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Morphological Abnormalities in the Gopher Frog (*Lithobates capito*) During a Headstarting Event

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Abstract.—**Conservation plans to protect Gopher Frog (*Lithobates capito*) populations commonly include headstarting to improve recruitment to the juvenile stage. Headstarting is being used across multiple federal, state, non-government, and academic organizations to augment and/or reintroduce Gopher Frog populations. In 2021, 99% of the 332 Gopher Frogs headstarted during the egg and larval stage at the University of Georgia Savannah River Ecology Laboratory in South Carolina displayed morphological abnormalities at metamorphosis. These included skin, eye, gill, and jaw abnormalities plus edema and redness of the skin. Skin abnormalities were the most prevalent, affecting 99.1% of frogs. Using a quantitative scoring system, we scored a subset of 92 frogs at metamorphosis and between 8–26 d after metamorphosis; all except eye abnormalities either partially or fully recovered. Based on photographs of early-stage tadpoles taken for a separate experiment, 79% of tadpoles had eye abnormalities as early as 16 d post-hatch. Except for gills and bloating, we found that models including mesocosm as a predictor had greater Akaike Information Criterion weights than those that did not have mesocosm as a predictor, suggesting the cause may be related to mesocosm-specific conditions. Since 2017, abnormalities in either Gopher Frogs or federally listed Dusky Gopher Frogs (*L. sevosus*) have been reported from at least six other headstarting facilities. It is unclear whether these abnormalities are related to captive conditions or are occurring in wild populations as well. Collection of additional data on rearing conditions will aid in determining relationships between headstarting environments and health of metamorphs.**

Key Words.—conservation; *Lithobates sevosus*; malformation; mesocosm; metamorph; *Rana capito*

Introduction

Intervention techniques are common (Soorae 2010) for species that rely on human management for continued persistence (Scott et al. 2005). One technique often used to counter declines of threatened and endangered species is headstarting. In headstarting programs, animals are collected from wild populations, reared in captivity during a vulnerable life stage, then released later in life as part of population augmentations and/or reintroductions (Bennett et al. 2017). Headstarting can help populations persist by improving juvenile recruitment (Thompson et al. 2020), increasing reproductive success (King and Stanford 2006), and maintaining genetic diversity (Hinkson 2015). Headstarting is currently used to support multiple at-risk and federally listed endangered amphibian species, and the number of headstarting programs continues to grow (Harding et al. 2016).

The Gopher Frog (*Lithobates capito*) is an at-risk species that is being considered for federal listing under the Endangered Species Act in 2025 (https://www.fws.gov/project/national-listing-workplan). Gopher Frogs breed in fishless, ephemeral ponds embedded within xeric Longleaf Pine (*Pinus palustris*) ecosystems (Enge et al. 2014). Historically, Gopher Frogs occurred throughout the southeastern USA coastal plain, but isolation of breeding ponds via habitat fragmentation, fire suppression, and the introduction of fish (Bailey 1991) has reduced the number of populations and necessitated active management of the species. Currently, Gopher Frogs are considered vulnerable in Florida, imperiled in Alabama, Georgia, and North Carolina, and critically imperiled in South Carolina and Tennessee (https://explorer.natureserve.org/ [Accessed 10 June 2023]). This species is likely extirpated from Tennessee where none have been collected since the 1990s (Richter et al. 2014).

Many Gopher Frog populations are thought to consist of relatively few breeding adults (Semlitsch et al. 1995) and are thus likely to experience population-level consequences of reproductive failure (Richter and Siegel 2002). In addition to few breeding adults, Gopher Frog populations often occur in areas with only one or a few breeding ponds (Cork 2019; Vanessa Kinney-Terrell and John Maerz, unpubl. report). In these isolated populations, persistence depends on consistent juvenile recruitment with low reproductive failure (Richter and Siegel 2002; Crawford et al. 2022b). Successful juvenile recruitment, however, is highly variable between and within ponds (Greenberg 2001), and stable juvenile recruitment to a population is made possible by having a network of multiple breeding ponds (Semlitsch and Bodie 1998).

To address these issues, translocations have been used for both wild-caught (Castellón et al. 2022) and captive-bred frogs (Roznik and Reichling 2021). The most common management strategy, however, involves headstarting wild-caught individuals followed by translocations to new sites (Vanessa Kinney-Terrell and John Maerz, unpubl. report), reintroductions to historic sites, and/or augmentations to existing populations (North Carolina Wildlife Resource Commission, unpubl. report). By collecting eggs and raising larvae in outdoor mesocosms, a subset of the population will likely survive to metamorphosis after a successful breeding event despite any harmful stochastic variation in breeding ponds (i.e., pond-drying, Richter et al. [2003]; and predation, Gregoire and Gunzburger [2008]). Therefore, headstarting is thought to be critical for the persistence of these isolated populations.

