

# Survival, movements and habitat use of captive-bred and reintroduced dusky gopher frogs

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## Keywords

amphibians; assisted reproduction; behavior; captive breeding; reintroductions; survival; release method; translocations.

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## Abstract

Despite the growing roles of assisted reproduction and captive breeding in reintroductions, we have a limited understanding of the viability of these animals once released. We radiotracked endangered dusky gopher frogs *Lithobates sevosus* for up to 24 days after they were released into the wild to (1) assess the viability of juvenile frogs produced using artificial fertilization and raised in captivity until four weeks post-metamorphosis, (2) investigate survival rates, movement patterns, and habitat use and (3) examine whether it is more beneficial to release frogs at the pond edge or into burrows in the uplands. Released frogs had a relatively high survival rate (76% overall, with mortality ceasing after 14 days) and behaved as expected, moving fairly long distances (up to 153 m daily and 426 m total) and locating suitable shelters, including burrows and stump holes. Frogs that sheltered underground had a 22% higher survival rate than frogs that did not, highlighting the importance of these habitat features. Compared to frogs released into burrows, frogs released at the pond had a 33% lower survival rate, spent less time underground and moved farther and more often, likely while searching for shelter. Frogs selected habitat characteristics that are associated with effects of fire, including an open canopy and abundant ground vegetation. Our study demonstrates that juvenile dusky gopher frogs successfully transitioned to their natural habitat, and that any effects of artificial fertilization or captivity on juvenile viability appear to be minimal. Releasing frogs into areas with abundant underground refuges and using frequent prescribed fire to maintain the habitat should benefit dusky gopher frog populations and improve the success of reintroductions.

## Introduction

Translocations have played important roles in the conservation of many species (Griffith *et al.*, 1989; Fischer & Lindenmayer, 2000; Germano & Bishop, 2009). Although translocations have been taxonomically biased toward vertebrates, especially mammals and birds (Seddon *et al.*, 2005), amphibian translocations have been increasing in number and success rate (Griffiths & Pavajeau, 2008; Germano & Bishop, 2009). The success of amphibian translocations is positively related to the number of animals released (Germano & Bishop, 2009). Often, wild-sourced animals are limited, and translocated animals must come from captive populations or breeding programs. However, many endangered species reproduce poorly or not at all in captivity, making it a challenge to reliably produce large numbers of offspring for reintroductions (Kouba *et al.*, 2009). Assisted reproductive technologies can be used to improve captive breeding programs by increasing breeding efficiency and maintaining genetic diversity in captive populations. These

technologies include hormone treatments for induced breeding or gamete collection and artificial fertilization (Kouba *et al.*, 2009; Clulow *et al.*, 2014; Silla & Byrne, 2019).

Despite the growing use of captive breeding and assisted reproductive technologies in managing populations and conserving species, potential effects of these techniques on offspring fitness are not well understood. For example, selecting pairs for artificial fertilization, rather than relying on female mate choice, could potentially reduce genetic compatibility between males and females, and thereby reduce offspring fitness (Dziminski *et al.*, 2008; Sherman *et al.*, 2008, 2010; Puurtinen *et al.*, 2009). In addition, storing sperm before performing fertilizations can have numerous effects on offspring fitness across taxa (Tarín *et al.*, 2000; White *et al.*, 2008; Crean *et al.*, 2012; Immler *et al.*, 2014; Gasparini *et al.*, 2017; Alavioon *et al.*, 2019; Evans *et al.*, 2019; Poo & Hinkson, 2020). Furthermore, the diet and environmental conditions (e.g. temperature, water, ultraviolet light) experienced by amphibians during development in captivity can also influence their fitness through numerous

effects on their morphology, behavior, physiology and immunology (Álvarez & Nieceza, 2002; Antwis & Brown, 2009; Mendelson & Altig, 2016; Knutie *et al.*, 2017).

Short- and long-term monitoring of animals that are released into the wild is essential for improving the overall success of reintroductions (Griffith *et al.*, 1989; Fischer & Lindenmayer, 2000; Germano & Bishop, 2009), and can be especially useful for examining the fitness of captive-bred animals. Post-release monitoring also can be used to examine survival rates and the factors that influence them. The survival of released animals depends on many factors, especially the amount and quality of habitat at reintroduction sites (Griffith *et al.*, 1989; Germano & Bishop, 2009). For species in which habitat requirements are not well understood, detailed field studies can be combined with monitoring activities to identify important habitat characteristics and inform actions to manage for them on the landscape. For example, pond-breeding amphibians require high-quality aquatic and terrestrial habitat. Postmetamorphic individuals spend most of their lives in terrestrial habitat and migrate to ponds only to breed; populations typically reside in the terrestrial habitat within 300 m of the pond, but this varies by species, with some individuals moving distances greater than 3.5 km (Semlitsch & Bodie, 2003; Humphries & Sisson, 2012). Studying the movements and habitat use of pond-breeding amphibians can thus provide valuable information on the amount and type of terrestrial habitat required, including important habitat features.

The dusky gopher frog *Lithobates sevosus* is a federally endangered (IUCN critically endangered) species that historically occurred throughout the southern Coastal Plain of the USA, before it drastically declined to one known population by 2000 (Hammerson *et al.*, 2004), prompting translocations and other conservation actions. Dusky gopher frogs are found in fire-maintained, open-canopy habitat dominated by longleaf pine *Pinus palustris* and a grassy, herbaceous understory (Hammerson *et al.*, 2004). Adult frogs spend most of their lives underground in stump holes and the burrows of small mammals and historically of gopher tortoises *Gopherus polyphemus* and migrate seasonally to isolated, temporary ponds to breed (Richter *et al.*, 2001). The major historic and current threat to the dusky gopher frog is habitat loss and alteration (Hammerson *et al.*, 2004). Urban development, forestry practices, and fire suppression have contributed to the decline of the longleaf pine ecosystem, of which less than two percent of original forest remains (Ware *et al.*, 1993). Translocations of dusky gopher frogs produced through natural and captive breeding are underway to establish and augment populations. Because efforts to facilitate natural breeding by dusky gopher frogs in captivity have not been successful, assisted reproductive technologies have been successfully developed for this species and a reintroduction program for captive-bred frogs has been established recently. Because this program is in its infancy, the short- and long-term viability of captive-bred animals once released is not known.

