0
Old Mill Stream (outflow from Sunken Gardens Pool)	2/23/2024 13:25	0	0
Old Mill Stream (outflow from Sunken Gardens Pool)	5/22/2024 11:30	0	0
Old Mill Stream (outflow from Sunken Gardens Pool)	9/4/2024 11:00	0	0
Upper Barton Spring	2/23/2024 9:30	4	0

Table 2. Environmental data for Barton Springs and Austin Blind salamander surveys from 2024. Data for Barton Springs Pool (Parthenia Spring) are recorded by the USGS.

Site	Date	Flow (ft^3/s)	Conductivity (μS/cm)	Dissolved oxygen (mg/l)	pH	Water Temp (°C)
Eliza Spring	2/5/2024	11.21	700	6.88	7.11	19.76
Eliza Spring	5/10/2024	5.07	703	5.41	7.24	21.56
Eliza Spring	8/20/2024	NA	705	4.79	7.28	22.04
Eliza Spring	8/21/2024	2.26	NA	NA	NA	NA
Eliza Spring	10/31/2024	2.68	NA	NA	NA	NA
Eliza Spring	11/1/2024	NA	767	4.17	7.4	21.86
Old Mill Spring	2/23/2024	6.84	830	6.3	7.08	20.36
Old Mill Spring	5/22/2024	2.55	796	4.77	7.19	21.75
Old Mill Spring	9/4/2024	0.70	893	3.72	7.29	21.89
Old Mill Spring	11/15/2024	0.05	1013	3.15	7.14	20.29
Upper Barton Spring	2/23/2024	1.22	615	6.46	7.13	21.6

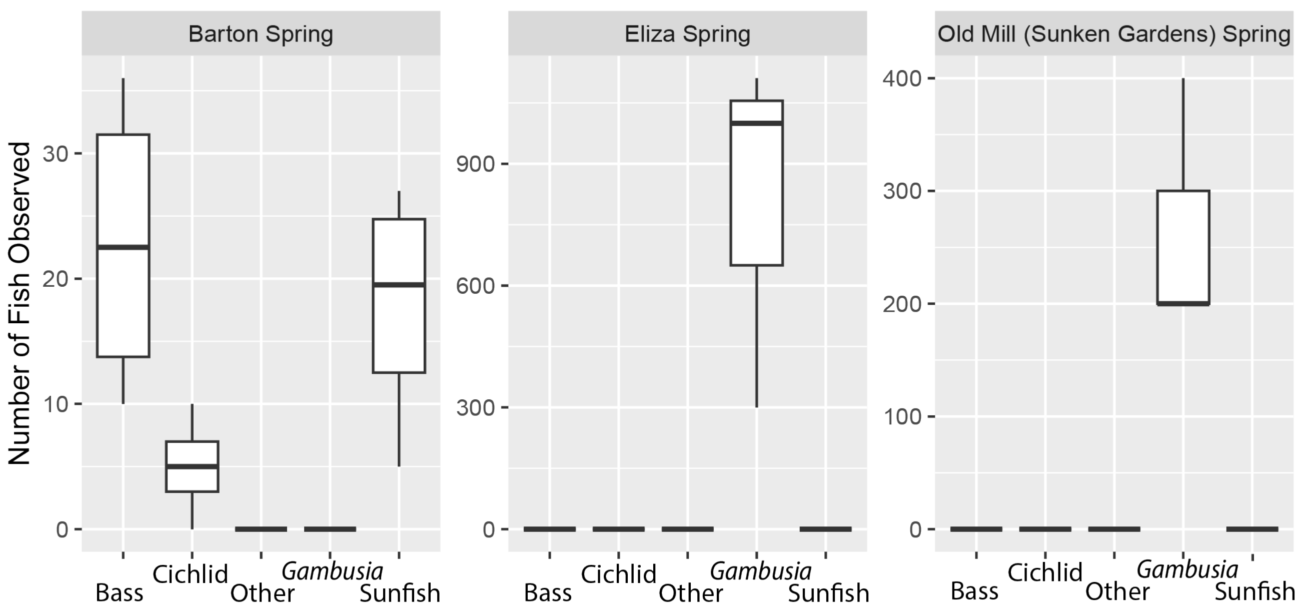

Figure 1: Box plots of fish counted during quarterly surveys in 2024. No fish were observed during the survey at Upper Barton Spring.

5) Species ID by Taxonomist
Not applicable.

6) Number of salamanders collected from the wild
Salamanders collected from the wild (salvaged from surveys, drift nets, or collected alive for captive propagation) are presented in Table 3, below.

7) Results of species identifications
See Table 1, above.

8) Number of salamanders handled and marked with elastomers.
None.

9) Observations of abnormal behavior or condition of salamanders handled/marked.
   None to report.

Table 3. Salamanders collected from the wild. Salvaged individuals were killed or injured during surveys or otherwise found dead. Individuals that were collected alive were done so in accordance with the City's captive population management plan.

Species name	Date	Site	Number Collected	Disposition	Method of Collection	Notes
E. waterlooensis	2/6/2024	Eliza Spring	1	Alive at captive breeding (ASCC)	capture-mark-recapture	<1" total length (TL)
E. sosorum	2/8/2024	Eliza Spring	2	Preserved in 95%+ EtOH	capture-mark-recapture	Both <1" TL. One didn't look injured but was bent backwards slightly and not moving. Was photographed and then preserved. The other one was not moving and didn't look in good condition and placed in the vial before being photographed.
E. sosorum	2/12/2024	Eliza Spring	1	Preserved in 70% EtOH	capture-mark-recapture	<1" TL, Found on the party barge after it had been removed from the water for at least 10 minutes.
E. waterlooensis	2/12/2024	Eliza Spring	1	Alive at ASCC	capture-mark-recapture	<1" TL
E. waterlooensis	2/23/2024	Old Mill Spring	1	Alive at ASCC	capture-mark-recapture	<1" TL, ventral side up, possible injury, recovered in captivity
E. waterlooensis	5/6/2024	Eliza Spring	1	Alive at ASCC	capture-mark-recapture	1-2" TL at collection
E. sosorum	5/9/2024	Eliza Spring	1	Preserved in 95%+ EtOH	capture-mark-recapture	~1" TL, Salamander found already dead while doing one of the last runs in the upstream end of the amphitheater.
E. waterlooensis	5/7/2024	Eliza Spring	1	Alive at ASCC	capture-mark-recapture	<1" TL
E. waterlooensis	8/21/2024	Eliza Spring	1	Alive at ASCC	capture-mark-recapture	<1" TL, collected from keyway
E. waterlooensis	8/26/2024	Eliza Spring	1	Alive at ASCC	capture-mark-recapture	< 1" TL
Eurycea sp.	11/5/2024	Eliza Spring	1	Alive at ASCC	capture-mark-recapture	1-2" TL, either *E. waterlooensis* or hybrid. Should get species ID from P. Diaz USFWS genetics project in 2025.

10) Results of any mark-recapture work

We performed capture-recapture surveys at three sites in 2024 using photographic identification methods[1]. No recaptures were found at Upper Barton or Old Mill springs, so we are unable to calculate estimates of abundance at those sites. Table 1 includes the total observed salamanders at these sites.

We conducted robust-design mark-recapture sampling at Eliza Spring in February, May, August, and November. Abundance estimates were generated from a hierarchical closed-population model using parameter-expanded data augmentation, modified from Kéry and Schaub[2]. The model included site, size-class (juvenile and adult) and temporal effects for each sampling period; parameters were estimated using Bayesian analysis in JAGS. Abundance estimates are provided below in Table 4.

Table 4. Estimates of abundance ($\widehat{N}$) and the 95% credible interval (CI) for capture-recapture surveys at Eliza Spring (spring pool and stream combined) from January 2024 through December 2024.

Period	Site	$\widehat{N}$ (95% CI)
Feb 2024	Eliza pool and stream	1449 (1390, 1515)
May 2024	Eliza pool and stream	1951 (1871, 2044)
Aug 2024	Eliza pool and stream	1681 (1625, 1744)
Nov 2024	Eliza pool and stream	975 (953, 1003)

11) Results of genetic research conducted as a result of tail-clipping

We are awaiting a final report for the project "Transcriptomic evaluation of chronic stress in Austin's endangered salamanders" from the University of Tulsa, PI Ronald Bonett.