Headstarting of Gopher Frogs is occurring in Georgia, North Carolina, and South Carolina across multiple federal, state, non-government, and academic organizations. In South Carolina, only two known metapopulations remain, one on the Francis Marion National Forest (FMNF) and one on the U.S. Department of Energy Savannah River site (SRS). On the SRS, at least three distinct metapopulations once occurred (Semlitsch et al. 1995), but two are now considered extirpated. The FMNF and SRS metapopulations may represent the last two Gopher Frog metapopulations in South Carolina and are critical to protect. Consequently, the South Carolina Department of Natural Resources and U.S. Fish and Wildlife Service began headstarting Gopher Frogs with the initial goal of augmenting populations and establishing national fish hatcheries as headstarting facilities. The first headstarting effort in South Carolina occurred in 2019 where Bears Bluff National Fish Hatchery reared Gopher Frog eggs from FMNF. In 2020, Orangeburg National Fish Hatchery (ONFH) headstarted eggs from the SRS in collaboration with the University of Georgia Savannah River Ecology Laboratory (SREL). The SREL is based on the SRS where it monitors Gopher Frog populations. In 2020, researchers (including authors AJM and SLL) noted that a small proportion of headstarted SRS frogs metamorphosed with skin and eye abnormalities. In 2021, both ONFH and SREL headstarted SRS eggs. At SREL, we headstarted Gopher Frogs as part of an experimental study designed to examine how rearing conditions affect juvenile survival; however, we ended the study when it became apparent that a majority of metamorphic frogs at both facilities had developmental abnormalities. We have since learned of similar abnormalities occurring at other headstarting facilities.

Between 2013–2015, researchers at the National Amphibian Conservation Center in Michigan headstarted closely related Crawfish Frogs (*L. areolatus*) and observed abnormalities all three years (Stiles et al. 2016). To our knowledge, this is the only published record of abnormalities in the Gopher Frog/Crawfish Frog complex. In addition to those observed at ONFH and SREL, however, researchers also observed abnormalities in headstarted Dusky Gopher Frogs (*L. sevosus*) in Mississippi in 2017 (Joe Pechmann, pers. comm.), in Gopher Frogs at two Georgia facilities in 2018 (John Maerz, pers. comm.), and in Gopher Frogs at one North Carolina facility in 2021 (Dustin Smith, pers. comm.). In all of those cases there are no quantitative data on the abnormalities, but they appear to have been less severe and less prevalent than the abnormalities we report here. The purpose of this paper is to provide the first formal documentation of the observed Gopher Frog abnormalities. Herein, we (1) describe the types of abnormalities we observed in 2021 at SREL, (2) report the proportions of abnormality types, (3) explore the impacts of these abnormalities on survival of metamorphs, (4) document the progression of abnormalities over time, (5) discuss inferences as to what may have caused the abnormalities, and (6) discuss their implications for the conservation of Gopher Frogs.

Materials and Methods

Egg collection.—We collected four partial Gopher Frog egg masses on 5 March 2021 from a wetland in Barnwell County, South Carolina, USA, for an experimental headstarting project (exact locality hidden for conservation concerns). We transported the eggs to the SREL greenhouse facility and reared them by clutch in 18.93 L (5-gallon) buckets with natal-pond water. On 9 March 2021, we moved the eggs to the SREL Animal Care Facility (ACF) and placed them in four separate large clear plastic bins (60 × 43 × 15 cm) filled with 11 L of natal pond water and water collected from another known Gopher Frog breeding site within the same metapopulation (Crawford et al. 2022a). We changed 25% of the water daily using water from established mesocosms (see *Aquatic mesocosms* section below). We fed algae wafers (Hikari USA Incorporated, Hayward, California, USA) to hatched larvae *ad libitum* and kept the larvae in bins (91.5 × 42.7 × 19.8 cm) until all larvae from each egg mass reached the free-swimming Gosner stage (GS) 25 (Gosner 1960). On 18 March 2021, we pooled larvae from all four clutches into a large aquarium (61 × 30.5 × 40.6 cm) filled with about 70 L of water. The next day we haphazardly assigned 25 larvae to 18 mesocosms for a total of 450 larvae across all mesocosms. We released all tadpoles that were not used in the study back at their natal pond.

Aquatic mesocosms.—For the mesocosms, we used 1,324 L polyethylene cattle tanks (n = 18) situated in an open canopied area outside the ACF. We filled each mesocosm with well water, 1 kg of air-dried Maidencane (*Panicum hemitomon*) collected and mixed from Dry Bay (exact locality hidden; Aiken County, South Carolina, USA) and Mona Bay (exact locality hidden; Barnwell County, South Carolina, USA), and 3.5 L of pond water inoculate collected from Flamingo Bay (exact locality hidden; Aiken County, South Carolina, USA). The headstarting protocol calls for collecting and air-drying senesced Maidencane to be used as substrate in the mesocosms (U.S. Fish and Wildlife Service, unpubl. report). Thus, we collected it in the fall, before we knew which wetlands would experience Gopher Frog breeding in the subsequent spring. We collected Maidencane from wetlands that had large stands and mixed it prior to adding to mesocosms. We collected inoculate from wetlands that appeared to have robust zooplankton populations. We covered each mesocosm with a 50% shade-cloth lid held in place by a tightly fitted strap (bungee cord) that also functioned for predator exclusion. All mesocosms aged with well water, dried Maidencane, and inoculate for at least two weeks prior to the addition of Gopher Frog larvae.