We radiotracked juvenile dusky gopher frogs that were produced using assisted reproductive technologies, raised in captivity through metamorphosis, and released into restored

longleaf pine forest. Our goals were to (1) assess the viability of juvenile frogs produced using artificial fertilization and raised in captivity through metamorphosis, (2) investigate survival rates, movement patterns, and habitat use and (3) examine whether it is more beneficial to release frogs at the pond edge or into burrows in the uplands. Previous field studies on adult and juvenile dusky gopher frogs at one site provide a baseline on their behavior and spatial ecology (Richter *et al.*, 2001; Tupy, 2012), but additional data from other sites are needed for a more comprehensive understanding of this endangered species. The findings of our study will provide an initial outlook on the captive-breeding and reintroduction program and will be used to guide future plans for producing and releasing dusky gopher frogs and improving their habitat.

## Materials and methods

### Captive breeding and husbandry

By 2000, the dusky gopher frog had declined to one known population in De Soto National Forest in Harrison County, MS (Hammerson *et al.*, 2004), which is approximately 50 km from our reintroduction site and within the historic range of this species. All extant wild and captive populations have originated from this source population. Founders of the captive populations are genetically representative of natural populations (Hinkson *et al.*, 2016), although genetic variation is low in all populations due to inbreeding and previous population bottlenecks (Hinkson & Richter, 2016; Hinkson *et al.*, 2016). Wild individuals have been regularly added to captive populations to maintain genetic diversity. Studbook records for captive adults were used to avoid breeding closely related individuals.

Because efforts to facilitate natural breeding by dusky gopher frogs in captivity have not been successful, assisted reproductive technologies were used to breed adults in the captive population at the Memphis Zoo to produce juvenile frogs for this study. This involved administering hormones to induce ovulation and spermiation, and fertilizing the eggs in vitro (Kouba *et al.*, 2012; Poo & Hinkson, 2019). To induce oviposition, two priming doses of 3.7 IU hCG/g body weight were administered 72 h apart (hCG: human chorionic gonadotropin; Sigma-Aldrich, St. Louis, MO), followed by an ovulation dose of 13.5 IU Hcg + 0.4 µg GnRH/g body mass 24 h after the second priming dose (GnRH: gonadotropin-releasing hormone; Sigma-Aldrich, St. Louis, MO). To induce spermiation, 10 IU hCG + 0.4 µg GnRH/g body weight were administered to males. Hormone dosages were based on Kouba *et al.* (2012) and Poo & Hinkson (2019). Sperm were collected through the urine using a 1-mm-diameter catheter one hour after hormone injections. Females were kept individually in covered 5.7-L boxes until oviposition began (24–72 h after the ovulation dose). Egg release was assisted by gentle squeezing and opening of the cloaca using a 1-mm inoculation loop. Females were held over cell culture dishes (150 × 25 mm), so that eggs could be deposited directly onto the dishes.

Immediately after eggs were deposited, fresh sperm samples were placed on eggs to fertilize them. If fresh sperm was not available, sperm stored for up to one hour at 4°C was used. After five minutes, the cell culture dish was filled with aged tap water at room temperature (23°C) to submerge the fertilized eggs. A total of 20 unique male-female crosses was performed; female egg masses were split among multiple males. Eggs were monitored until hatching (5–6 days) and tadpoles were transferred to 10-gallon aquaria (51 × 25 × 31 cm) filled with aged tap water at room temperature (23°C) in groups of no more than 20 tadpoles per aquarium and fed a spirulina-based gel (Soilent Green, Repashy Ventures, Inc., Oceanside, CA). After metamorphosis, juvenile frogs were transferred to two large (178 × 81 × 36 cm) communal enclosures at room temperature (23°C) containing moist sphagnum moss, ultraviolet lighting and a pool of aged tap water. Vitamin-dusted crickets were provided daily. The largest frogs were selected for the radiotelemetry study at approximately four weeks post-metamorphosis and the remaining frogs were released at the reintroduction site after the study had ended.

### Reintroduction site

The radiotracking study took place in restored longleaf pine forest at Ward Bayou Wildlife Management Area in Jackson County, MS (Fig. 1). The reintroduction area consists of a 19-ha burn unit containing two isolated ponds. This unit is surrounded mainly by bottomland hardwoods lying in the alluvial floodplain of the Pascagoula River. Restoration efforts for the reintroduction area included removing slash pines *Pinus elliotii*, applying prescribed fire at regular intervals, restoring one pond (Mayhaw Pond, 800 m<sup>2</sup>) and creating a new pond (Gil's Pond, 400 m<sup>2</sup>). Elevation at the site is 15–23 m above sea level. The restored habitat is dominated by longleaf pine and a grassy, herbaceous understory and contains an abundance of underground shelters for dusky gopher frogs, including stump holes and burrows excavated by gopher tortoises and small mammals.

### Radiotracking

We released and radiotracked 53 juvenile frogs that had metamorphosed approximately four weeks prior to the study. Frogs were tracked for up to 24 days during the study period (mean: 10 days, 24 July – 18 August 2018). The mean snout-vent length (SVL) was 40.6 mm (range: 37–50 mm), and the mean mass was 5.4 g (range: 4.3–9.8 mm). Frogs were released at dusk either at the edge of Mayhaw Pond ( $n = 25$ ), which held water for the duration of the study, or into gopher tortoise burrows ( $n = 28$ ). These burrows were located 42–529 m from the pond, and all were in similar habitat that consisted of grassy, herbaceous vegetation among longleaf pines (Fig. 1). Although dusky gopher frogs also use other types of underground shelters (e.g. stump holes) that are abundant at the reintroduction site, the dimensions (e.g. width, length, depth) are difficult to determine and standardize, so we released frogs into gopher tortoise burrows

because they have similar characteristics. Initially, 15 frogs were released at the pond and 15 frogs were released into burrows on 23 July 2018. Recovered transmitters from frogs that did not survive ( $n = 13$ ), slipped their belts ( $n = 17$ ), or developed abrasions ( $n = 3$ ) were re-used to track additional frogs when sufficient battery life remained; these additional frogs were released when transmitters were available (typically staggered every few days), and the release location alternated between pond and burrow. Data for frogs that left the study early were right-censored for survival analyses and we controlled for the number of days monitored in other analyses when appropriate. A total of 20 burrows were used for release; due to limited availability, some burrows were re-used, but only when the previous frog released there had died or left the area.