12) Results of any research or management activities authorized by this permit and approved through the submission of study plans to the CPI Branch of the Austin ESFO

a. City of Austin monitors water quality at the Barton Springs complex under this permit to meet the requirements of the Habitat Conservation Plan contained in the USFWS 10(a)(1)(B) permit PRT-839031 and the Texas Pollutant Discharge Elimination System permit WQ0004705000 (EPA NPDES TXS000401). Tested parameters include total suspended solids, volatile suspended solids, nitrate plus nitrite, ammonia, ortho-phosphorus, *E.coli*, temperature, dissolved oxygen, pH, and specific conductivity. Quarterly sampling includes monthly parameters plus alkalinity, chloride, sulfate, fluoride, total organic carbon, and the following metals: As, B, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Na, Ni, Pb, Sr, Zn. TPDES annual sampling includes the above plus total petroleum hydrocarbons, organophosphorus and organochlorine pesticides, herbicides, polychlorinated biphenyls, volatiles, and semi-volatiles. Monthly sampling is performed at Barton Springs Pool only, while all springs are sampled quarterly (Parthenia, Eliza, Upper Barton, Old Mill, Backdoor and Cold springs). Parthenia, Eliza, and Old Mill are sampled for the more comprehensive annual sample. Additionally, the City of Austin in cooperation with the United States Geological Survey (USGS) maintains continuous monitoring for spring discharge and physiochemical parameters at Barton Springs. Data are available here.

[1] Bendik, N. F., T. A. Morrison, A. G. Gluesenkamp, M. S. Sanders, and L. J. O'Donnell. 2013. Computer-assisted photo identification outperforms visible implant elastomers in an endangered salamander, *Eurycea tonkawae*. PLoS ONE 8:e59424.

[2] Kéry, M., and M. Schaub. 2012. Bayesian Population Analysis using WinBUGS. Academic Press.

b. U.S. Geological Survey deploys and maintains water quality sampling equipment in Parthenia Spring. Equipment was periodically serviced by USGS dive teams. Data are available here.

Captive Breeding Annual Reporting Requirements

1) The number of *Eurycea sosorum, E. waterlooensis,* and *E. tonkawae* held at the captive breeding facility (including the number of wild-caught and captive-bred individuals from each spring-site collected; see Table 5).

Table 5. Inventory of salamanders in the captive breeding program 01/01/2025. WC = wild-caught, CB = captive-bred.

Species	Spring of Origin	WC	CB
Eurycea sosorum	Parthenia Spring	21	78
	Old Mill Spring	1	38
	Eliza Spring	33	88[1]
	Upper Barton Spring	0	1
	Zara Well	1	0
	Total	56	205
E. waterlooensis	Old Mill Spring	4	NA[2]
	Eliza Spring	24	NA[2]
	Total	28	82
E. tonkawae	Bull Creek	0	1
	McDonald Well	0	1
	Total	0	2

[1] Includes 21 F3 progeny of F2's obtained from SMARC (San Marcos Aquatic Resources Center, U.S. Fish and Wildlife Service facility) for an experiment. These will be used for education.
[2] *E. waterlooensis* have not been separated and bred according to spring site of origin because the species is primarily aquifer-dwelling.

2) Number of observations of courtship behavior, spermatophores, spermatophore depositions, sperm transfers, and ovipositions.

Courtship behavior was observed in both wild-caught and captive-bred salamanders at the captive breeding facility. Spermatophores are small and not easily observed. In 2025, we will describe courtship behavior and spermatophore deposition in *Eurycea sosorum* and *E. waterlooensis* in a paper to be submitted to a scientific journal. Because salamanders can store sperm, observed courtship behavior does not necessarily result in immediate egg-laying. Each oviposition with viable offspring represents at least one spermatophore transfer, and possibly multiple transfers. Oviposition data are presented in Table 6.

Table 6. Ovipositions in captivity 1/01/2024–12/31/2024. Individuals in reproductive groups are recorded to follow actual or potential dams and sires. "P" denotes groups from Parthenia, "E" from Eliza, "WC" wild-caught salamander, and "F1/F2" first- or second-generation captive-bred salamanders.

Oviposition Date	Reproductive Group (parent spring site, generation, group ID)	Clutch Size	No. Hatched	Comments
		Eurycea sosorum		
1/2/2024	P WC Grp 1	11	7 Culled[1]	
1/19/2024	P WC Grp 2	12	11	
3/5/2024	P WC Grp 3	25	see comment	3/5 and 3/12 clutches combined, culled 35; 18 hatched
3/5/2024	E WC Grp 1	21	18	
3/12/2024	P WC Grp 3	36	see comment	3/5 and 3/12 clutches combined, culled 35; 18 hatched
~3/14/2024	P WC females only	23	Culled[1]	Oviposited 32 days after being separated from males; viable but females had already reproduced.
4/25/2024	P WC Grp 3	20	Culled[1]	
4/30/2024	P WC Grp 3	26	Culled[1]	
		Eurycea waterlooensis		
2/1/2024	F1 Grp 1	38	12	
2/23/2024	F1 Grp 2	19	7	
3/1/2024	F1 Grp 3	25	15	
3/15/2024	F1 Grp 3	10	0	
5/8/2024	F1 Grp 2	14	0	
8/6/2024	WC X F2 Grp 1	16	5	
9/28/2024	WC X F2 Grp 1	36	1	
11/4/2024	WC X F2 Grp 1	24	11	
12/4/2024	F1 females only	22	NA	Not viable[2]
12/23/2024	F1 females only	19	NA	Not viable[2]

[1]Preserved to manage the population size and genetic diversity (prevent a disproportionate number of offspring produced from any single reproductive group, or to minimize inbreeding).
[2] Females not housed with males in over six months

3) <u>Information on clutch sizes (range, mean, and standard deviation) and hatching success (range, mean, and standard deviation)</u> are shown in Table 7.

Table 7. Salamander clutch size and hatching success from 12/01/2022–12/31/2023.

Clutch Parameter	Min-Max Range	Mean	Standard Deviation
	Eurycea sosorum		
Clutch Size	11–36 (N=8)	21.7	7.80
Percent Hatched	64-92 (N=4)	77.6	13.28
	E. waterlooensis		
Clutch Size	10–38 (N=10)	22.3	8.98
Percent Hatched	0–60 (N=8)	26.0	22.73

4) Salamander mortalities including age and cause of death, if known, are shown in Table 8.

Table 8. Salamander mortalities from 01/01/2024-12/31/2024. WC = wild-caught, CB = captive-bred.

Wild-Caught or Captive-Bred	Age[1] (years)	No. Mortalities	Notes, Cause of Death (health condition observed), if known
		Eurycea sosorum	
WC, CB	3-5	1, 2	
CB	9-11[2]	2	
CB	11-13[2]	8	1 with edema euthanized
CB	13-15[2]	10	2 with scoliosis euthanized
WC, CB	15–17[2]	2, 6	1 WC with growth in mouth, 1 CB with edema, both euthanized
WC, CB	17-19[2]	1, 1	
		Eurycea waterlooensis	
CB	7-8	1	

[1]Age of wild-caught salamanders is estimated based on size at collection, with a maximum estimated age of 1.5 years for salamanders > 2 inches total length at collection.
[2] > 50% survivorship of age 7.5 years
Chamberlain DA. 2019. Barton Springs Salamander (*Eurycea sosorum*) and Austin Blind Salamander (*Eurycea waterlooensis*) captive breeding population management plan. City of Austin Watershed Protection Department. SR-20-03.

5) Information on Obvious Health Conditions or Behavioral Aberrations

We observed an 18-year-old wild-caught male with the still-attached hind right foot of a 3-year-old wild-caught male in his mouth. The body and total lengths of the older salamander were 40 mm and 70 mm, only 2 mm and 3 mm, respectively, longer than the younger salamander. Since the salamanders are suction feeders, it’s not clear if this was an act of aggression or an accidental intake of the foot. The younger male struggled for over 10 minutes to get away while his foot was still in the mouth of the older salamander. Eventually, a complete break occurred above the foot (Fig. 2) as the younger salamander moved away. By approximately 51 days later, the salamander had regenerated a small foot (Fig. 3).

Figure 2. Right hind limb after foot bitten off.

Figure 3. Regenerated foot approximately 51 days later.

6) Research Activities

The report "The effect of variable pH and calcium concentration on *Eurycea sosorum* toe length" is attached.

General Annual Reporting Requirements for Jollyville Plateau Salamanders

One research project is ongoing for Jollyville Plateau salamanders (*Eurycea tonkawae*) (JPS), COA WPD project 646: Old Lampasas Dam JPS Monitoring. All raw tabular data are publicly available from this link. This tributary was mostly dry for most of the year.

We performed surveys of Old Lampasas Dam spring and tributary. We observed ten salamanders at two previously documented sites (Table 9).

Personnel

Sampling personnel included Nathan F. Bendik, Sarah Donelson, Radmon Rice, and Matthew Westbrook.