Original experiment.—We originally headstarted these Gopher Frogs as part of an experiment to test whether larval exposure to snake-predator cues influences growth, survival, or behavior of larvae and/or post-metamorphic Gopher Frogs. Thus, we assigned 18 mesocosms to one of three treatment groups in a randomized block design. To create predator cues, we collected four adult Northern Cottonmouths (*Agkistrodon piscivorous*), put each into a nylon mesh bag, and then partially submerged each bag in a separate aquarium containing 20 L of soft water (made by dissolving 2.4 g $NaHCO_3$, 1.5 g $CaSO_4$, 1.5 g $MgSO_4$, and 0.1 g KCl in 50 L of 18 MΩ-cm Milli-Q® water; MilliporeSigma Company, Burlington, Massachusetts, USA) following procedures approved by the University of Georgia (UGA) Institutional Animal Care and Use Committee (IACUC; AUP A2020 12–010). We used soft water to remove the possibility of other cues entering the system and confounding the experiment. Soft water provides minerals removed through purification of Milli-Q® water. After 4 h (Moore et al. 2004), we removed the snakes and released them back at their capture sites. We combined water from the four tanks and then aliquoted it into 50 mL conical tubes and froze them at -20° C until use. At the same time, we made aliquots of soft water that had not come into contact with snakes for our control treatments. We added 50 mL of treatment water to each tank three times per week. The control treatment received control water three times per week, the low cue treatment received control water twice and predator water once per week, and the high cue treatment received predator water three times per week. Cues were added to all mesocosms through 16 June 2021.

Husbandry, larval photographs, and metamorphosis.—Throughout development we supplementally fed larvae algae wafers following an established protocol used in recent years by most headstarting facilities with minor adjustments (U.S. Fish and Wildlife Service, unpubl. report). On 3 April 2021 and 11 May 2021, we photographed four larvae from each mesocosm. We originally took photographs for measurements as part of the experiment described above. On both dates, we haphazardly dip-netted four larvae from each mesocosm (n = 72) and photographed them in clear acrylic viewing containers. On 3 June 2021, we set two minnow traps in each mesocosm to capture metamorphosing Gopher Frogs. We checked traps every morning and collected recently metamorphosed individuals (metamorphs) that had reached GS 42. We transferred metamorphs to the ACF in 18.9 L buckets with mesocosm water and then moved them into individual 0.47 L (16-ounce) Pro-Kal® deli containers (Fabri-Kal Corporation, Kalamazoo, Michigan, USA) with a layer of moist Spagmoss (Besgrow Limited, Bishopdale, Christchurch, New Zealand). After complete tail resorption (typically 1–2 d), we weighed and measured snout-vent length (SVL) of each metamorph.

Table 1. Descriptions of observed abnormalities in headstarted Gopher Frogs (*Lithobates capito*), adapted from Johnson et al. (2001), Schotthoefer et al. (2003), and Stiles et al. (2016).

Condition	Description
CEPHALIC AND AXIAL	
Microphthalmia	One or both eyes are small, often sealed by a transparent layer of skin.
Abnormal eye pigmentation	Discolored or speckled pupil, sclera, or iris.
Brachygnathia	Overbite; shortened lower jaw.
SKIN	
Cutaneous hypopigmentation	Skin is clear with a variable number of chromatophores and granular glands; feels sticky to the touch.
Erythema	Skin appears red across the body or in specific areas (i.e., face, sides, legs).
Gill slit exposed	Gill slit not resorbed upon metamorphosis.
Edema	Subcutaneous swelling in torso and/or limbs.

Recording abnormalities.—The first frogs to emerge showed evidence of either cutaneous hypopigmentation (hereafter skin abnormality), microphthalmia (hereafter eye abnormality), or both, and many frogs suffered from additional abnormality types including exposed gill slits (terminology from Stiles et al. 2016; hereafter gill abnormality), brachygnathia (hereafter jaw abnormality), edema (hereafter bloating), and erythema (hereafter redness; Table 1). We did not know if we were dealing with a pathogenic infection, so we instituted additional biosecurity measures, treating each frog as if it was infected with an unknown pathogen, and devised methodologies to describe symptoms and progression. To quantify the condition of each frog, we developed a scoring system (Table 2) to rank the types and severity of observed abnormalities. We scored, photographed, and measured weight and SVL for each metamorph at full tail resorption (4 June 2021 to 20 July 2021), but we did not begin recording gill, jaw, or bloating abnormalities until 11 June 2021. To assess the progression of these abnormalities, we re-scored 92 metamorphs on 13 July 2021 that had been individually scored between 18 June 2021 to 6 July 2021. We also reared 97 frogs with various abnormality types for several months past metamorphosis in 25.55 L (27-quart) latchable containers (Sterilite Corporation, Townsend, Massachusetts, USA) with soil substrate and artificial burrows following procedures approved by IACUC (A2021 06–014) to understand if the abnormalities had any effect on survival. These 97 frogs came from multiple headstarting facilities including SREL, ONFH, and the North Carolina Zoo. Each facility followed an established headstarting protocol with minor adjustments (U.S. Fish and Wildlife Service, unpubl. report). The North Carolina Zoo reared larvae from a population in North Carolina (Sandhills Gamelands) while both the ONFH and SREL reared frogs from the Savannah River Site in South Carolina. We obtained their frogs within one week of metamorphosis and then reared all frogs under the same conditions. We fed frogs crickets (*Gryllodes* sp.) dusted with calcium and vitamin D powder (Zoo Med Laboratories, San Luis Obispo, California, USA) and vitamin A supplement (Repashy Ventures, Oceanside, California, USA) ad libitum. We did not systematically re-score these frogs for abnormalities, but we kept frogs with eye abnormalities separate from those without eye abnormalities so that all frogs would have a chance to eat the crickets.