Frogs were fitted with radiotransmitters (model A1015, 0.55 g, 29-day maximum battery life, Advanced Telemetry Systems, Isanti, MN) attached to frogs using an external belt made of elastic thread and glass seed beads (Muths, 2003). We tracked frogs using a R410 (Advanced Telemetry Systems, Isanti, MN) and R1000 (Communications Specialists, Inc., Orange, CA, USA) receiver with a handheld three-element Yagi antenna. Each frog was located once daily throughout the tracking period. We examined frogs every few days when possible to check for possible skin abrasions caused by the transmitter belt. When we found abrasions on a frog, we immediately removed the transmitter and released the frog. At the end of the study, we removed transmitters from all recaptured frogs ( $n = 8$  of 20 frogs); remaining transmitter belts were expected to deteriorate and detach from frogs within the next few weeks.

### Survival rates

We located frogs daily to monitor their survival and attempt to determine the cause of death for non-surviving frogs. While frogs were in underground shelters, they often could be observed visually and their transmitter signals periodically changed positions, which we used as evidence that frogs were still alive. We used a Cox proportional hazards model to examine survival rates and how they were affected by release site (pond vs. burrow) and SVL. We used a second Cox proportional hazards model to directly examine how survival rates were affected by the use of underground refuges, when controlling for release site and SVL. A hazard ratio was calculated for significant factors in each model; this value indicates the ratio of the hazard rates corresponding to the conditions described by the two levels of the explanatory variable. Analyses were performed using the *survival* package (Therneau, 2015) in R statistical software (R Core Team, 2017).

### Movement patterns

We recorded the latitude and longitude for each unique frog location using a GPSMAP 64st GPS unit (Garmin, Olathe, KS; accuracy within 5–10 m), and we imported these coordinates into ArcGIS 10.6 to measure the distances between



**Figure 1** Reintroduction site for the dusky gopher frog at Ward Bayou Wildlife Management Area in Jackson County, MS. The 19-ha burn unit (bordered by fire breaks) is dominated by longleaf pine and a grassy, herbaceous understory and contains two isolated ponds. Frogs were released at Mayhaw Pond (star) and at gopher tortoise burrows (open circles) and they moved to their final locations (closed circles). A patch of dense, early-successional vegetation may have altered frog movements (crescent-shaped polygon adjacent to Mayhaw pond).

successive locations and sum the total distance each frog moved during the study period. We also calculated the daily movement probability for each frog as the number of days the frog changed locations divided by the total number of days that the frog was monitored. We marked each frog location with a wire flag and noted daily whether it had moved; we considered a change in position that was greater than 1 m to be a movement. We used a linear mixed-effects model to examine daily movement distances and how they were affected by release site (pond vs. burrow), SVL, movement number (defined as the sequential order of a frog's movements, from first to last), and the interaction between release site and SVL. Frog identity was used as a random effect to control for any effects of individual frogs. We also used linear models to examine the effects of release site, SVL, the number of days monitored, and the interaction between release site and SVL on the total distance moved during the study (with one outlier of 426 m removed from the dataset) and on daily movement probability. Analyses were performed in R statistical software; models were constructed using the *nlme* package (Pinheiro *et al.*, 2017) or built-in *lm* function (R Core Team, 2017), the *car* package (Fox & Weisberg, 2011) was used to calculate probability values, and the *visreg* package (Breheny & Burchett, 2017) was used to generate partial residual plots to examine relationships between variables.

## Habitat use

### Underground refuge use

Each time we located a frog, we recorded whether it was on the surface of the ground or in an underground refuge. We used a linear model to examine how the proportion of days spent underground was related to release site (pond vs. burrow), SVL and number of days monitored. To help explain any differences in underground refuge use between sites, we used ANOVAs to compare the numbers of underground refuges at the pond versus uplands (see below for sampling details) and examine whether the number of underground refuges varies with distance from pond. Analyses were performed in R statistical software; the built-in *aov* and *lm* functions (R Core Team, 2017) were used to construct the models, the *car* package (Fox & Weisberg, 2011) was used to calculate probability values for the linear model, and the *visreg* package (Breheny & Burchett, 2017) was used to generate partial residual plots to examine relationships between variables.

### Coarse-scale habitat use

The most recent prescribed fire at the reintroduction site did not carry well through the terrestrial habitat immediately surrounding Mayhaw Pond, and consequently, goldenrod (*Solidago* sp.) had densely colonized a large area (7863 m<sup>2</sup> within 50–100 m of the pond center) and excluded other plant species (Fig. 1). We investigated whether frogs released at the pond avoided this patch of dense, early-successional

vegetation using a coarse-scale approach in ArcGIS 10.6. We first created a 115-m buffer around the pond, which encompassed 95% of the locations of all frogs released from the pond ( $n = 128$ , excluding seven outliers). We delineated the areas of dense goldenrod and sparser vegetation (grasses and herbaceous plants) within the buffer, using GPS points taken on the ground and aerial photographs as a guide. We then generated a number of random points that was equal to the number of points representing frog locations, and we counted the number of points that fell into the polygons delineating the two habitat types. To determine whether frogs used habitat types in proportion to their availability, we evaluated expected and observed numbers of frog locations and random points in dense and sparse habitat using a contingency table analysis in SYSTAT 13.0.

### Fine-scale habitat use

To investigate whether frogs selected attributes of their habitats at a finer scale, we also sampled habitat characteristics at locations used by frogs and at random locations that were not used by frogs. We used different methods to select random points near the pond and in the upland release area due to the configuration of the reintroduction area. Random points in the pond area were located every 10 m along transects in each cardinal direction that began at the pond edge and ended at the edge of the reintroduction area or at a maximum of 200 m. Random points in the uplands were located 50 m in each cardinal direction from each gopher tortoise burrow where a frog was released. We sampled habitat characteristics at 195 locations used by frogs (99 near pond and 96 in uplands) and 152 random points (64 near pond, and 88 in uplands).

