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Role of Reptile-Invertebrate Interactions in Enigmatic Reptile Declines

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Biology

by

Meredith Swartwout
Virginia Tech
Bachelor of Science in Biology, 2014

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This dissertation is approved for recommendation to the Graduate Council.

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Abstract

Reptile populations are declining world-wide and the mechanisms behind many of these declines remain enigmatic. Food web interactions (i.e., reduced prey availability or increased predation) have been implicated behind some reptile declines. However, relatively little is known about predation on lizard and snake eggs, despite egg survival being important for population dynamics of some species. Ants are important predators of squamate reptile eggs in tropical and temperate systems. In Costa Rica, long-term declines in terrestrial anole lizards were linked with reduced leaf litter depth, a factor that could influence egg vulnerability to ant predation. Fire ants (genus *Solenopsis*) are aggressive generalist predators that are known to depredate reptile eggs. Over the past few decades, red imported fire ants (RIFA; *Solenopsis invicta*) have been introduced from South America to the U.S., where they have caused substantial ecological damage in their invasive range. RIFA invasion has coincided with population declines of terrestrial snake species in the southeastern U.S., but direct links between RIFA and snake declines remain primarily anecdotal. I used a tropical system (lowland tropical rainforest, Costa Rica) and a temperate one (southeastern U.S.) to test whether ant predation on reptile eggs could be driving enigmatic declines in squamate reptiles. I used a combination of field experiments, observational studies, laboratory incubation, and review of published literature to determine whether squamate reptile eggs were vulnerable to ant predation under different conditions. At La Selva Biological Station, Costa Rica, I tested whether leaf litter depth, nest microhabitat, or forest type influenced lizard egg predation rates and predatory ant activity. In the southeastern U.S., I tested whether eggs of different snake species were vulnerable to predation by RIFA at different points in incubation, and whether ecology and life history variables relating to vulnerability to ant predation (i.e., oviparity, geographic range overlap with RIFA, terrestrial/underground nests, etc.) predicted

declining status across snake species. I found no support for leaf litter depth, nest microhabitat or forest type influencing ant predation in Costa Rica, but the results indicated that lizard and snake eggs were vulnerable to ant predation. Additionally, I found evidence that RIFA invasion is a major driver of snake population declines in the southeastern U.S. Although the mechanisms tested in this study remain uncertain, this study provides a baseline for future studies of ant predation on reptile eggs and highlights the need for additional studies on squamate reproduction and food web interactions.

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List of Published Papers

Chapter 1

Swartwout, M. C. and J. D. Willson. In review. Leaf litter depth does not influence survival of neotropical lizard eggs. *Tropical Ecology*.

Chapter 2

Swartwout, M. C. and J. D. Willson. In review. Factors influencing tropical lizard reproduction vary by microhabitat but not forest type. *Journal of Tropical Ecology*.

Chapter 3

Swartwout, M. C. and J. D. Willson. In review. Southeastern U.S. snake species are vulnerable to egg predation by red imported fire ants (*Solenopsis invicta*). *Herpetologica*.

Introduction

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Reptile populations are declining worldwide, and many of these declines are enigmatic, meaning that there is no known cause. According to current IUCN data, approximately 18% of all assessed reptile species are threatened, endangered or extinct in the wild, and 14% remain data deficient (IUCN 2021). The global reptile declines are concerning because without knowledge about population status or causes of decline, it is difficult to prioritize conservation or management actions. Although conclusive causal relationships are lacking for mechanisms driving enigmatic reptile declines, the primary factors identified as likely contributors are habitat loss, overharvesting, pollution, climate change, invasive species, and disease (Todd et al. 2010). Such factors can also have indirect effects on reptile populations by altering predator-prey relationships and food web interactions (e.g., Zhang et al. 2017).