Pathology.—On 15 June 2021, we brought nine metamorphs headstarted at the ONFH and with varying degrees and types of abnormalities to the Southeastern

Table 2. Scoring system developed to quantitatively evaluate presence and severity of abnormalities observed in headstarted Gopher Frogs (*Lithobates capito*).

	Score				
Condition	0	1	2	3	4
Eye	Normal	One eye partially emergent and/or concealed by a layer of skin.	Both eyes partially emergent or one eye not visible, either completely covered by skin or absent.	One eye partially emergent and/or concealed by a layer of skin and one eye not visible, either completely covered by skin or absent.	Both eyes not visible, either completely covered by skin or absent.
Skin	Normal	Patch that surrounds front limbs.	Band that extends across lateral sides.	Band that extends across lateral sides and onto back.	
Gill	Normal	Gills retained on one side.	Gills retained on both sides.		
Bloating (edema)	Normal	Bloated on any part of the body.			
Jaw	Normal	Overbite; shortened lower jaw.			

Cooperative Wildlife Disease Study (SCWDS) wildlife research and diagnostic service at the UGA College of Veterinary Medicine. They performed gross and histopathologic examination on three metamorphs, ranavirus PCR on a pooled sample of three livers, and *Batrachochytrium dendrobatidis* (*Bd*) PCR on two individual skin samples. They submitted samples to the California Animal Health and Food Safety Laboratories (Davis, California, USA) for mass-spectrometry organic-compound screening (pesticides, environmental contaminants, drugs, and natural products) on a pooled sample of four livers and heavy-metal screening on a pooled sample of four carcasses absent the liver.

Data analysis.—We performed all statistical analyses using R open software (R Development Core Team 2022). To evaluate the predictors of abnormalities, we adjusted our scoring system so that all ordinal categories were on a binary scale. Thus, we lumped the different levels of severity for abnormalities into a single level so that scores were treated as either normal or abnormal. We conducted tests to determine whether an association existed between both mesocosm and abnormality presence and predator cue treatment and abnormality presence using Fisher's Exact Tests (Kim 2017). Using the AICcmodavg package (Mazerolle 2020), we then built two sets of models for each abnormality response using the original scores on ordinal and binary scales and compared the predictive power of our models using Akaike Information Criterion (AIC) weights (Wagenmakers and Farrell 2004) to further elucidate which predictor variable (mesocosm or treatment) might be explaining the differences in abnormality response. We assessed the progression of abnormality scores using either a paired two-tailed Wilcoxon Signed-Rank Test with continuity correction for abnormalities on an ordinal scale or McNemar's Test with continuity correction for abnormalities on a binary scale. We tested and met the assumptions for all statistical tests prior to analyses. We used $\alpha = 0.05$ for all statistical comparisons.

Results

Prevalence of abnormalities and larval survival.—We scored 332 of 450 headstarted frogs at tail resorption. We scored all 332 frogs for skin and eye abnormalities, and we also scored 249 of the 332 for gill, jaw, and bloating abnormalities. Overall, almost all metamorphs (99.1%) had some degree of skin abnormality, and the prevalence of other abnormalities varied greatly (Table 3). The

Table 3. Composition of post-metamorphic abnormalities for Gopher Frogs (*Lithobates capito*) across predator cue treatments and mesocosms during a headstarting event in 2021. For each abnormality type we present the number with the abnormality scored in that mesocosm and the percentage in parentheses. The sample sizes varied for each abnormality and mesocosm and the range is indicated for each column.