At each sampling point, we measured the average height of the understory (grasses and herbaceous plants) and visually estimated the percentage of ground covered by vegetation, leaf litter and bare soil within circular plots with 1 m diameter. We also sampled the basal area of trees using a 10 basal area factor wedge prism, and we counted the number of underground refuges (>3 cm diameter within 3 m) and ant nests (within 3 m). We also took a hemispherical photograph of the canopy over each point using the Fisheye Pro application on a smartphone, and we measured canopy cover from these photographs using Gap Light Analyzer software (Frazer *et al.*, 2000). We used a principal components analysis (PCA) to combine the eight habitat variables into fewer factors that weighted variables according to their importance. This generated principal components (PCs) with a given loading value for each habitat variable, which designated its importance to the PC (ranging from  $-1.0$  to  $+1.0$ ). We used linear models to determine whether used versus available habitat differed on each PC that had an eigenvalue > 1 (Quinn & Keough, 2002), when controlling for release area (pond vs. uplands). We used 0.50 as the minimum loading value for a habitat variable when interpreting a given PC. Analyses were performed in R statistical software; the PCA was performed using the built-in *prcomp* function (R Core Team, 2017) and the *factoextra* package (Kassambara &

Mundt, 2017), the linear model was constructed using the built-in *lm* function (R Core Team, 2017), the *car* package (Fox & Weisberg, 2011) was used to calculate probability values and the *visreg* package (Breheny & Burchett, 2017) was used to generate partial residual plots to examine relationships between variables.

## Results

### Survival rates

Of 53 total frogs tracked, 75.5% survived until the end of the study ( $n = 20$ ), their transmitter belts were lost ( $n = 17$ ), or their belts were removed due to abrasions ( $n = 3$ ). Of all frogs tracked, 24.5% ( $n = 13$ ) died before the end of the study; eight frogs appeared to have been preyed on by ants (including invasive fire ants, *Solenopsis invicta*), one frog was preyed on by a black racer snake *Coluber constrictor*, one frog was trampled by a feral hog (*Sus scrofa*) and the cause of death could not be determined for three frogs.

Survival rates were affected by release site ( $z = 2.0940$ ,  $P = 0.0363$ ), but were not affected by body size ( $z = -0.048$ ,  $P = 0.9621$ ). Frogs released into burrows had a 22% higher survival rate than frogs released at the pond (Fig. 2a); the survival rates for frogs released at burrows and the pond were 86% and 64%, respectively. The hazard ratio was 3.6, which indicates that the risk of mortality for frogs released at the pond was 3.6 times higher than that of frogs released into burrows. When controlling for the effects of release site and body size, frogs that used at least one underground refuge had a 33% higher survival rate than frogs that did not shelter underground ( $z = 1.9870$ ,  $P = 0.0469$ , Fig. 2b). The survival rates for frogs that used at least one refuge ( $n = 36$ ) or no refuges ( $n = 17$ ) were 86% and 53%, respectively. The hazard ratio was 8.6, which indicates that the risk of mortality for frogs that did not shelter underground was 8.6 times higher than that of frogs that used at least one underground refuge.

### Movement patterns

Although some frogs remained sedentary, most moved along relatively linear paths and did not appear to settle into home ranges. Of the 25 frogs released at the pond, nine frogs moved to the uplands (i.e. beyond the treeline), and 16 frogs remained at the pond until their deaths ( $n = 8$ ) or the end of the study ( $n = 8$ ). Frogs moved up to 153 m in one day, up to 426 m total throughout the study, and up to 323 m straight-line distance from their release point. On average, frogs moved 25 m between locations, a maximum distance of 43 m between locations, and a total distance of 91 m during the study, and frogs changed locations an average of 50% of days monitored. Frogs released at the pond moved greater daily and total distances and had higher daily movement probabilities than frogs released into burrows (Table 1, Fig. 3a-c). Daily distance, but not total distance or daily movement probability, was positively related to body size (Table 1, Fig. 3d-f). Daily distance was negatively related to

movement number (the sequential order of a frog's movements, from first to last), indicating that successive movements by frogs tended to decrease in distance (Table 1, Fig. 3g). Total movement distance was positively related to the number of days tracked, and daily movement probability was negatively related to the number of days tracked (Table 1, Fig. 3h-i), indicating that frogs continued moving throughout the study, but changed locations less often over time. There was not a significant interaction between release site and SVL for any movement metric examined (Table 1).