Locations

Table 9. Sum of all *Eurycea tonkawae* salamanders observed at each site during the reporting period. Observations of zero salamanders were excluded. For negative data and other ancillary information, please follow the raw data link above.

Site Name	Latitude	Longitude	Date	Total Observed
JPS Occupancy site 267	30.42146	-97.7988	3/26/2024	7
JPS Occupancy site 269	30.42103	-97.7986	3/21/2024	1
JPS Occupancy site 279	30.42859	-97.7997	3/21/2024	1
JPS Occupancy site 279	30.42859	-97.7997	4/2/2024	1

General Annual Reporting Requirements for Karst Invertebrates

During hydrogeological work and void inspections, City of Austin permitted staff entered several caves that may harbor protected karst invertebrates (Table 10).

Table 10. Caves entered by City of Austin staff. ES = endangered species. KZ = karst zone.

Date	Cave or Property	Purpose	Habitat Observations	Karst Zone	Personnel
1/9/2024	Apple Campus	Inspection of karst voids/caves encountered during construction	Karst void encountered while trenching for temporary power supply. Terracon is hiring a consultant to determine extent of cave. A tape used by the contractors extends 16 feet.	1	Michael Markowski, Joaquina Guevara
2/9/2024	Pennie's Cave	Geology field trip and inspection of protective buffer area	Cave with multiple rooms, observed cave crickets	3	Lindsey Sydow, Scott Hiers, Michael Markowski, Joaquina Guevara, other WPD staff
3/8/2024	Bill Russell Karst Preserve	Clean up old irrigation hoses and tree cages remaining on-site from recharge restoration project	Visited Blowing Sink Cave and several other caves on the property but did not enter them this day.	3	Lindsey Sydow, Scott Hiers, Michael Markowski, Joaquina Guevara, other WPD staff
4/22/2024	Hays Commons	Recharge Zone Boundary verification	Inspected pits to verify rock units on-site. No habitat observed, although there is a recharge feature elsewhere on the site.	3	Lindsey Sydow, Michael Markowski, Scott Hiers, other WPD staff
6/6/2024	Wildflower Cave and LaCrosse Cave	Nature Nights outreach	cave crickets	3	Michael Markowski, Tyson McKinney
6/14/2024	4711 Spicewood Springs Road	Inspection of karst voids/caves encountered during construction	Dry void approximately 7 feet below ground surface but with blowing air. Connected across 2 trenches, but when final excavation extent was reached was no longer blowing.	1	Lindsey Sydow other WPD staff
7/26/2024	Gragg East	Recharge Zone Boundary verification	No habitat observed	3	Lindsey Sydow, Scott Hiers, other WPD staff
12/27/2024	Rogers Tract	Geology field trip and investigation of closed depressions.	Cave discovered with an ~8' opening, leading to a small chamber; large (~30' in diameter) sinkhole also identified, but largely filled in and overgrown with vegetation.	4	Tyson McKinney, Scott Hiers, Joaquina Guevara, other WPD staff

The Effect of Variable pH and Calcium Concentration on *Eurycea sosorum* Toe Length

RR-24-05
September 2024

Dee Ann Chamberlain, Environmental Scientist Senior
Nathan Bendik, Environmental Scientist Senior
Sarah Donelson, Environmental Scientist
Matthew Westbrook, Environmental Scientist
City of Austin, Watershed Protection Department
Justin Crow, *U. S. Fish and Wildlife Service, San Marcos Aquatic Resources Center*

Abstract

Eurycea sosorum individuals in the City of Austin (COA) salamander assurance colony have experienced a reduction in toe, foot, and sometimes limb length. Because pH and calcium concentrations in captivity differed from conditions in the wild, we experimentally tested whether they caused appendage reduction. We tested two replicates of three combinations of "low" (~7.1) and "high" (~8.1) pH with "low" (~35 mg/L) and "high" (~100 mg/L) Ca^{2+} concentration. Two controls were fed with groundwater to replicate pH and Ca^{2+} conditions in the wild. We stocked each tank with 12 adult captive-reared *E. sosorum*. The salamanders were visually assessed quarterly and photographed at the start and every six months for 1.5 years. Measurements of total length, body length, and toe length of each foot were made at the start and the end of the experiment. Over 1.5 years, appendage reduction did not occur. Our results indicate that the tested water chemistry changes of low calcium, the combination of high pH and low calcium, or a static system mimicking the conditions in the wild do not result in appendage reduction in *E. sosorum*.

Keywords: salamander, *Eurycea sosorum*, toe length, pH, calcium, captivity, appendage reduction

Introduction

The City of Austin (COA) Watershed Protection Department (WPD) maintains an assurance colony of the endangered Barton Springs salamander (*Eurycea sosorum*) consisting of a genetically diverse population in captivity that can be used to produce offspring for repatriation if the species were to be extirpated from primary spring sites in the wild (Chamberlain 2019). Over the course of two decades, we have observed toe and foot reduction in *E. sosorum*, the cause of which is not known. This occurred to less than 10% of the population during the first 10 years of the captive program with reduction stopping along the toes in most cases. The condition later became extreme, affecting most of the population and causing truncation extending to the base of the foot and into the limb in some cases. Given that the colony in captivity serves as a safety net for the species, understanding health issues and how they may be related to water quality in captivity is paramount for the long-term care and maintenance of a healthy population.

Toe reduction, which has not been observed in this species in the wild, occurred to various degrees in wild-caught and captive-raised salamanders over a range of ages and residence times in captivity, including salamanders that had been healthy for years (Chamberlain 2016). The condition appeared as a reduction across all of the toes of a given foot over weeks to months, starting with normal feet and toe tips (Figures 1, 2) and ending in a range of states, resulting in short toes from missing distal phalangeal elements (Figure 3), no toes, no foot (Figure 4), or, in less common cases, partial limb loss, leaving a stump just below the shoulder. The skin of the tips of the toes or the distal stump of the foot or limb typically appeared intact with no obvious indications of infections. Pathological investigations did not uncover the cause (Chamberlain 2016). Reduction was often followed by regeneration and further processes of loss and regeneration over a period of years (Figure 5). In one case, the salamander regressed from four feet with "short" toes to missing all toes on 3 feet in 35 days (Chamberlain 2016); in other cases, individuals maintained short toes for a year or more.

Figure 1. *Eurycea sosorum* with normal feet.

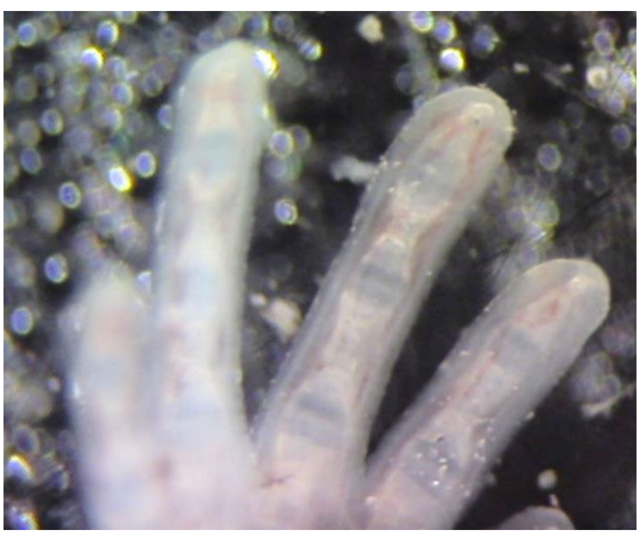

Figure 2. Ventral view – hind foot with normal toes which terminate with bone; phalangeal segments are visible in toes 2–4.

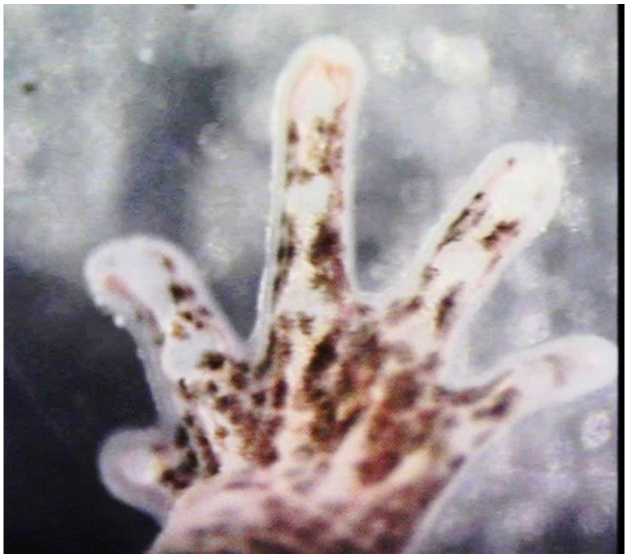

Figure 3. Dorsal view of hind foot as an example of “short” toes with missing distal phalanges. The toe tips appear to terminate with cartilage, as opposed to bone, except for a tiny section of bone at the tip of the middle toe.