		Abnormality type				
Mesocosm	Treatment	Skin n = 13–22	Eye n = 13–22	Gill n = 11–20	Bloating n = 11–20	Jaw n = 6–20
1	Low	19 (100)	16 (84)	7 (58)	9 (75)	1 (8)
2	High	18 (100)	16 (89)	7 (54)	11 (85)	1 (8)
3	Low	17 (100)	17 (100)	7 (58)	8 (67)	6 (50)
4	Low	18 (100)	15 (83)	7 (47)	12 (80)	5 (33)
5	High	22 (100)	12 (59)	4 (20)	13 (65)	1 (5)
6	High	21 (95)	9 (41)	3 (21)	6 (43)	0 (0)
7	Control	19 (100)	18 (95)	10 (63)	16 (100)	6 (38)
8	Control	21 (100)	16 (76)	3 (21)	11 (79)	5 (36)
9	Control	19 (95)	1 (5)	2 (11)	10 (56)	1 (6)
10	Low	18 (100)	14 (77)	5 (33)	10 (67)	4 (27)
11	Control	16 (94)	4 (24)	2 (15)	8 (62)	1 (8)
12	Low	19 (100)	14 (74)	5 (33)	11 (73)	3 (20)
13	High	17 (100)	8 (47)	1 (9)	9 (82)	1 (9)
14	High	13 (100)	12 (92)	3 (50)	6 (100)	3 (50)
15	Control	15 (100)	15 (100)	6 (67)	9 (100)	6 (67)
16	High	20 (100)	7 (35)	5 (31)	10 (63)	0 (0)
17	Control	21 (100)	13 (62)	4 (24)	8 (47)	1 (6)
18	Low	16 (100)	10 (63)	4 (31)	11 (85)	0 (0)

Table 4. Akaike Information Criterion (AIC) model comparisons to examine predictors of observed Gopher Frog (*Lithobates capito*) abnormalities across predator cue treatments (Tx) and mesocosms. Abbreviations are K = number of parameters used in the model, AICc = information score of the model, Delta_AICc = difference in AIC score between the best model and the current model, AICcWt = AICc weight, Cum.Wt = sum of the AICc weights, LL = log-likelihood.

Response	Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
Skin	Mesocosm + Block	21	544.81	0.00	1.00	1.00	-249.91
	Tx + Block	6	587.64	42.83	0	1.00	-287.69
Eye	Mesocosm + Block	22	767.67	0.00	1.00	1.00	-360.20
	Tx + Block	7	833.56	65.89	0	1.00	-409.61
Gills	Tx + Block	5	387.59	0.00	0.88	0.88	-188.67
	Mesocosm + Block	20	391.56	3.96	0.12	1.00	-173.94
Bloating	Tx + Block	4	313.87	0.00	0.89	0.89	-152.85
	Mesocosm + Block	19	318.05	4.18	0.11	1.00	-138.37
Jaw	Mesocosm + Block	19	219.61	0.00	0.92	0.92	-89.15
	Tx + Block	4	224.56	4.95	0.08	1.00	-108.20

prevalence of some abnormalities differed significantly among our mesocosms. In particular, the proportion of metamorphs with eye (Fisher's Exact Test, $P < 0.001$), gill (Fisher's Exact Test; $P = 0.009$), jaw (Fisher's Exact Test, $P < 0.001$), and bloating (Fisher's Exact Test, $P = 0.039$) abnormalities differed significantly among mesocosms, but the proportion with skin abnormalities (Fisher's Exact Test, $P = 0.790$) did not. Predator-cue treatment groups differed in the proportion of metamorphs with eye (Control: 0.59; Low: 0.80; High: 0.58; Fisher's Exact Test; $P < 0.001$) and jaw (Control: 0.41; Low: 0.41; High: 0.34; Fisher's Exact Test, $P = 0.008$) abnormalities but not those with skin (Control: 0.97; Low: 1.0; High: 0.99; Fisher's Exact Test, $P = 0.780$), gill (Control: 0.47; Low: 0.56; High: 0.48; Fisher's Exact Test, $P = 0.130$), and bloating (Control: 0.77; Low: 0.79; High: 0.75; Fisher's Exact Test, $P = 0.620$). For eye and skin AIC models, mesocosm predicted the presence of abnormalities much better than treatment (Table 4). For the jaw model, mesocosm predicted abnormality presence slightly better than treatment, but for the gill and bloating models, treatment predicted abnormality presence better than mesocosm. Despite the prevalence of abnormalities, survival rate from the larval stage to complete metamorphosis was 94.7%, with a range of 84–100% across mesocosms.

Earliest signs of abnormalities.—The larval photographs we took provided us the opportunity to assess whether any abnormalities were observable prior to metamorphosis. From photographs of the 72 larvae taken on 3 April 2021, 16 d post-hatch, 79% had at least one abnormally pigmented eye (Table 1) and/or abnormal skin around the eye (Fig. 1). By 11 May 2021 (54 d post-hatch), 85% of larvae we photographed had at least one abnormally pigmented eye and/or additional abnormalities including microphthalmia and abnormal configuration of the eye parts (Fig. 1). We did not see any other abnormalities from the photographs.