Human-induced changes to climate and habitat can alter the outcome and intensity of interspecific interactions in food webs (Gilman et al. 2010, Valiente-Banuet et al. 2014). Food web interactions can be strong drivers of reptile population dynamics. Declines of tropical anole lizards are correlated with declines of invertebrate prey (Lister and Garcia 2018), and population dynamics of the Costa Rican leaf litter anole (*Anolis humilis*) are strongly influenced by prey availability (Guyer 1988). Invertebrates have important food web interactions with reptiles as predators, prey, or competitors (i.e., Spiller and Schoener 1990). Spiders are likely to be important predators of small lizards in the Neotropics (Reyes-Olivares et al. 2020), and ants are reported to depredate adults, hatchlings, and eggs of reptile species (Montgomery 1996, Allen et al. 2004, Thawley and Langkilde 2016). Invertebrate predators may be an often overlooked but important source of mortality for reptiles (Nordberg et al. 2018).

Many reptile species experiencing declines are oviparous. Eggs are a relatively vulnerable life stage, and in some short-lived species, egg survival can be an important driver of population

dynamics (Andrews 1982). Yet, data on nesting and reproduction of reptiles, particularly squamate reptiles (lizards and snakes), remains scarce. Known sources of squamate egg mortality include fungal infection, predation by invertebrates and vertebrates, and desiccation (Lopes Moreira and Barata 2005, Reedy et al. 2013). Ants are major predators of tropical anole lizard eggs in Panama (Andrews 1982). Species from four genera of ants (*Crematogaster*, *Pheidole*, *Solenopsis*, *Wasmannia*) are reported to depredate squamate reptiles or their eggs (i.e., *Crematogaster* in Jamaica [Vogel 1983]; *Pheidole* in Hawaii and Taiwan [Huang 2008, Fisher and Ineich 2012]; *Solenopsis* in Panama and the U.S. [Andrews 1982, Thawley and Langkilde 2016]; and *Wasmannia* in New Caledonia and Galapagos [Jourdan 2001, Williams and Wilson 1988 – in Patterson 1994]). Rates of egg predation may be influenced by environmental factors and changes in that predator-prey relationship could potentially drive reptile declines.

In Panama, increased rainfall resulted in higher predation rates on lizard eggs by *Solenopsis* ants (Chalcraft and Andrews 1999). Nest location or microhabitat also influences egg survival (DeSana et al. 2020). Abundance of small terrestrial lizards is positively correlated with litter depth and microhabitats that contain greater litter depth (Whitfield and Pierce 2005, Whitfield et al. 2014). Mechanisms explaining why litter depth is important to terrestrial lizard populations remain unclear, but one possibility is that litter depth influences egg predation risk. Other possible mechanisms include reduced prey availability, increased predation risk for adults and juveniles, and loss of favorable microclimate in deep leaf litter.

In tropical rainforests, spatial heterogeneity in habitat conditions is very high (Pianka 1966). Species richness is also higher in the tropics compared to temperate regions (Stevens 1989), resulting in complex community dynamics that vary across landscapes. Understanding spatial

scale of community interactions is important for investigating mechanisms behind enigmatic declines of reptiles and amphibians.

In tropical regions, ants are often the most abundant leaf litter invertebrates and many species depredate squamate reptile eggs (e.g., *Wasmannia auropunctata* [Williams and Wilson 1988 – in Patterson 1994], *Solenopsis* [*Diplorhoptrum*] spp. [Andrews 1982]; Lieberman and Dock 1982). Many predatory ant species are also highly successful and damaging invaders that have spread worldwide through international transport of soil (Tsutsui and Suarez 2003). In the U.S., most research on ant predation on reptile eggs has focused on invasive species, to understand the effects they might have on native species as their invasive range continues to expand. I focused on determining the role that variation in ant predation and egg survival might play in enigmatic reptile declines at a tropical site (La Selva Biological Station, Costa Rica) and a temperate one (Southeastern U.S.A.). In the following two sections, I provide background information for each of the study systems.