Figure 4. Hind limb with no foot.

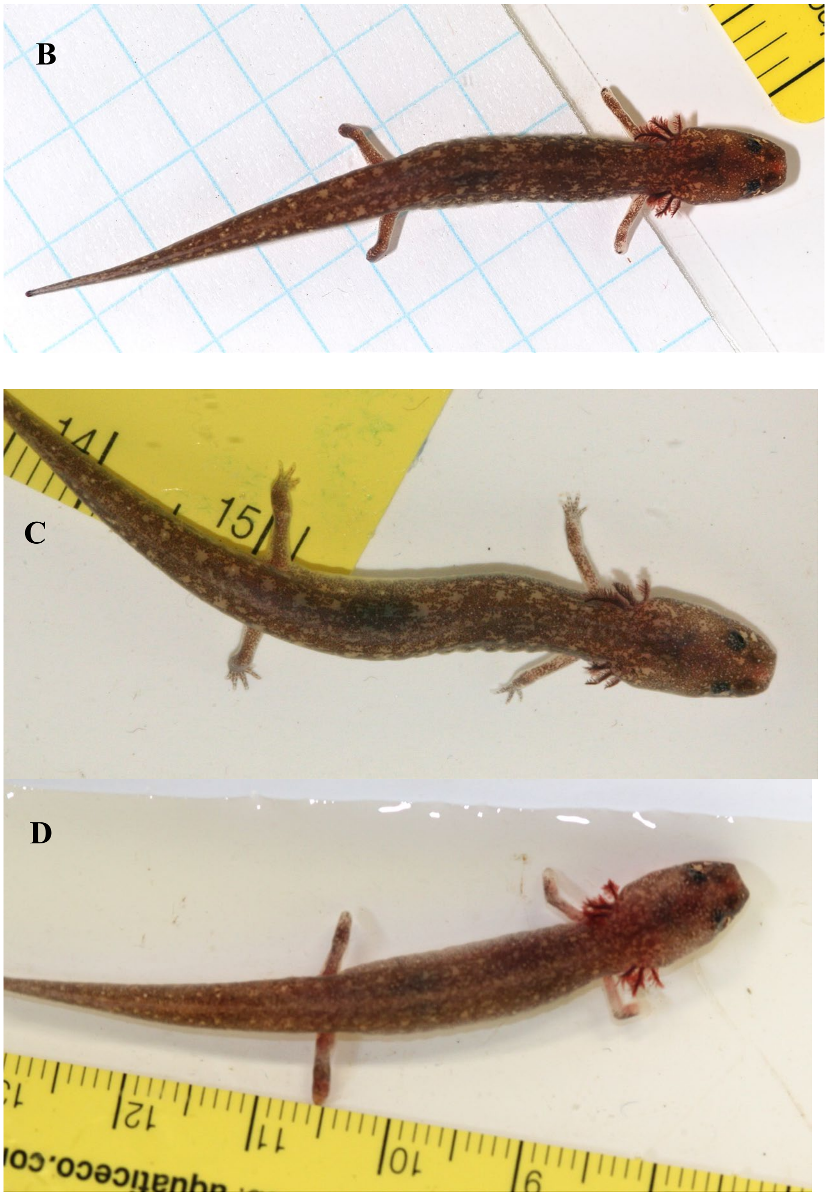

Figure 5. Photos of same individual show changes in feet over a period of 34 months. **A**: Normal feet. **B**: Missing four feet at six months after photo A. **C**: Regenerated toes at three months after photo B. **D**: Missing four feet at 24 months after photo C.

Concurrent with the period of the most extreme toe reduction, approximately 15% of the wild-caught population developed other health conditions including abnormal body curvature (scoliosis, kurtosis, lordosis), foot dragging, edema, and limb stiffness (Chamberlain 2016). Although a cause of abnormal curvature may be low calcium (K. Wright, pers. communication), pathological investigations on individuals did not determine the cause of the problems (Chamberlain 2016). Foot samples tested positive for *Batrachochytrium dendrobatidis* (Bd), which might be an incidental finding or might be a factor in secondary infections that result in the loss (A. Pessier, pers. communication). Salamanders in the wild and in captivity have tested positive for Bd with no apparent symptoms or health problems (Gaertner et al. 2009, City of Austin unpubl. data). Still, bacteria with antimicrobial properties that inhibit Bd (e.g., *Chromobacterium*) have been found on the foot of a salamander in captivity (Chamberlain 2016) and could be present in the groundwater going into the tanks. Currently, it's unclear if the flow regime could play a role and affect the balance of these microorganisms, affecting the health of the salamanders.

Observations of toe reduction in wild and captive central Texas salamanders are almost entirely limited to anecdotal accounts. Sweet (1978) observed *Eurycea pterophila* in the wild with phalangeal (bone segments in the foot) reduction and suggested that the loss was due to the bacteria *Aeromonas* sp.; however, clinical signs of an *Aeromonas* infection include lethargy, emaciation, ulcers, and cutaneous pinpoint hemorrhages (Kahn and Line 2005), none of which were observed in the *E. sosorum* with toe reduction. Furthermore, *Aeromonas* is ubiquitous, opportunistic, and typically not the underlying cause of a health problem (A. Pessier, pers. communication). Dallas Aquarium staff reported toe reduction and scoliosis in a group of *E. rathbuni* and assumed it was due to the high levels of phosphate measured in the closed system tanks housing the salamanders (B. Huntington, pers. communication); after the problems were noted, staff switched the tank systems to flow-through systems using well water with no further problems. There have also been observations of short toes made at other facilities that house central Texas *Eurycea*, including Aquarena (M. West pers. communication) and San Marcos Aquatic Resources Center (SMARC) (pers. observation). For example, SMARC staff observed toe and foot loss in some *E. nana* and assumed it was due to conspecific aggression. They switched to flow-through systems and similar problems in those tanks were not reported later (T. Brandt, pers. communication). Similarly, we observed a single male biting the hind foot off another male, but it is not clear if this was aggressive behavior or a feeding mishap, catching the foot in his mouth and not releasing. We do not believe this behavior accounts for the many observations of reduction, however. Since switching to a flow-through system, we have housed over 100 males in same-sex groups with no new cases of reduction. In addition, salamanders housed alone have also exhibited reductions.

Given the lack of an explanation from pathological findings or literature, we postulated that toe reduction may result from water chemistry differences between captive versus natural conditions. When water emerges from the aquifer, dissolved CO_2 equilibrates with the atmosphere, resulting in an increase in pH and a decrease in calcium due to the reduced solubility of calcium at higher pH. Without a constant influx of groundwater in captivity, we see an increase in pH from approximately 7 to 8.1, and a decrease in the calcium ion concentration by about two thirds from ~95 mg/L to ~35 mg/L.

Calcium is integral in a multitude of biological processes. Amphibians living in water with low ambient calcium might lose calcium through cutaneous diffusion (Hillman et al. 2009, Wright and Whitaker 2001) or might experience a low influx of calcium. For example, laboratory studies on *Rana pipiens* showed that, when ambient calcium is low, the influx of calcium into a hypocalcemic (calcium deficient) frog is not significantly different than that of a normal frog; however, when the ambient calcium is high, the influx into a hypocalcemic frog is twice as high as that of a normal frog (Stiffler 1996). A calcium deficiency can result in an imbalance with phosphorous, magnesium, Vitamin D3, and/or other vitamins and minerals (Wright and Whitaker 2001), causing metabolic bone disease (MBD). MBD can result in demineralization of the skeleton, including decalcification of digits (Bruce and Parkes 1950). However, pathological examinations in *E. sosorum* with toe loss problems did not exhibit evidence of this condition (Chamberlain 2016). Calcium is also important for electrical signaling of nerves (Kahn and Line 2005), and when nerves are experimentally severed in conjunction with amputation along the same limb, salamanders exhibit progressive regression of the remaining portion of the limb without regeneration (Schotte and Butler 1941, Olsen-Winner 1989). Our observations in captive *E. sosorum* resemble characteristics of limb resorption described in these studies. Salamanders take calcium from storage in bone and endolymphatic sacs (Hillman et al. 2009; Figure 6), as needed, for physiological processes including maintaining the concentration in the blood necessary for survival (Stiffler 1993). We therefore speculated that salamanders may mobilize calcium from their toes in a low calcium environment, possibly contributing to the toe loss phenomenon we have observed.