Progression of abnormalities.—We found that most abnormalities scored at tail resorption improved over time (Figs. 2 and 3). We compared the total initial scores and re-scores for all the frogs (n = 92). We found that re-scores for skin ($W = 559.0$; $P < 0.001$), gill ($W = 1378.0$; $P < 0.001$), bloating (McNemar's Test, $P < 0.001$), and jaw (McNemar's Test, $P = 0.008$) abnormalities were significantly lower than initial scores. We found that re-scores for eyes, however, were not significantly different than initial scores ($W = 119.5$; $P = 0.380$). Over the course of seven to 25 d, 98% of metamorphs with gill abnormalities completely resorbed their gills,

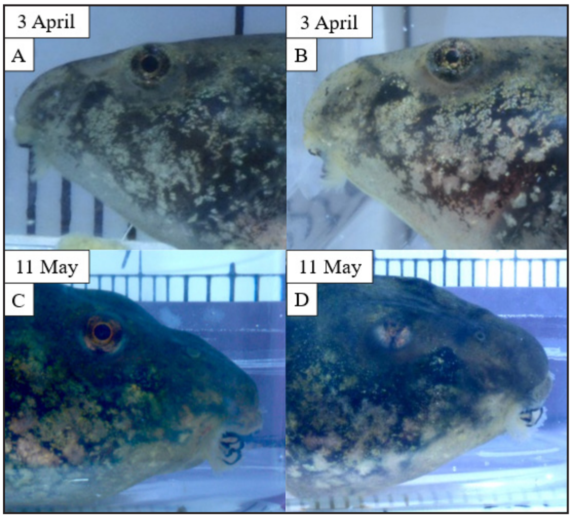

Figure 1. Photographs of Gopher Frog (*Lithobates capito*) larvae with normal eyes (A and C) and abnormal eyes (B and D). Note the speckled iris of tadpole B compared to the normally pigmented iris in tadpole A and the irregularly shaped eye parts of tadpole D compared to tadpole C. (Photographed by Jason O'Bryhim).

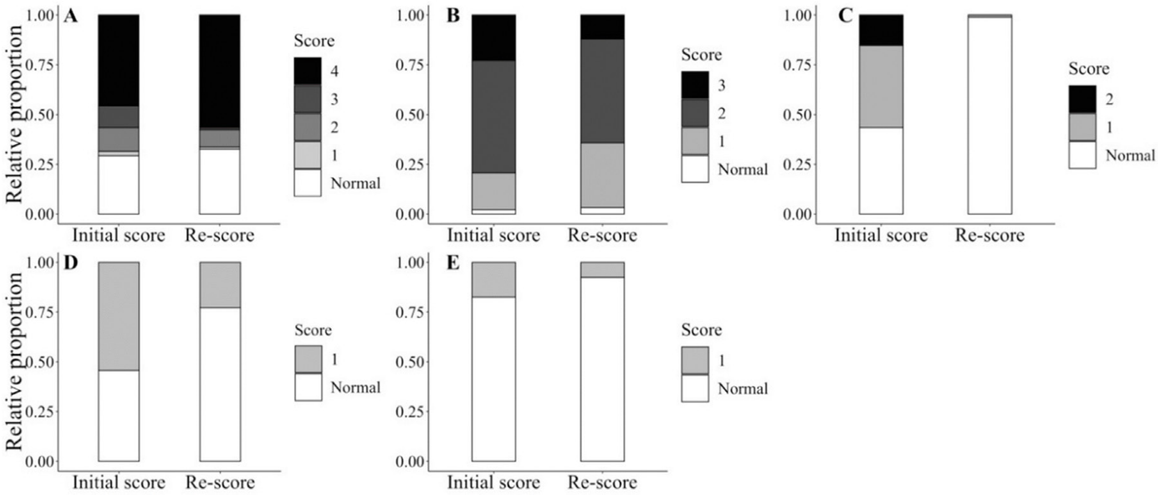

Figure 2. A subset of Gopher Frogs (*Lithobates capito*) was scored twice to document the progression of symptoms over time. The abnormality scores for 92 metamorphs were initially recorded at tail resorption (18 June 2021 to 6 July 2021), then re-scored on 13 July 2021. For each abnormality type (A = eye, B = skin, C = gill, D = bloating, E = jaw), the relative proportions of each score are presented by severity (1–4 = abnormal, increasing in severity).

58% of metamorphs that were bloated recovered (no visible signs of the abnormality), and 56% of frogs with jaw abnormalities developed normal jaws (Fig. 2). The severity of skin abnormalities also decreased by 13%, but eye abnormalities increased by 3% (Fig. 2).

Pathology.—A diagnostician performed postmortem examinations but did not identify any underlying infectious or inflammatory cause. In addition to the aforementioned abnormalities, the diagnostician discovered scoliosis in two of the three metamorphs examined. Researchers did not detect Ranavirus and *Bd* in samples from three pooled liver samples and two individual skin samples, respectively. From a pooled liver sample, toxicological analysis detected flunixin, a non-steroidal anti-inflammatory drug used in horses and cattle (Moses and Bertone 2002), and ethyl anthranilate/ anthranilic acid, a repellant for mosquitos used in topical repellants (Afify et al. 2014) and as food additives (U.S. National Archives and Records Administration. Code of Federal Regulations. 21 CFR 172.515. Available from https://www.ecfr.gov [Accessed 9 August 2023]). Toxicologic analysis did not detect other toxic compounds. Heavy-metal screening detected copper (2.4 ppm, reporting limit = 0.3 ppm), iron (17 ppm, reporting limit = 1 ppm), manganese (0.47 ppm, reporting limit = 0.1 ppm), and zinc (20 ppm, reporting limit = 0.3 ppm) in the pooled carcass sample, but not arsenic, cadmium, lead, mercury, or molybdenum.