Anole Lizard Declines at La Selva Biological Station, Costa Rica

Declines over 35 years in a terrestrial lizard community in primary forest at La Selva Biological Station, Costa Rica, were correlated with reduced leaf litter depth and increasing temperature and rainfall (Whitfield et al. 2007). Subsequent experimental manipulations and observational studies provided additional evidence that leaf litter depth affected abundance of the common leaf litter anole, *Anolis humilis* (Whitfield et al. 2014, Whitfield and Pierce 2005). Reductions in leaf litter depth could result in increased vulnerability to predation, reduced availability of prey, and changes to microclimate for leaf litter lizards. Previous studies have investigated reptile thermal limits, finding that lizards occupying forest habitats have lower critical thermal maxima (CT_{max}), although this is likely to be associated with closed canopy as

well as leaf litter (Brusch et al. 2015). However, my studies are the first to test a food web mechanism: whether predation rates on lizard eggs increase with decreasing leaf litter depth.

Tropical anole lizards primarily lay their eggs terrestrially beneath the leaf litter layer (Andrews 1988), so their reproductive success could also be tied to leaf litter depth. Thief ants (*Solenopsis [Diplorhoptrum]* spp.) were the primary predators of anole lizard eggs in Panama (Andrews 1988). I hypothesized that decreased leaf litter depth could increase susceptibility of lizard eggs to ant predation through increased probability of detection by predators. However, ant density and leaf litter depth and structure vary across La Selva Biological Station (McGlynn et al. 2009, Donoso et al. 2010). Additionally, lizard egg survival differs by habitat and microhabitat (Schlaepfer 2003), leading me to hypothesize that lizard egg survival would differ across forest types and microhabitats. I also predicted that lizard egg survival and abundance would be negatively correlated with predatory ant abundance across La Selva, due to increased egg predation in locations that predatory ants are common.

Enigmatic Declines of Southeastern U.S. Snakes

The red imported fire ant (RIFA; *Solenopsis invicta*) is native to Central and South America but has invaded the southeastern United States where it is responsible for an estimated \$600 million per year in environmental damages (Pimentel et al. 2000). RIFA also negatively affect wildlife through predation, competition for prey, behavioral changes and sublethal effects of envenomation (Allen et al. 2004). RIFA depredate eggs, hatchlings, and adult reptiles (Wojcik et al. 2001, Todd et al. 2008), and there are multiple studies about RIFA predation on nests of turtles and tortoises (Moulis 1997, Buhlmann and Coffman 2001, Allen et al. 2001, Dziadzio et al. 2016), lizards (Mount et al. 1981, Newman et al. 2014, Thawley and Langkilde 2016), and crocodilians (Reagan et al. 2000, Parachú Marcó et al. 2015). However, despite apparent

correlations of species declines with RIFA invasion, relatively few studies have observed RIFA predation on snake nests (Conners 1998, Thawley 2014).

Invasion and spread of RIFA is cited as a possible cause for enigmatic reptile declines in the southeastern United States. Local population declines of the common kingsnake (*Lampropeltis getula* complex) at one site in Florida coincided with RIFA invasion (Kauffeld 1957, Bartlett 1997). *Lampropeltis getula* complex has declined across southeastern portions of its range, including protected areas where habitats have been relatively undisturbed by humans (Krysko and Smith 2005, Winne et al. 2007). Other species such as the southern hognose snake (*Heterodon simus*) have also experienced enigmatic declines in the southeastern portion of their range, possibly due to RIFA invasion (Tuberville et al. 2000). Across these same regions, some terrestrial oviparous snakes such as racers (*Coluber constrictor*) and ratsnakes (*Pantherophis* spp.) remain common and have apparently stable populations. Observations of declines in some snake species but not others led us to hypothesize that differences in species reproduction and life history (e.g., nest microhabitat, time between pipping and hatching) could influence snake species vulnerability to RIFA predation and predict declining status. Previous studies of snakes have found that geographic range size, vertebrate prey, aquatic habitat use, slow life history, ambush foraging, and lack of male–male combat correlate with extinction risk or sensitivity to land use change (Webb et al. 2002, Reed and Shine 2002, Böhm et al. 2016, Todd et al. 2017). However, these studies did not investigate range overlap with invasive species or explicitly test reproductive variables that might influence vulnerability to egg predation, such as nest microhabitat and incubation time. To assess contributions of RIFA invasion to snake declines in the southeastern U.S., I conducted a field experiment examining RIFA predation on eggs from