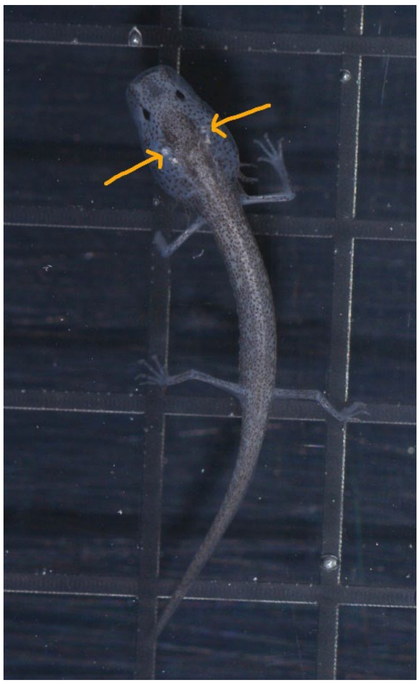

Figure 6. Endolymphatic sacs visible in juvenile *Eurycea waterlooensis*. This individual has fewer melanophores to obstruct the view compared to *E. sosorum*.

To understand whether the water chemistry differences in calcium and pH cause toe reduction, we tested the effects of combinations of two treatments of pH and calcium concentrations on *E. sosorum* toe length. These treatments represent the pH and the calcium concentration in the wild versus in water systems used in captivity. We used continuous flow from the groundwater well as a control and, since the microorganisms coming in from the aquifer could provide a beneficial microbiome on the feet, we also tested for a difference between flow-through and closed systems. In this study, we addressed the following questions: 1) Does the water chemistry that had been used during the extreme toe loss with pH ~8.1 and low calcium (< 50% of that in the wild) cause toe reduction? 2) Does low calcium (< 50% of that in the wild) with normal groundwater pH cause toe reduction? 3) Does a closed system with normal pH and calcium concentration result in toe reduction?

Methods

Study Overview

We placed groups of 12 *Eurycea sosorum* in eight tanks representing two replicates of three treatments and a control. The treatments consisted of combinations of two levels of pH and two levels of calcium based on the pH and calcium concentrations in the wild and in tanks in captivity in previous years. We measured the pH and calcium weekly/biweekly and photographed the salamanders at the start and every six months for 1.5 years. We measured total length, body length, and the length of toe #3, counting from the inner toe, on each foot from photographs using ImageJ software and analyzed for changes in toe length over time.

Tank Environment and Water Source

Each of the eight tanks was a 65-gallon rectangular glass tank (23” W X 47” L X 14” H) filled to 8.5” (final water volume of 40 gallons). The lid was comprised of a 2 in-wide polypropylene frame that was never removed to eliminate the possibility of salamander escapes over the rim as well as additional polypropylene pieces placed on top of the frame to reduce evaporation. Two submersible water pumps enclosed in mesh bags to exclude salamanders provided ~20 l/min circulation below the surface as well as mechanical and biological filtration. The substrate consisted of a layer of acrylic-coated pebbles and cover items fabricated out of acrylic and PVC plastic pipe. Every tank had an outlet that was fitted with a 2 in diameter pipe with the opening covered with mesh to exclude salamanders. The control tanks had a continuous influx of groundwater at a rate of about 1.5 l/min with the water exiting the 2 in pipe. The outlets of two airline tubes weighed down by acrylic habitat provided air and CO_2 as required per treatment for pH adjustments. For lighting, facility windows allowed light to enter for a natural day/night schedule.

The water used in the experiment was pumped from the Austin Nature and Science Center groundwater well which is tapped into the Cold Springs basin of the Barton Springs segment of the Edwards Aquifer (Hauwert 2009). Prior to use, the water passed through a 0.5-micron carbon block filter. The temperature of the treatment tanks was maintained by the building air temperature, which was set at 70°F (21°C), and the temperature of the flow-through control tanks was influenced by the air temperature as well as the temperature of the groundwater, which averages approximately 70°F (21°C).

Treatments and Control

To produce the treatments to be tested, we manipulated the groundwater to change the pH and calcium while maintaining other water chemistry constituents. The control and three water chemistry treatments (Table 1) consisted of combinations of relatively low and high pH and low and high calcium concentrations, approximating the levels found in the wild and in a closed system in captivity. A “closed” water system is one in which water is periodically removed and added only via water changes.

Table 1. Tank Water Treatments.

Treatment (pH, Ca^{2+} levels)	Target pH	Target Ca^{2+} Concentration (mg/L)	Method to Maintain Chemistry
Control[1]	7.0–7.2	90–110	~1.5 l/min influx of groundwater
Low pH-High Ca	7.0–7.2	90–110	CO_2 injection system with pH probe
Low pH-Low Ca	7.0–7.2	< 45	CO_2 injection system with pH probe
High pH-Low Ca	8.0–8.4	< 45	No manipulation

[1]Low pH-High Ca

For the Control, we mimicked the water chemistry in the wild ("Low pH-High Ca") by supplying a constant influx of groundwater at ~1.5 l/min into the tank. To test if the constant addition from the aquifer makes a significant difference, we also mimicked the water chemistry in the wild ("Low pH-High Ca") in a closed water system using a CO_2 injection system. To produce the water chemistry in a closed system in captivity in which the CO_2 has equilibrated with the atmosphere resulting in "High pH-low Ca," we used submersible pumps to circulate groundwater resulting in an increase in pH to ~8 and a decrease in calcium by more than ~50%. To tease apart pH and calcium and test low calcium as the sole change ("Low pH-Low Ca"), we added a CO_2 injection system to "High pH-Low Ca" water to lower and maintain the pH while the calcium remained low. Due to the lower solubility of calcium at higher pH, a High pH-High Ca treatment was not feasible.

We included a replicate of each treatment and control for a total of eight tanks. We adjusted all the treatment water in food grade plastic barrels prior to transfer to the salamander tanks. After each water change, we emptied the barrels, submersible pumps, and tubing and rinsed with vinegar to remove $CaCO_3$ precipitate.

We used American Marine Inc. Pinpoint pH Controller CO_2 injection systems to maintain "low" pH in the "Low pH-High Ca" and "Low pH-Low Ca" treatments, including backup water. This allowed for a set range of 0.2 pH units at the narrowest. For example, to mimic the pH of the groundwater with this system, the pH range could be set to 7.00–7.20. We set it to 7.05–7.25 to avoid an overcorrection causing acidity that might dissolve any calcium precipitate. The CO_2 injection system monitors pH continuously via a probe in the tank. When the pH approaches the upper set point, the solenoid on the CO_2 regulator opens to allow flow of CO_2 into the tank until the pH drops; if the pH were to drop below the lower set point of 7.05 the solenoid controlling the CO_2 would be closed and power would automatically be sent to an aerator and the resulting aeration in the tank would then cause the CO_2 to come out of solution faster to increase the pH. We calibrated CO_2 injection system pH probes per device instructions and exchanged them for clean, disinfected probes weekly to avoid fungal growth which can cause accuracy errors.

Study Salamanders

We obtained one hundred adult captive-raised (second generation) *Eurycea sosorum* from SMARC and transported them to COA's Austin Salamander Conservation Center (ASCC) where the experiment was conducted. For transport, we placed the salamanders in mesh bags submerged in coolers filled with groundwater at a density not greater than two salamanders per liter and five salamanders per bag. Pieces of mesh in the bags provided habitat. To maintain a water temperature within a few degrees Fahrenheit of the resident tanks at SMARC, we floated frozen plastic "ice" blocks enclosed in plastic containers in the coolers, as needed.

Upon arrival at ASCC, we acclimated the salamanders by changing ~25% of the water in each cooler with facility groundwater in 15-minute intervals. Then, we sorted the salamanders by size class and sex and placed them in groups of four in three-gallon tanks plumbed into a flow-through system with a continuous drip of groundwater to provide a pH of ~7.1 so they could acclimate to the environment at ASCC. The substrate in these tanks consisted of the glass tank bottom and pieces of mesh. The salamanders were housed in these tanks for months since Covid-19 restrictions delayed the start of the experiment.

We later exchanged approximately 40% for additional salamanders from SMARC because they exhibited signs of health issues such as scoliosis or mild/moderate toe loss soon after arrival. In addition, some individuals were large for this species. The average body length of males and of females kept for the experiment was 40.9 mm each, which is in the 99.9th percentile of body length of salamanders at Eliza Spring, including all age classes. The average total length of study females (82.7 mm) and males (84.1 mm) is about 10mm longer than salamanders measured at Eliza Spring (City of Austin unpubl. data).

We photographed ninety-six salamanders and placed them into the eight tanks with approximately equal numbers of females and males (Table 2). Prior to distributing them into the tanks, we placed them in acclimation buckets filled with treatment water and conducted incremental water changes over the course of at least an hour. We temporarily removed the salamanders from the tanks every six months for photos and every three months between photo sessions for a visual assessment to determine if major changes had occurred in the feet.