Figure 3. Photographs of one Gopher Frog (*Lithobates capito*) metamorph taken 19 d apart showing progression of brachygnathia (A to B) and cutaneous hypopigmentation/ microphthalmia/ exposed gill slits (C to D) over time. (Photographed by Christian Swartzbaugh).

Observations from long-term rearing.—Of the 97 frogs that we reared for several months after metamorphosis, 78 survived for at least 171 d (1 September 2021 to 18 February 2022). Within a few weeks, all abnormalities appeared to recover except for eye abnormalities. Although we did not take any quantitative data on frog behavior, it appeared that the eye abnormalities influenced behavior. Frogs without eye abnormalities and frogs with abnormalities affecting only one eye appeared to spend most of their time in burrows. When we approached enclosures to mist or feed, frogs with at least one normal eye would be in their burrows but would emerge when we opened the lid. By contrast, the frogs with abnormalities in both eyes would already be on the surface when we approached. Survival was similar between frogs with abnormalities in one or both eyes (83%) and those without eye abnormalities

(79%). Frogs with eye abnormalities gained an average weight of 1.9 ± 0.70 (standard deviation) g and frogs without eye abnormalities gained an average weight of 1.90 ± 1.76 g from 23 November 2021 to between 19 January 2022 and 18 February 2022, and the crickets disappeared from the enclosures, indicating the frogs were eating.

Discussion

Overall, we observed five distinct abnormalities in our headstarted Gopher Frogs. The most prevalent abnormalities affected the skin and occurred in almost every frog, but eye abnormalities were also common. Unfortunately, we were not aware of the abnormalities until the first metamorphic individuals emerged, so we are not able to establish when they began. Examination of photographs of early-stage tadpoles, however, indicated that some abnormalities were present by 16 d post-hatch. Although we saw some influence of predator-cue treatment on prevalence of abnormalities, the control and low-cue treatments had a higher prevalence than high-cue treatments. Thus, we do not think exposure to predator cues had a direct impact on abnormalities. Rather, it is possible that these results were spurious due to the effect of mesocosm on abnormalities. By rearing juveniles for at least 5 mo, we determined that all but the eye abnormalities improved with time. Abnormalities in amphibians are not new, but the suite of abnormalities we observed is not common (Ouellet 2000). More commonly, abnormalities affecting the limbs have been documented (Ouellet et al. 1997; Ouellet 2000). Abnormalities of the limbs and/or digits collectively represent over 77% of the abnormalities of amphibians seen in the wild (Ruhl and Sanders 2022). Some of the abnormalities we observed have been seen in other species. For example, eye abnormalities, jaw abnormalities, and bloating have been observed *in situ* in numerous species in the U.S. (Ouellet 2000; Vandenlangenberg et al. 2003; Reeves et al. 2013) and globally (Ouellet 2000; Gurushankara et al. 2007; Peltzer et al. 2011; Henle et al. 2017), affecting multiple families of anurans and caudates.

It is challenging to retroactively determine causes of abnormalities; in fact, there is still much uncertainty around causes of abnormalities in the wild (Stocum 2000; Blaustein and Johnson 2003; Ruhl and Sanders 2022). There have been many proposed causes of amphibian abnormalities including chemical contaminants (Burkhart et al. 1998; Fort et al. 1999), parasites (Johnson et al. 1999), ultraviolet radiation (UV; Ankley et al. 1998), nutritional deficiencies (Densmore 2007; Ferrie et al. 2014), and disruption of the retinoid signaling pathway (Gardiner and Hoppe 1999). Contaminants such as synthetic chemicals and heavy metals can cause craniofacial (Tietge et al. 2000) and eye abnormalities (Britson and Threlkeld 1998), edema, and gill displacement (Harris et al. 1998) in frogs, although we did not find an association between the compounds detected in this population and abnormalities in the literature. Nonetheless, in our case, possible sources of contamination include chemical leaching from mesocosms (Weir et al. 2014) or input from a contaminated water source. We have used the same mesocosms in an open-canopied area with shade cloth lids and pond water inoculate for a previous amphibian study using larval Southern Toads (*Anaxyrus terrestris*) and never observed abnormalities (Rumrill et al. 2016). Further, several facilities that used different mesocosms and water sources observed similar abnormalities. A diagnostician from the California Animal Health and Food Safety Laboratories detected some heavy metals in postmortem tissue samples, but without published information on appropriate mineral ranges for Gopher Frogs, it is difficult to determine whether the levels detected are abnormal. Parasitic trematodes (Frog-Mutating Flatworms, *Riberoia ondatrae*) are often associated with amphibian abnormalities (Johnson et al. 1999). Trematodes, however, primarily cause limb deformities (Johnson and Sutherland 2003), which we did not observe. Additionally, we covered mesocosms with a shade cloth during the larval period and found no evidence of snails, which are necessary intermediate hosts before the trematodes can infect amphibian larvae (Sessions and Ruth 1990). Both ranaviruses (frog virus 3) and Amphibian Chytrid Fungus (*Bd*), the causative agent of chytridiomycosis, have been documented on the SRS (Love et al. 2016) and the bacterium *Aeromonas hydrophila*, the causative agent of red-leg syndrome, has been found at a former cooling reservoir on the SRS (Hazen 1979). Redness and bloating are common symptoms of these pathogenic infections (Kulp and Borden 1942; Green 2001; Yaw and Clayton 2018). Histopathologic evaluations and molecular testing, however, failed to detect any of these pathogens, and they are not known to be associated with the other abnormalities we observed. Further, we saw almost no mortality and the redness and bloating quickly subsided. We cannot, however, rule out the presence of any of these, or other untested pathogens that could have contributed to redness and edema.