### Habitat use

#### Underground refuge use

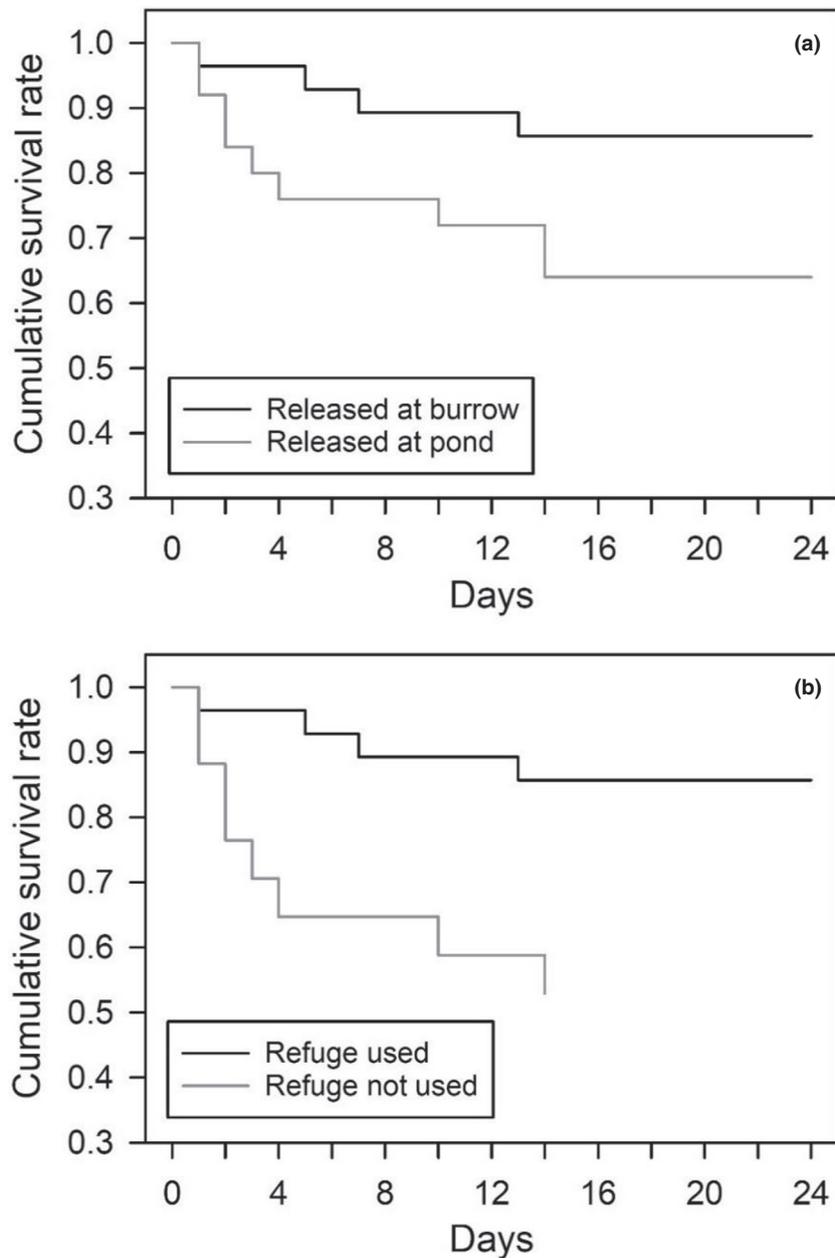
Frogs sheltered on the surface of the ground in vegetation and leaf litter, and underground in gopher tortoise burrows, small mammal burrows, and other holes associated with stumps and roots. Of all frogs released into gopher tortoise burrows, 85% left those burrows, and 52% of the frogs that left located a new underground refuge. In contrast, only 32% of frogs released at the pond located an underground refuge. The proportion of days that frogs used underground refuges was affected by release site ( $F = 52.5965$ , d.f. = 1,  $P < 0.0001$ ) and the number of days monitored ( $F = 7.3126$ , d.f. = 1,  $P = 0.0094$ ), but not by body size ( $F = 0.4713$ , d.f. = 1,  $P = 0.4956$ ). Frogs released into burrows spend more time underground than frogs released at the pond (Fig. 4a), and the number of days underground was positively related to the number of days monitored (Fig. 4b). There were slightly more underground refuges present near the pond than in the uplands overall ( $F = 4.1240$ , d.f. = 1,  $P = 0.0441$ ; Fig. 5a), but the number of refuges depended on distance from the pond ( $F = 22.7000$ , d.f. = 1,  $P < 0.0001$ ; Fig. 5b). More refuges were located at farther distances from the pond.

#### Coarse-scale habitat use

When we compared points representing locations of frogs released at the pond ( $n = 128$ ) to an equal number of random points, we found that frogs used a patch of dense, early-successional habitat 11.7% less often than expected, based on availability ( $\chi^2 = 6.6397$ , d.f. = 1,  $P = 0.0100$ ).

#### Fine-scale habitat use

A PCA incorporating eight fine-scale habitat variables resulted in four PCs with eigenvalues  $> 1$  that together explained 81% of variation among habitat variables (Table 2). While controlling for release site, used and available habitat differed significantly for PC1 ( $F = 10.3580$ , d.f. = 1,  $P = 0.0014$ ), and PC3 ( $F = 39.1640$ , d.f. = 1,  $P < 0.0001$ ), but not PC2 ( $F = 0.0144$ , d.f. = 1,  $P = 0.906$ ) or PC4 ( $F = 1.6729$ , d.f. = 1,  $P = 0.1967$ ). For PC1, frogs selected locations that had lower values than available locations (Fig. 6a); selected habitat was positively associated with ground vegetation and understory height, and negatively



**Figure 2** Cumulative survival rates for (a) frogs released at the pond or gopher tortoise burrows and for (b) frogs that used at least one underground refuge or remained on the surface of the ground during the study period.

associated with tree basal area, canopy cover and bare soil. For PC3, frogs selected locations that had lower values than available locations (Fig. 6b); selected habitat was positively associated with bare soil and ant nests, and negatively associated with canopy cover, tree basal area and ground vegetation.

## Discussion

Juvenile dusky gopher frogs produced using artificial fertilization and raised in captivity through metamorphosis experienced relatively high survival rates during the transition to

the wild and behaved as expected. We found that 76% of frogs survived the study period (up to 24 days, with mortality ceasing after 14 days), and frogs were able to move fairly long distances (up to 153 m daily and 426 m total) and locate suitable shelters, including burrows and stump holes. Our overall survival rate was much higher than that of a similar study on closely related gopher frog (*L. capito*) juveniles that were produced through natural breeding and emigrated from their natal ponds (Roznik & Johnson, 2009a); only 4 of 32 frogs (13%) frogs survived their first month in the terrestrial habitat, mainly due to predation by snakes. All four juvenile dusky gopher frogs monitored by