At the end of the experiment, we shipped the salamanders to Dr. Allan Pessier (Washington State University) for pathological work, including Bd qPCR analysis, to expand the veterinary knowledge on the species.

Table 2. Number of females and males in each treatment tank and control.

Treatment	Replicate 1		Replicate 2	
	n females	*n* males	*n* females	*n* males
Control	8	4	6	6
Low pH-Low Ca	7	5	6	6
Low pH-High Ca	7	5	6	6
High pH-Low Ca	7	5	6	6

Husbandry

To minimize the spread of pathogens, each tank had dedicated equipment, and all tanks, substrate, habitat, and supplies were disinfected prior to use. Salamanders were fed thawed commercially available frozen chironomidae (fly larvae) twice weekly with occasional supplementation with live amphipods, *Hyallela azteca*. We monitored the salamanders on a regular basis, removing any dead individuals. We siphoned uneaten food out of the tanks as needed and siphoned debris from underneath the pebbles on a quarterly basis when salamanders were removed for assessment and photographs. Every two weeks, we pumped out 15–20% of the water from each tank and refilled it using back-up water for each treatment and the influx of groundwater for the "Low pH-High Ca" flow-through controls.

Water Chemistry Monitoring

We measured water chemistry parameters weekly for 15 months then biweekly for four months and samples were analyzed by a commercial laboratory every six-months for 1.5 years (Table 3). Water testing was conducted at least 48 hours after a water change. To ensure that nutrients and temperature were within safe levels, we regularly tested ammonia, nitrite, and nitrate using commercial test strips and/or a LaMotte ammonia test kit 3304 and measured temperature using a Hydrolab DS4A.

Table 3. Water chemistry data collection.

Parameter	Sample Location	Method
Ca^{2+}, Mg, Total Hardness	Each tank and backup barrel	Hach Spectrophotometer DR3900
pH, specific conductivity	Each tank and backup barrel	Hydrolab DS4A
Temperature, dissolved oxygen	Each tank	Hydrolab DS4A
Ca^{2+}, Mg, Na, K, Cl, SO_4, CO_3, HCO_3, alkalinity (total, bicarbonate, carbonate)	Each tank	Commercial lab (LCRA[1], DHL[2])

[1]LCRA = Lower Colorado River Authority Environmental Laboratory Services, Austin, Texas.
[2]DHL = DHL Analytical, Austin, Texas.

Evaluating Change in Toe Length

We photographed each salamander at the start of the trial and every six months thereafter for 1.5 years for the purposes of individual identification by matching melanophore and iridophore patterns, documentation of body condition, and measurements as warranted per quarterly evaluations. We used Nikon D7100 and D850 cameras mounted on photo stands and placed each salamander into a clear acrylic photo cartridge with a 5 mm etched grid sitting on a black background. After all the salamanders from a given tank were photographed, we disinfected each cartridge.

We used the program ImageJ to obtain length measurements from photographs. Measurements included total length (snout to tail tip) (Figure 7), body length (snout to intersection of an imaginary line drawn between the posterior margin of the back limbs), and the length of toe #3 (Figure 8) on each foot, which we measured from the center of an imaginary line between the extension points of the toe at the base of the foot to the toe tip. We conducted measurements at the start and end of the experiment. Measurements in intervening periods were not warranted based on quarterly evaluations.

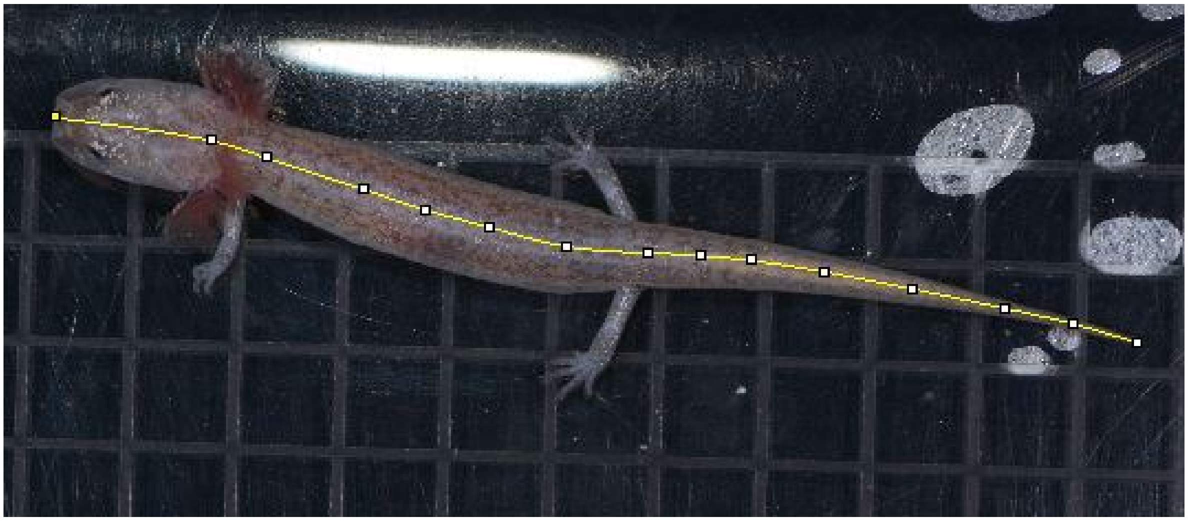

Figure 7. Example photograph and measurement line of total length of salamander.

To standardize toe size measurements, we divided each toe length by the concurrent body size measurement. We then took the difference for each standardized toe size between the start and end of the study and calculated the mean of these values. We only calculated changes in toe size for individuals that survived the entire 1.5-year study period.

We assessed whether our treatments had a statistically significant effect ($\alpha = 0.05$) on the average difference in standardized toe size by performing an analysis of variance in program R v 4.3.2 (R Core Team 2023). We also performed analyses of variance for each toe location individually. We used the package NADA v 1.6-1.1 (Helsel 2004) in R v 4.3.2 (R Core Team 2023) to test for differences between treatments using survival curves.

Figure 8. Example photograph of left hindlimb and measurement line of Toe #3.

Results

We achieved the target water chemistry (Ca^{2+} and pH) for each treatment (Appendix A) and did not find substantial differences between the calcium concentrations measured with the spectrophotometer and the concentrations measured by the commercial laboratory (Appendix B).

We did not find evidence for an overall treatment effect on average standardized toe length based on the analysis of variance (F = 1.831, P = 0.15). Looking at each toe location separately (Figure 9), only the front-right toe analysis was significant (P = 0.048). In this case, the largest difference was between treatments High pH-Low Ca and Low pH-High Ca (diff = 0.0059), but this comparison was not statistically significant using Tukey's HSD test for multiple comparisons (P = 0.055).

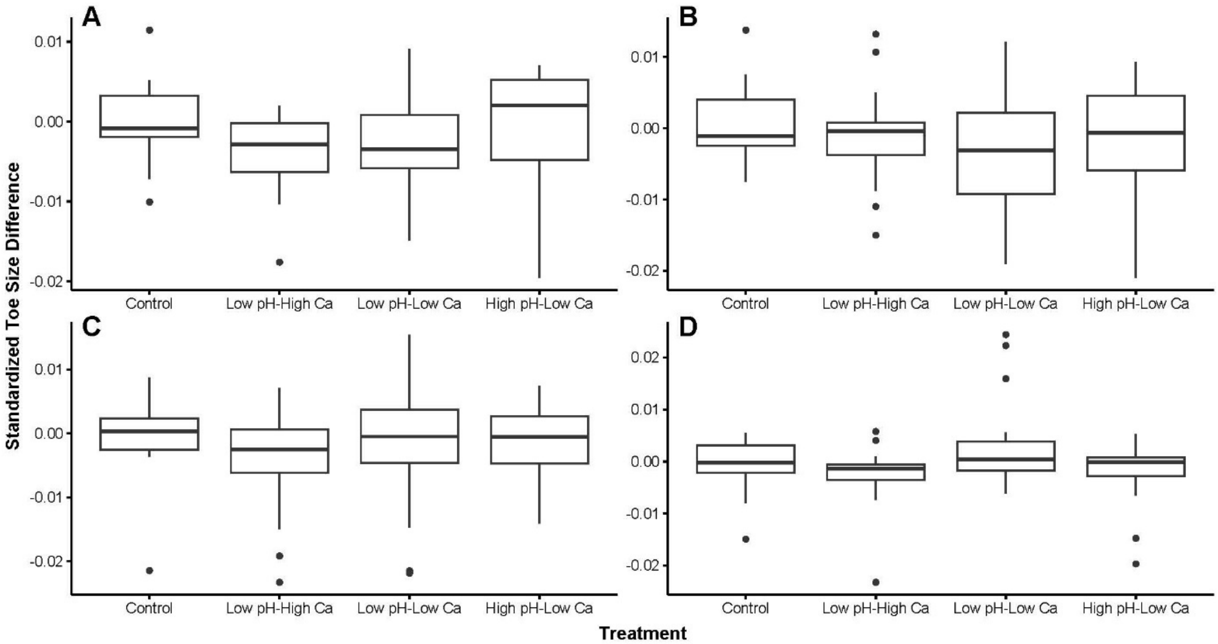

Figure 9. Boxplots of standardized toe size differences by toe location (A= back left foot, B = back right foot, C = front left foot, D = front right foot) and treatment.