Exposure to UV can cause eye abnormalities (Ankley et al. 2002) and bloating (Hays et al. 1996; Blaustein et al. 1997) in amphibians. All of our mesocosms were in an area of full sun, but with 50% shade cloth. While we did observe abnormalities in every mesocosm, other facilities with the same setup only observed them in one or two mesocosms (John Maerz and Dustin Smith, pers. comm.), making it unlikely that UV is the primary cause. Abnormal levels of dietary vitamin A can cause abnormalities of epithelial skin cells (Li et al. 2009) in toads and eye abnormalities (Alsop et al. 2004) in frogs; however, we followed a feeding protocol that is used by most headstarting facilities, many of which have never seen abnormalities. Thus, if nutrition were responsible, we would expect to see more consistency in abnormalities between mesocosms and across headstarting facilities. Nutritional deficiencies are known to cause abnormalities in captive amphibians (Wright and Whitaker 2001), though, and represent an area in need of further investigation.

We cannot rule out the possibility that the abnormalities we observed are, in part, a result of genetic bottlenecking and inbreeding depression in Gopher Frog populations. Other species that occur in small, isolated populations have developed developmental issues related to inbreeding depression (Madsen et al. 1996; Hitchings and Beebee 1998; Mansfield and Land 2002). Recent genetic work on Gopher Frogs found relatively high heterozygosity and allelic richness and low within population relatedness (Devitt et al. 2023); however, their work primarily focused on more robust Florida populations. Given the importance of mesocosm in our study and that other facilities only saw abnormalities in some, but not all mesocosms, it is likely that if genetics are at play it is manifested as a gene by environment interaction.

At this point we do not know what caused the abnormalities. We believe the most likely cause, however, is disruption of the retinoid signaling pathway. Retinoids, a group of vitamin A metabolites, play a role in differentiation of the limbs, epithelial and mucosal tissues, craniofacial features, eyes, and several other organ systems (Gardiner and Hoppe 1999; Gardiner et al. 2003; Das et al. 2014). Retinoids can cause abnormalities in mammals, birds, and amphibians that resemble the ones reported here for Gopher Frogs including abnormalities of the eyes (Sulik et al. 1995; Emmanouil-Nikoloussi et al. 2000; Alsop et al. 2004), skin (Weissman et al. 1963; De Luca et al. 1985; Aşkın et al. 2021), craniofacial features (Emmanouil-Nikoloussi et al. 2000; Gardiner et al. 2003) and bloating (Tey and Theng 2006). Because retinoids are involved in a wide range of developmental pathways, they could explain each abnormality type observed in the Gopher Frogs. The timing of experimental exposure to retinoic acid alters the toxicity and development of abnormalities in a dose-dependent fashion (Degitz et al. 2000). In other ranid species, exposure to retinoic acid early in development can result in craniofacial effects including micropthalmia and anophthalmia (Degitz et al. 2000). Teratogenic retinoid-like exudates can be produced during cyanobacterial harmful algal blooms (Wu et al. 2012, 2013; Jonas et al. 2014; Sehnal et al. 2019) at levels that can affect amphibian development (Smutná et al. 2017). Each headstarting facility adds plant material collected from local wetlands to their mesocosms. It is possible that cyanobacteria could have been brought in on the plant substrate. Under the right environmental conditions, a harmful algal bloom could occur in the mesocosms. Future investigations will be aimed at identifying whether algal blooms may contribute to these symptoms.

The cause of the Gopher Frog abnormalities remains unclear, and we do not know whether these abnormalities are restricted to headstarting environments or if the abnormalities occur in wild populations. If the abnormalities are related to harmful algal blooms, they have the possibility of occurring in the wild. Under headstarting conditions, the observed abnormalities appeared to have no negative effects on survival and all but the eye abnormalities recovered after metamorphosis. The likelihood that wild frogs survive to metamorphosis with these abnormalities, however, may be much lower than what we observed in a protected environment. Additionally, we do not know whether the abnormalities influence the post-release survival of these frogs or if there are any negative reproductive effects. Thus, there is still a need to understand the underlying cause of the abnormalities so that we know whether they pose a threat to wild populations. Whether they directly affect wild populations or not, the abnormalities can hinder headstarting and conservation efforts. It is important for headstarting facilities to collect quantitative data on abnormalities to allow for a better understanding of possible causes. Further, collection of additional data on rearing conditions (i.e., type of plant substrate used, water source, water quality, feeding regime, etc.) will aid in determining relationships between headstarting environments and health of metamorphs.

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