six snake species and reviewed existing snake ecological and life history data in relation to vulnerability to RIFA predation.

Summary

In both the southeastern U.S. and lowland Costa Rica, ants are important egg predators and reptiles have experienced enigmatic declines potentially linked with changes in food web interactions (e.g., Costa Rica – decreased leaf litter depth increasing predation risk, U.S. – introduction of a predatory ant species). Understanding factors influencing egg predation in lizards and snakes and the role of ants as squamate reptile egg predators is an important step towards determining mechanisms behind enigmatic declines. My dissertation provides novel investigations of ant predation on lizard and snake eggs and will inform future research into mechanisms behind enigmatic reptile declines. In Chapter 1, to test a possible food web mechanism (reduced lizard egg predation) linking leaf litter depth to lizard population dynamics, I manipulated leaf litter depth in field plots at La Selva Biological Station, Costa Rica, and monitored survival of experimentally placed lizard (*Anolis humilis*) eggs, ant abundance, and predatory ant activity. In Chapter 2, I examined variation in predation on lizard eggs, predatory ant activity, ant abundance, prey availability and the number of lizards and lizard eggs encountered across four different forest types (abandoned agroforestry, abandoned plantation, secondary forest, primary forest) and three microhabitats (buttress, fallen log, leaf litter) in lowland tropical rainforest at La Selva Biological Station. In Chapter 3, I used short term (12 hour) field trials early in incubation to evaluate whether RIFA could successfully depredate intact eggs of six species of terrestrial oviparous snakes native to the southeastern U.S. and an artificial nest field experiment at the end of incubation to test whether RIFA predation differed between a species that has declined and one that remains common. In Chapter 4, I tested whether

ecological and life history variables relating to RIFA invasion were correlated with southeastern snake declines. Specifically, in the chapters listed, I set out to answer the following questions about reptile-invertebrate interactions, in order:

- 1) Does reduced leaf litter depth affect lizard egg survival through changes in ant predation rates?
- 2) Do lizard egg survival and predatory ant activity differ across forest types and microhabitats in lowland tropical rainforest?
- 3) Do red imported fire ants (RIFA) depredate eggs of native snake species and do species differ in vulnerability to egg predation?
- 4) What ecological and life history variables correlate with snake population declines in the southeastern U.S. and do those variables relate to RIFA invasion?

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Chapter 1:

Leaf litter depth does not influence survival of lizard eggs at La Selva Biological Station

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Abstract

Accumulation of leaf litter and detritus on the forest floor is important for the detrital food web but may be affected by human-induced changes to climate and habitat. Long-term declines in leaf litter depth have been correlated with terrestrial lizard and frog population declines in the neotropics, but mechanisms driving this relationship remain uncertain. Ants are important predators of lizard eggs and changes in that predator-prey relationship could potentially drive lizard population declines. To test a possible food web mechanism (reduced lizard egg predation) to explain why deep litter is important for terrestrial lizard populations, we manipulated leaf litter depth in field plots at La Selva Biological Station, Costa Rica, and monitored survival of lizard (*Anolis humilis*) eggs, ant abundance, and predatory ant activity. Leaf litter depth did not affect lizard egg survival. However, predatory ant activity was positively correlated with egg predation, providing evidence that ants are important predators of lizard eggs. We also suggest that mass loss from protein-based bait stations could be used to rapidly assess probability of ant predation on lizard eggs. We recommend more studies on interacting food web and climate effects to determine what specific mechanisms are driving tropical lizard population declines.