The number of mortalities (Figure 10) was lowest in "High pH-Low Ca" and highest in the "Low pH-High Ca" but there were no significant differences in survival between the treatments (X^2 = 0.4 (df = 3), P = 0.9). Multiple health conditions were observed in every treatment group, including the controls (Appendix C), and no single problem dominated.

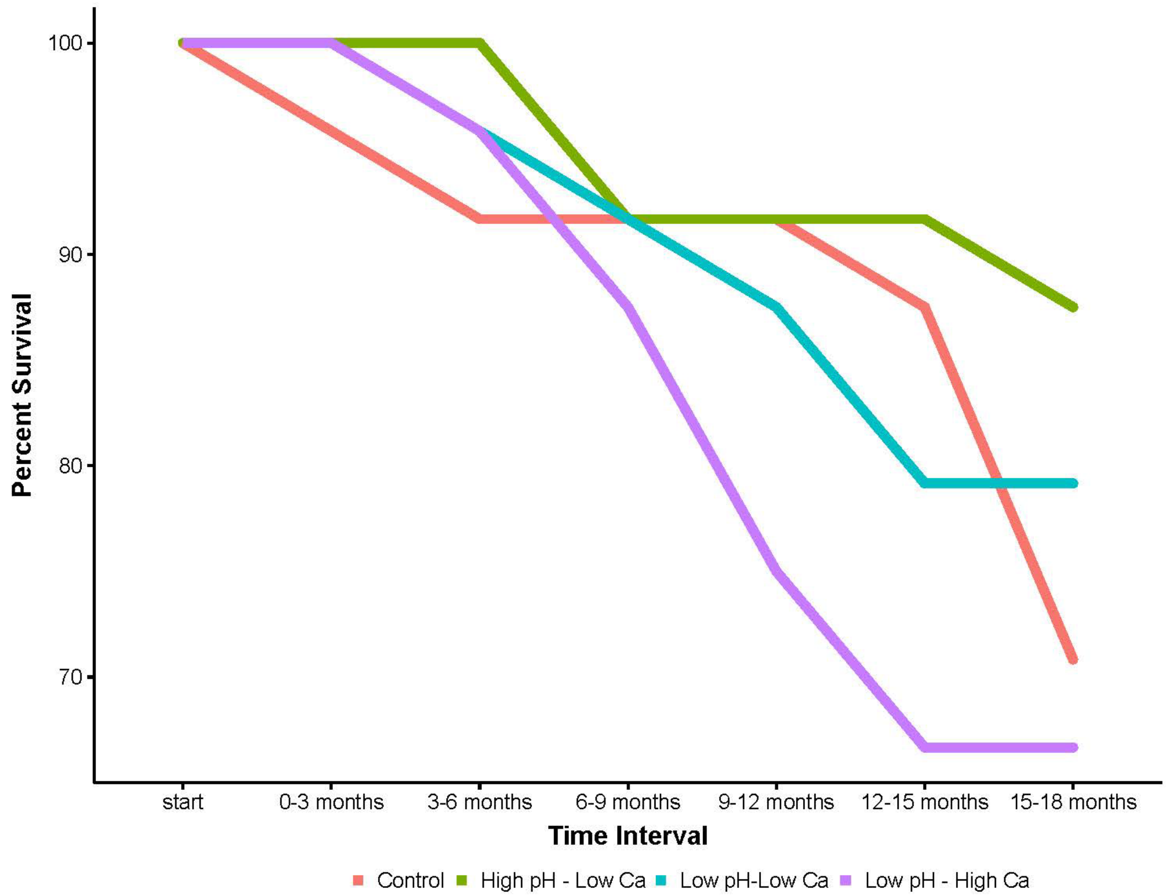

Figure 10. Survival per treatment over 1.5 years.

Although reproduction occurred in each of the treatments, only 6% of the total clutches were in the High pH-Low Ca treatment, while ~30% occurred in each of the other two treatments and controls. This is likely unimportant as samples sizes were small, and salamanders in the assurance colony have bred readily in high pH, low calcium conditions, similar to the experimental High pH-Low Ca treatment.

Discussion

Toe reduction did not occur during the study. This indicates that neither the "low calcium" treatment nor the "high pH, low calcium" treatment cause toe reduction over 1.5 years. In addition, a "closed system" with normal groundwater pH and calcium concentration ("low pH, high calcium" treatment) maintained with CO_2 augmentation was not more likely to result in toe reduction compared to a system with a constant influx of groundwater.

The results do not explain why the reduction in the assurance colony stabilized, with no new problems, after the water system for the aquaria was changed to a flow-through system with a constant influx of groundwater. One explanation may be an insufficient length of time for an effect on the feet of the study salamanders. In previous years, most salamanders in the assurance colony developed problems within 1.5 years, but it took some *Eurycea sosorum* as well as all the *E. tonkawae* housed at ASCC more than two years to develop the problem after being housed in low calcium, high pH water. Given the large average size at the start, perhaps, the study salamanders were heartier with more stored nutrients, requiring more time to deplete those nutrients. Still, the smaller individuals did not exhibit reduction by the end of the experiment, so size is unlikely a factor.

Another explanation may be a disease or an infection that affected the salamanders in the assurance colony in the past but not the study salamanders. Given the lack of information on salamander health, there could be diseases or pathogens of which we are unaware, so we did not test for them. In addition, there may have been effects of the microbial community on the feet of the salamanders in the assurance colony compared to the study salamanders. For example, *E. sosorum* has been found to harbor Bd as well as bacteria that inhibit Bd. Because we did not see an effect of toe reduction, we did not investigate differences in Bd load or other differences in the microbiome on the feet across treatments. Since Bd was documented in preliminary investigations, this could have been a factor in the past and could have been a reflection of the microbial community in the aquaria and/or the aquifer at the time of water collection. Bd, a fungus that can impair electrolyte transport in the skin, attacks keratinized skin which is found on the salamanders' toes. While there is currently no clear mechanism explaining how a Bd infection could cause toe reduction, there is not sufficient information with which to rule it out (A. Pessier pers. communication). Given that Bd has been found on the toes and the toe loss starts at the toe tips, Bd (or the presence/absence of Bd-inhibiting bacteria) could have been a contributing factor.

In the future, we will consider the results of this study in determining acceptable water chemistry for salamanders in captivity, depending on the purpose. For example, it may be acceptable to use "High pH-Low Ca" water, which is easier to maintain but differs from the water in the wild, in educational displays or on a short-term basis in an emergency. For long-term maintenance of the assurance colony, mimicking the groundwater is still the preferred method.

If toe reduction happens again, this study could be helpful in determining investigations to further eliminate possible causes. Characterization of the microbiome on the feet could prove informative as this information was not feasible when the problem occurred in the past. In addition, to quantify bone loss, affected specimens could be cleared and stained to identify the presence/absence of phalangeal segments. Such additional information could lead to a greater understanding of this problem and the animals' physiological and environmental needs that will help protect the species in the long term, both in the wild and in captivity.

References

Bruce HM, Parkes AS. 1950. Rickets and osteoporosis in *Xenopus laevis*. Journal of Endocrinology 7(1):64–81.

Chamberlain DA. 2016. Investigations of foot reduction and other concurrent health problems in *Eurycea sosorum* in captivity. City of Austin Watershed Protection Department. SR-16-12.

Chamberlain DA. 2019. Barton Springs Salamander (*Eurycea sosorum*) and Austin Blind Salamander (*Eurycea waterlooensis*) captive breeding population management plan. City of Austin Watershed Protection Department.

Gaertner JP, Forstner MRJ, O'Donnell L, Hahn D. 2009. Detection of *Batrachochytrium dendrobatidis* in endemic salamander species from central Texas. EcoHealth, 6(1):20–26.

Hauwert NM. 2009. Groundwater flow and recharge within the Barton Springs Segment of the Edwards Aquifer, southern Travis County and northern Hays County, Texas [dissertation]. University of Texas at Austin.