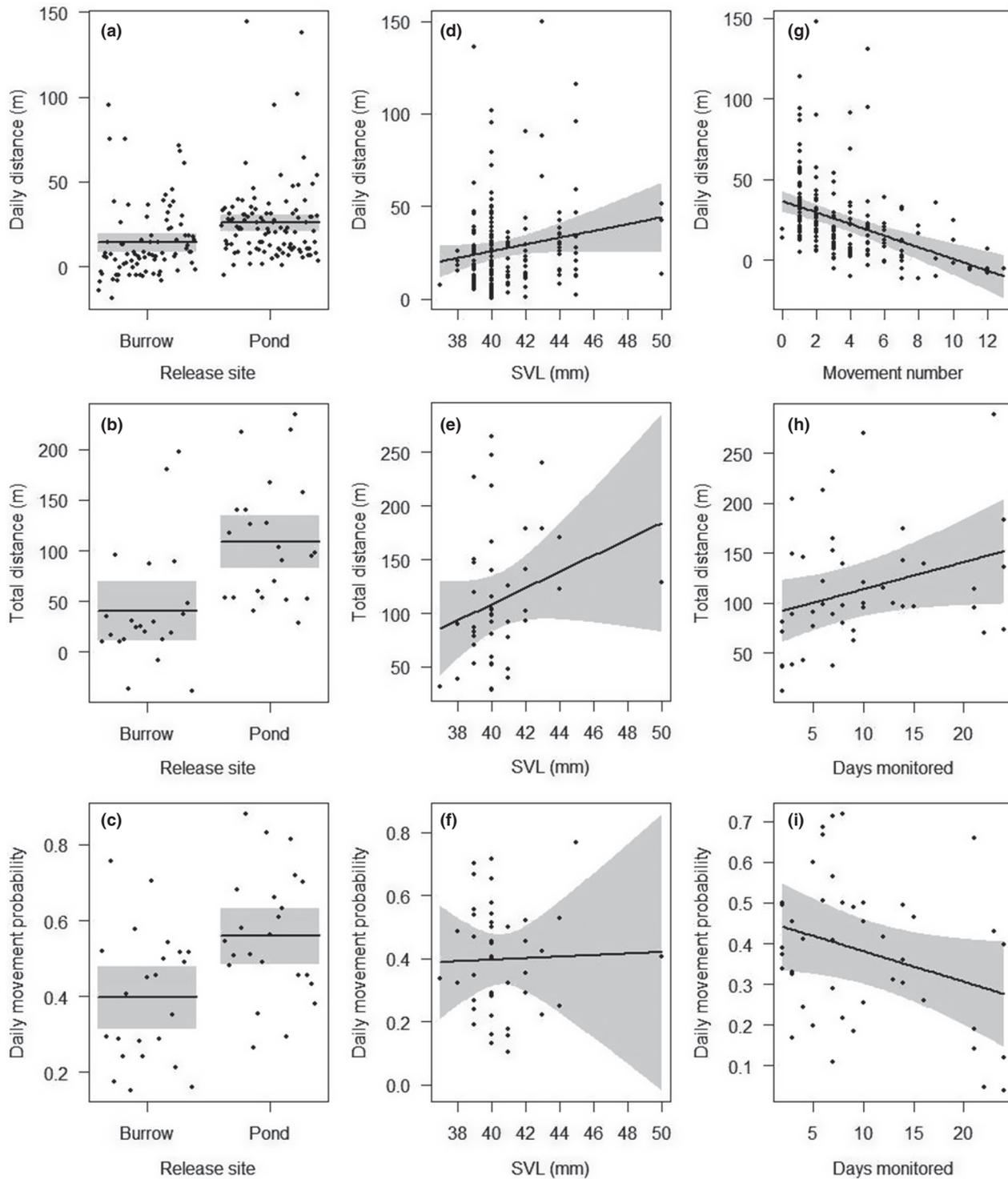
**Table 1.** Statistical results for a linear mixed-effects model used to examine the effects of release site (pond vs. burrow), SVL, movement number (the sequential order of a frog's movements, from first to last), and the interaction between release site and SVL on daily movement distance (individual frog identification was used as a random effect; d.f. = 1, test statistic =  $\chi^2$ ), and linear models used to examine the effects of release site, SVL, number of days monitored, and the interaction between release site and SVL on the total distance moved during the study, and the daily movement probability (d.f. = 1, test statistic = F)

Effect	Estimate	SE	Test statistic	P
<b>Daily distance</b>				
Release site (pond)	169.0843	97.9423	5.0116	0.0252
SVL	6.1810	1.9901	9.7003	0.0018
Movement number	-3.8622	0.6835	31.9284	<0.0001
Release site (pond) × SVL	-3.8893	2.4061	2.6128	0.1060
<b>Total distance</b>				
Release site (pond)	-8.9120	437.5730	13.2405	0.0008
SVL	5.6630	9.5350	2.5199	0.1203
Days monitored	2.7190	1.3970	3.7850	0.0588
Release site (pond) × SVL	1.9170	10.8030	0.0315	0.8601
<b>Daily movement probability</b>				
Release site (pond)	-0.4449	1.0833	10.2146	0.0027
SVL	0.0024	0.0226	1.1545	0.2889
Days monitored	-0.0075	0.0039	3.6657	0.0625
Release site (pond) × SVL	0.0152	0.0267	0.3239	0.5724

Tupy (2012) survived the month-long study. In the present study, only one frog was preyed on by a snake, although others appeared to have been preyed on by ants (including invasive fire ants), trampled by a feral hog, or died of unknown causes. Controlling invasive species may be beneficial to gopher frogs and other species, particularly in disturbed areas (e.g. roads, clearings) where fire ants are more abundant (Stiles & Jones, 1998; Todd *et al.*, 2008). We found that frog mortality ceased after the first 14 days, suggesting that survival rates should increase after the initial adjustment to the habitat, which would be necessary to establish a population. The rate at which adult dusky gopher frogs returned to a pond to breed between years was 16-22% (Richter & Seigel, 2002), suggesting that annual survival may be low. Estimated annual survival probabilities for other ranids vary within 7-82% for all postmetamorphic life stages, including 7-72% for juveniles (reviewed by Howell *et al.*, 2016). Given our survival rates and behavioral observations, any effects of artificial fertilization or captive rearing on offspring fitness appear to be minimal in the juvenile stage. However, longer-term data and direct comparisons with frogs produced through natural breeding would be useful to examine potential effects of captive breeding on offspring fitness. Importantly, a review of amphibian translocations worldwide found that releases of wild and captive frogs had similar success rates (Germano & Bishop, 2009).

Because gopher frogs rely on underground refuges for protection from extreme temperatures, dehydration, predators and fire (Richter *et al.*, 2001; Roznik & Johnson, 2007, 2009a, 2009b; Roznik *et al.*, 2009; Tupy, 2012), we tested whether releasing frogs directly into gopher tortoise burrows would be a beneficial release strategy. We found that survival rates were relatively high for groups of frogs released into burrows and at the pond (>64%), but they were 22% higher for frogs released into burrows (Fig. 2a), even though most frogs left those burrows. The higher survival rates for frogs released into burrows may be caused by a greater availability and use of underground refuges in the uplands than in the immediate pond area. There were more refuges with increasing distance from the pond, so frogs were more likely to encounter a refuge as they moved away from the pond edge (Fig. 5b). Furthermore, frogs avoided a large patch of dense, early-successional vegetation between the pond and uplands (Fig. 1), which may have impeded their movements away from the pond. Consequently, over half of the frogs that left their release burrows were able to locate new gopher tortoise burrows or other underground refuges, compared to 32% of frogs released at the pond that found underground refuges. When controlling for release site, the risk of mortality for frogs that did not shelter underground was 8.6 times higher than that of frogs that used at least one underground refuge (Fig. 2b). Juvenile dusky gopher frogs raised in outdoor enclosures with more burrows had higher growth and survival rates than individuals with access to fewer burrows (Nacy, 2019), emphasizing the importance of these habitat features in the landscape. Furthermore, survival rates of juvenile *L. capito* are also strongly related to their ability to locate and use underground refuges (Roznik & Johnson, 2009a). Releasing gopher frogs into areas with high densities of underground refuges and facilitating access to them from the pond area appear important to juvenile survival. Increasing burrow densities using artificial burrows (Nacy, 2019) or using delayed-release techniques (often called soft releases), such as temporary enclosures around burrows (e.g. Tuberville *et al.*, 2005; Mitchell *et al.*, 2011), to acclimate released frogs to burrows may also be useful management techniques to test in the future.