Introduction

Human-induced changes in plant communities, temperature, rainfall, and storm frequency and intensity may alter the amount of leaf-fall and the litter decomposition rate on forest floors, affecting the standing leaf litter layer that forms the base of detrital food webs (Vitousek 1994, Aerts 1997, Vitousek et al. 1997). Small lizards, frogs and salamanders make up a significant amount of biomass in temperate and tropical forests (i.e., Burton and Likens 1975, Bullock and Evans 1990) and are important for leaf litter dynamics because they are highly efficient at accumulating biomass, provide nutrients through their waste, and are intermediate predators that may regulate litter decomposition by consuming litter invertebrates (e.g., Wyman 1998, Sin et al. 2008, Hocking and Babbitt 2014). Long-term reductions in litter depth were strongly correlated with declines in terrestrial lizard and frog populations at La Selva Biological Station, Costa Rica (Whitfield et al. 2007). Furthermore, abundances of small terrestrial lizards were positively correlated with litter depth when manipulated experimentally and were higher in microhabitats with deep leaf litter (Whitfield and Pierce 2005, Whitfield et al. 2014). However, mechanisms explaining why litter depth is important to terrestrial lizard populations remain unclear.

Mechanisms such as lower prey availability, increased predation, and lack of suitable microclimate could explain how reduced litter depth can drive terrestrial lizard declines. Recent studies on lizards at La Selva primarily have focused on how litter depth might be influencing microclimate and thermoregulation of adults (e.g., Brusch et al. 2016), overlooking potential food web effects and mechanisms involving lizard reproductive success. Egg survival is important for population dynamics of short-lived lizard species such as tropical anoles (Andrews 1988) and because they lay eggs terrestrially their reproductive success may be tied to leaf litter depth. In a previous study surveying for *Anolis* eggs, it was estimated that 99.5% of eggs are laid

on the ground and all but one of the eggs found terrestrially were located on the soil surface beneath the litter (Andrews 1988). Additionally, female anoles in a nesting choice experiment selected for areas with both soil and leaf cover over bare soil or just leaf litter (Socci et al. 2005). Because most oviparous reptile species lay their eggs terrestrially, leaf litter may be an important nesting microhabitat that provides shelter from predation and favorable incubation conditions.

All food webs are made up of a network of interspecific interactions, the outcome and intensity of which can be affected by changes in habitat and climate (Gilman et al. 2010, Valiente-Banuet et al. 2014). Changes in rainfall and soil moisture resulted in increased vulnerability of lizard eggs to predation by ants at Barro Colorado Island in Panama (Andrews 1988, Chalcraft and Andrews 1999). Ant predation on lizard eggs has been reported for several lizard species and may have significant effects on short-lived species where egg survival is an important driver of population dynamics (Andrews 1988). Species from four genera of ants found or introduced in the Neotropics (*Crematogaster*, *Pheidole*, *Solenopsis*, *Wasmannia*) have been reported to depredate tropical lizards or their eggs (i.e., *Crematogaster* in Jamaica [Vogel 1983]; *Pheidole* in Hawaii and Taiwan [Fisher and Ineich 2012, Huang 2008]; *Solenopsis* in Panama [Andrews 1982]; and *Wasmannia* in New Caledonia and Galapagos [Jourdan 2001, Williams and Wilson 1988 – in Patterson 1994]). Ants are often the most abundant macro-invertebrates in tropical leaf litter and their abundance is positively correlated with leaf litter depth (Lieberman and Dock 1982). Therefore they are likely to be the most common lizard egg predators in tropical leaf litter, though deeper and more complex litter may protect lizard eggs by increasing the surface area ants have to traverse to encounter eggs.