Helsel DR. 2005. Non-detects and data analysis; statistics for censored environmental data. John Wiley and Sons, USA, NJ.

Hillman SS, Withers PC, Drewes RC, Hillyard SD. 2009. Ecological and environmental physiology of amphibians. Oxford University Press, Inc., New York.

Kahn CM, Line S, editors. 2005. The Merck veterinary manual. Merck and Co., Inc., Whitehouse Station, NJ.

Olsen-Winner C. 1989. Ability of various injuries to promote resorption of denervated, nerve independent regenerates of adult newts. Journal of Experimental Zoology 249:23–30.

R Core Team. 2023. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Available at https://www.R-project.org/ (accessed on 5 December 2023).

Schotte OE, Butler EG. 1941. Morphological effects of denervation and amputation of limbs in urodele larvae. Journal of Experimental Zoology 87:279–322.

Stiffler DF. 1993. Amphibian calcium metabolism. Journal of Experimental Biology 184: 47–61.

Stiffler DF. 1996. Exchanges of calcium with the environment and between body compartments in amphibia. *Physiological Zoology*. Vol. 69, No. 2 (Mar. – Apr. 1996), pp. 418–434.

Sweet S. 1978. On the status of *Eurycea pterophila* (Amphibia: Plethodontidae). Herpetologica 34(1):101–108.

Wright K, Whitaker BR. 2001. Amphibian medicine and captive husbandry. Malabar (FL): Krieger Publishing Company.

APPENDIX A

Water chemistry per treatment tank. Ca^{2+} (mg/L), pH, specific conductivity (μS/cm), magnesium, total hardness, and temperature mean, standard deviation, and number of samples. Ca^{2+}, magnesium, and total hardness measured with Hach DR3900 spectrophotometer and pH, conductivity, temperature, and dissolved oxygen measured with a Hydrolab DS4A. Rep. = Replicate tank.

	Control Low pH-High Ca		**Low pH-High Ca**		**Low pH-Low Ca**		**High pH-Low Ca**	
	Rep. 1	**Rep. 2**	**Rep. 1**	**Rep. 2**	**Rep. 1**	**Rep. 2**	**Rep. 1**	**Rep. 2**
pH (standard units)	7.1 ± 0.16 N = 74	7.0 ± 0.15 N = 74	7.1 ± 0.13 N = 74	7.1 ± 0.13 N = 74	7.2 ± 0.16 N = 74	7.2 ± 0.15 N = 74	8.1 ± 0.14 N = 74	8.1 ± 0.14 N = 74
Ca^{2+} (mg/L)	102 ± 5.1 N = 72	102 ± 5.6 N = 72	104 ± 6.0 N = 72	103 ± 6.3 N = 72	35 ± 3.9 N = 72	36 ± 4.1 N = 72	33 ± 5.3 N = 72	33 ± 6.1 N = 72
Specific Conductivity (μS/cm)	695 ± 19.0 N = 74	693 ± 10.5 N = 74	715 ± 13.7 N = 74	711 ± 13.4 N = 74	451 ± 11.4 N = 74	452 ± 11.4 N = 74	434 ± 13.4 N = 74	432 ± 15.6 N = 74
Mg (mg/L)	26 ± 2.6 N = 72	26 ± 2.6 N = 72	26 ± 2.4 N = 72	26 ± 2.4 N = 72	24 ± 2.5 N = 72	24 ± 2.5 N = 72	23 ± 2.4 N = 72	23 ± 2.6 N = 72
Total Hardness as $CaCO_3$ (mg/L)	361 ± 7.39 N = 72	361 ± 8.2 N = 72	368 ± 11.6 N = 72	366 ± 12.6 N = 72	185 ± 7.6 N = 72	187 ± 7.9 N = 72	178 ± 7.9 N = 72	179 ± 10.4 N = 72
Dissolved Oxygen (mg/L)	6.8 ± 0.37 N = 19	6.6 ± 0.37 N = 19	8.3 ± 0.27 N = 19	8.4 ± 0.33 N = 19	8.3 ± 0.34 N = 19	8.3 ± 0.39 N = 19	8.5 ± 0.34 N = 19	8.4 ± 0.35 N = 19
Temperature (°C)	21.6 ± 0.86 N = 74	21.7 ± 0.78 N = 74	22.2 ± 1.15 N = 74	21.9 ± 1.39 N = 74	21.9 ± 1.21 N = 74	22.0 ± 1.11 N = 74	22.1 ± 1.25 N = 74	22.1 ± 1.18 N = 74

APPENDIX B

Laboratory[1] analysis of water chemistry per treatment tank. Parameter mean, standard deviation, and number of samples. Rep. = Replicate tank.

	Control Low pH–High Ca		Low pH-High Ca		Low pH-Low Ca		High pH-Low Ca	
	Rep. 1	**Rep. 2**	**Rep. 1**	**Rep. 2**	**Rep. 1**	**Rep. 2**	**Rep. 1**	**Rep. 2**
Total Alkalinity ($CaCO_3$) (mg/L)	300 ± 6.5 N = 4	302 ± 9.3 N = 4	300 ± 12.4 N = 4	297 ± 12.5 N = 4	135 ± 4.4 N = 4	135 ± 3.7 N = 4	131 ± 11.2 N = 4	131 ± 13.0 N = 4
Bicarbonate Alkalinity (mg/L)	300 ± 6.5 N = 4	302 ± 9.3 N = 4	300 ± 12.4 N = 4	297 ± 12.5 N = 4	135 ± 4.4 N = 4	135 ± 3.7 N = 4	128 ± 11.1 N = 4	127 ± 11.5 N = 4
Calcium Total (mg/L)	106 ± 0.5 N = 4	101 ± 5.0 N = 4	106 ± 0.5 N = 4	103 ± 1.7 N = 4	36 ± 4.2 N = 4	35 ± 4.0 N = 4	33 ± 3.5 N = 4	34 ± 3.7 N = 4
Carbonate Alkalinity[2] (mg/L)	0 ±0 N = 3	0 ±0 N = 3	0 ±0 N = 3	0 ±0 N = 3	0 ±0 N = 3	0 ±0 N = 3	4.80 ±2.541 N = 3	4.91 ±2.379 N = 3
Chloride (mg/L)	26.4 ±2.07 N = 4	26.5 ±2.04 N = 4	29.0 ±1.89 N = 4	28.8 ±2.08 N = 4	30.8 ±0.87 N = 4	31.0 ±0.47 N = 4	29.8 ±1.46 N = 4	29.6 ± 1.82 N = 4
Magnesium Total (mg/L)	23.6 ± 1.39 N = 4	23.6 ± 1.40 N = 4	25.3 ± 0.75 N = 4	24.7 ± 0.51 N = 4	25.1 ± 1.25 N = 4	25.5 ± 1.07 N = 4	24.5 ± 1.01 N = 4	24.7 ± 0.45 N = 4
Potassium Total (mg/L)	1.45 ±0.248 N = 4	1.45 ±0.226 N = 4	2.12 ±0.169 N = 4	2.10 ±0.325 N = 4	2.26 ±0.179 N = 4	2.30 ±0.120 N = 4	1.79 ±0.311 N = 4	1.68 ±0.168 N = 4
Sodium Total (mg/L)	13.8 ±1.96 N = 4	13.7 ±2.02 N = 4	15.2 ±1.52 N = 4	14.9 ±1.68 N = 4	15.8 ±1.42 N = 4	16.0 ±1.20 N = 4	15.6 ±1.49 N = 4	15.1 ±1.37 N = 4
Sulfate (mg/L)	32.2 ±1.91 N = 4	32.4 ±1.94 N = 4	35.3 ±2.03 N = 4	35.0 ±2.24 N = 4	37.0 ±0.98 N = 4	37.1 ±0.42 N = 4	36.4 ±1.97 N = 4	36.1 ±2.21 N = 4

[1] Three samples analyzed by LCRA Environmental Laboratory Services and one sample analyzed by DHL Analytical in Austin, Texas.
[2] 4th sample result < 10 mg/L for each tank.

APPENDIX C

Health problems per treatment.

Treatment	*n* salamanders with health problems	*n* health problems
Control Low pH–High Ca	5	4[1, 2, 3, 11]
Low pH–High Ca	8	6[2, 3, 6, 7, 8, 9]
Low pH–Low Ca	6	3[2, 4, 5]
High pH – Low Ca	8	6[1, 2, 3, 5, 7, 10]

[1] abnormal body curvature, [2] stiff foot/dragging toes/foot, [3] short toes on at least 1 foot, [4] coiled position (straightened later), [5] red toes, [6] dragging back legs, [7] edema, [8] missing limb, [9] fungus on gills, [10] excess blood and swelling around heart, [11]bump on tail