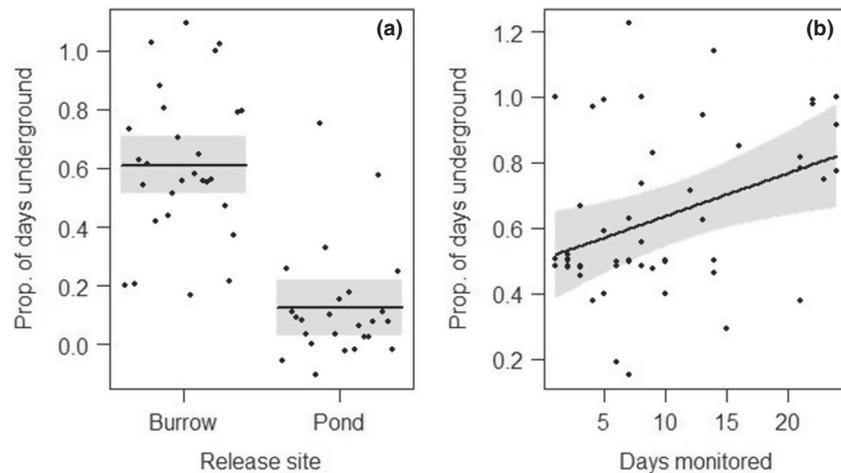
Juvenile dusky gopher frogs are capable of moving fairly long distances. Frogs in our study moved up to 153 m in one day, up to 426 m total throughout the study, and up to 323 m straight-line distance from their release point. In pond-breeding amphibians, juveniles leave the pond after metamorphosis and disperse into terrestrial habitat; some disperse short distances and eventually return to their natal pond to breed, whereas others disperse long distances and breed at other ponds when they reach maturity (Pittman *et al.*, 2014; Sinsch, 2014). Among pond-breeding amphibians, gopher frogs are known for their capacity for long-distance movements (Richter *et al.*, 2001; Blihovde, 2006; Roznik *et al.*, 2009; Roznik & Johnson, 2009b; Humphries & Sisson, 2012; Tupy, 2012). Adult *L. capito* can travel at least 3.5 km during breeding migrations (Humphries & Sisson, 2012) and dispersing juveniles can move at least 691 m from natal ponds (Roznik & Johnson, 2009b). The movement



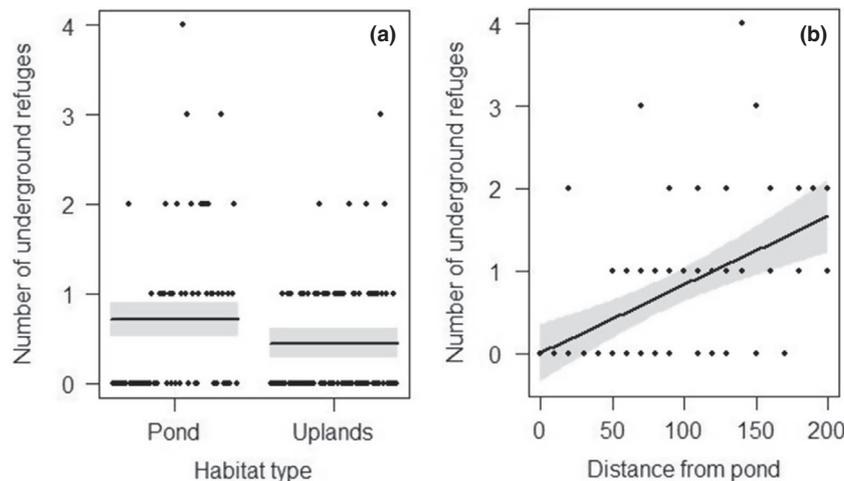
**Figure 3** Partial residual plots with 95% confidence bands (shaded) showing the relationship between daily movement distance, total movement distance, and daily movement probability and (a-c) release site (pond vs. burrow), (d-f) SVL, and (g) movement number (the sequential order of a frog’s movements, from first to last) or (h-i) number of days monitored. Partial residual plots hold constant all factors in the statistical model that are not being displayed.

ecology of dusky gopher frogs has been studied previously during two studies that took place at one site (Glen’s Pond); Richter *et al.* (2001) reported adults moving up to 299 m from the

pond, and Tupy (2012) reported maximum movement distances from the pond of 240 m for adult females, 217 m for adult males, and 194 m for juveniles. Movement rates may be related



**Figure 4** Partial residual plots with 95% confidence bands (shaded) showing the relationship between the proportion of days frogs spent in underground refuges and (a) release site and (b) number of days monitored. Partial residual plots hold constant all factors in the statistical model that are not being displayed.



**Figure 5** Partial residual plots with 95% confidence bands showing (a) the number of underground refuges available in the two release areas and (b) the relationship between the number of underground refuges and distance from the pond. Partial residual plots hold constant all factors in the statistical model that are not being displayed.

to landscape resistance or availability of underground refuges. For example, Richter *et al.* (2001) suggested that movement distances by adult dusky gopher frogs may have been constrained by a recent clearcut on adjacent private property. We found that frogs released at the pond moved greater daily and total distances and had higher daily movement probabilities than frogs released into burrows (Fig. 3a-c). This pattern may have been driven by higher densities of underground refuges at farther distances from the pond (Fig. 5b); frogs released at the pond may have needed to travel longer distances and change locations more often to find suitable shelter, compared to frogs released into the burrow-dense uplands. As frogs moved away from the pond over time, they spent more time underground (Fig. 4b) and tended to move less often (Fig. 3i) and move shorter distances between locations (Fig. 3g), which is

consistent with this hypothesis. Although some frogs remained sedentary, many moved along relatively linear paths and did not appear to settle into home ranges. A longer-term study would be necessary to examine the transition from dispersal behavior to the establishment of a home range.

Frogs tended to select locations with characteristics that are associated with effects of fire, including an open canopy, few trees, abundant grasses and herbaceous vegetation, scarce leaf litter, and bare soil associated with burrows (Table 2, Fig. 6). This finding is consistent with the results of other studies that have examined microhabitat selection by gopher frogs or compared areas where populations are present and absent (Thurgate, 2006; Roznik *et al.*, 2009; Roznik & Johnson, 2009b; Tupy, 2012). Amphibians tend to select characteristics of the ecosystems in which they evolved,

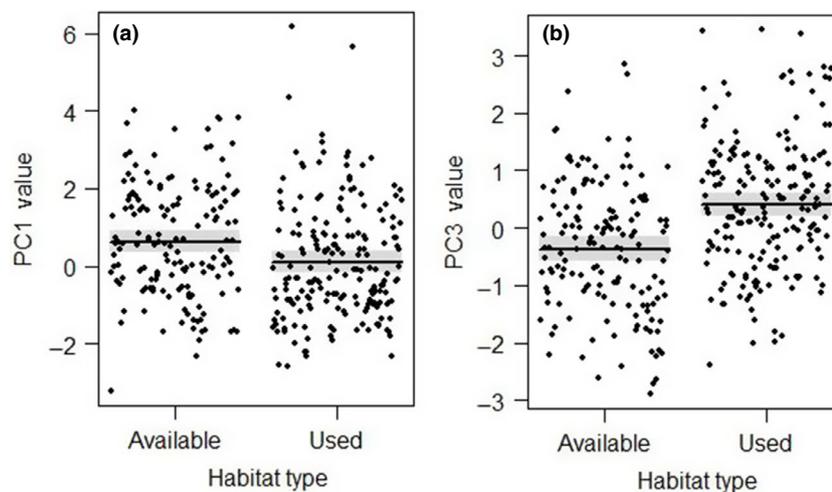
**Table 2.** Eigenvalues and loading values from a principal components analysis for habitat characteristics at the dusky gopher frog reintroduction site. This analysis resulted in four principal components (PC) with eigenvalues > 1, which together explained 81% of the variation among habitat characteristics

Eigenvalues and loading values	PC1	PC2	PC3	PC4
Eigenvalues	2.413	1.475	1.431	1.122
Variation explained (%)	30.159	18.433	17.884	14.029
Loading values				
Basal area (m <sup>2</sup> /ha)	0.733	-0.128	-0.522	0.145
Canopy cover (%)	0.737	-0.219	-0.466	0.072
Understory height (m)	-0.671	-0.360	-0.041	0.314
Vegetation (%)	-0.654	0.318	-0.656	0.081
Leaf litter (%)	0.151	-0.930	0.191	-0.131
Bare soil (%)	0.586	0.516	0.555	0.048
Underground refuges (#)	0.269	0.140	-0.138	0.686
Ant nests (#)	-0.124	-0.171	0.380	0.708

which increase their fitness. For example, forest-associated amphibians select closed-canopy areas and avoid areas with an open canopy, such as fields and clearcuts, which increase the risk of desiccation (deMaynadier & Hunter, 1999; Rothermel & Semlitsch, 2002; Rittenhouse & Semlitsch, 2006). Gopher frogs evolved in fire-maintained longleaf pine forests that are dominated by an open canopy and herbaceous, grassy understory. Gopher frogs avoid patches of fire-suppressed habitat and orient toward fire-maintained habitat (Roznik *et al.*, 2009; Roznik & Johnson, 2009b; Tupy, 2012). One explanation for their preference for open habitat is that these areas are associated with higher densities of underground refuges (Roznik & Johnson, 2009b; Tupy, 2012; Nacy, 2019). Fire contributes to the formation of holes associated with stumps and roots, and fire also maintains an herbaceous, grassy understory that provides quality habitat

and forage for gopher tortoises and small mammals that excavate burrows (Funderburg & Lee, 1968; Boglioli *et al.*, 2000). Gopher frogs may also select fire-maintained habitat because open habitat provides thermoregulatory benefits to frogs by allowing sunlight to reach the ground, which increases frog growth rates (Nacy, 2019). Invertebrate abundance appears to be similar in fire-maintained and fire-suppressed habitats (Nacy, 2019), so habitat selection by frogs may not be related to prey availability.

In conclusion, our study demonstrates that juvenile dusky gopher frogs produced using artificial fertilization and raised in captivity through metamorphosis had relatively high survival rates and behaved as expected during the transition to their natural habitat. These findings are encouraging for our reintroduction and others, especially given concerns regarding the low level of genetic diversity present in this species due to inbreeding and previous population bottlenecks (Hinkson & Richter, 2016; Hinkson *et al.*, 2016). Our findings emphasize the importance of underground refuges to the survival of gopher frogs. We found that survival rates were higher for frogs released into burrows than at the pond, likely due to higher densities of underground refuges in the uplands and a patch of dense, early-successional vegetation that may have impeded frog movements around the pond. We recommend releasing gopher frogs into areas with high densities of underground refuges and facilitating access to them from the pond. Increasing burrow densities using artificial burrows (Nacy, 2019) or using delayed-release techniques (e.g. temporary enclosures around burrows) to acclimate released frogs to burrows may also be useful management techniques to test in the future. Our findings also highlight the importance of fire in maintaining longleaf pine habitat for gopher frogs. Frogs selected habitat characteristics that are associated with effects of fire, which are more likely to contain more underground refuges than areas that have been fire suppressed (Roznik & Johnson, 2009b; Tupy,



**Figure 6** Partial residual plots with 95% confidence bands (shaded) showing values from a principal components (PC) analysis for (a) PC1 and (b) PC3 in habitat available to and used by frogs. Partial residual plots hold constant all factors in the statistical model that are not being displayed. Habitat variables included in the principal components and their loading values are presented in Table 2.

2012), and which also contribute to increased growth and survival rates of frogs (Nacy, 2019). Because natural fires no longer occur at a scale and frequency to maintain the species composition and structure of longleaf pine forests, it is important for prescribed fires to be conducted at a season, frequency, and intensity that maintain the vegetative characteristics of this ecosystem (Glitzenstein *et al.*, 1995, 2003).

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