Herein we test a potential food web mechanism, egg predation, linking litter depth to lizard abundance and provide novel data on egg survival of a common tropical lizard (*Anolis*

humilis Peters 1863). Specifically, the objectives of this study were to determine (1) whether leaf litter depth affects lizard egg mortality; (2) how much lizard egg mortality is due to predation; and (3) whether litter depth affects ant abundance and predatory ant activity. To meet these objectives, we monitored lizard egg survival in experimentally manipulated leaf litter field plots at La Selva Biological Station. We hypothesized that (1) egg survival would be highest in deep leaf litter because of favorable microhabitat conditions and reduced probability of ants encountering lizard eggs (measurable as reduced predatory ant activity); (2) predatory ant activity would be positively correlated with predation on lizard eggs because ants are the most abundant egg predators in tropical leaf litter; and (3) ant abundance would be highest in deep leaf litter because ant abundance is positively correlated with litter depth.

Methods

Study Site

The study was conducted at La Selva Biological Station, a 15 km² biological reserve in the lowland wet forest of Sarapiquí, Costa Rica (10°25'54.192" N, 84°0'25.4052" W; datum = WGS84; 35–137 m elevation above sea level). The site was located on alluvial soil along the Puerto Viejo River, on a tract of abandoned agroforestry acquired by the station in 1968. *Hura crepitans* L., *Virola* spp., *Euterpes precatoria* Mart. (Mart.) A. J. Hend, and *Geonoma cuneata* H. Wendl. ex spruce trees were common at the site. *Piper* and palm species were prevalent in the understory. Average litter depth measured by setting a ruler on the ground and recording the distance to top of the litter for the site prior to litter manipulation (on 7 April 2017) was 6.20 ± 3.11 SD mm, based on 270 measurements. The study was conducted from 3 April to 4 July 2017, during the transition from dry to wet season. Rainfall and air temperature increases during the wet season, resulting in higher litter decomposition rates (Cusack et al. 2009, Wieder et al.

2009). Air temperatures recorded by La Selva's weather station every 15 minutes during the study period ranged between 19.2 and 41.9 °C (mean = 26.4 ± 0.04 °C). The average daily rainfall was 11.07 ± 2.182 mm, and the maximum daily rainfall was 85.85 mm.

Experimental Plots

We used $30 - 2 \times 2$ m plots, spaced at least 2 m apart from each plot edge, and manipulated litter in three randomly assigned treatments ($N = 10$ per treatment): litter removal (L-); litter addition (L+); and control (L0). On 10 April 2017, we removed litter from L- plots and placed it in L+ plots, and disturbed litter in control plots (L0) as if removing it, following the methods of Whitfield et al. (2014). Then we left the plots for three weeks to allow ants to respond to litter manipulations, and renewed litter manipulations every four weeks to maintain treatments. Our manipulations were less frequent than in a previous litter manipulation experiment (every two weeks; Whitfield et al. 2014), but we were concerned about disturbing plots too frequently while eggs were incubating within them. When renewing litter manipulations, we froze litter from L- at -20 °C for a minimum of five hours to limit translocation of ant colonies into L+ plots and then returned the litter to L+ plots no more than two days after collection. To characterize each plot, we recorded the following variables before litter manipulations and at the end of the experiment: average litter depth ($N = 9$ measurements taken in the approximate center of nine 0.67-m^2 quadrants of the plot), average number of leaf litter layers (number of leaves pierced by a wire; $N = 5$ measurements from corners and center of each plot), microhabitat availability (visually estimated percent ground cover of tree buttress, palm, leaf litter, bare ground, or fallen log), canopy cover using a spherical densiometer (Lemmon 1956; Spherical Crown Densiometer, Concave Model C, Forestry Suppliers), and understory stem density (number of stems at ground level in the plot). We measured relative soil

moisture by using a hand feel and soil appearance method from a subsurface soil sample, with four possible categories (wet, moist, slightly moist, and dry), and accounting for high clay content and medium to fine texture of alluvial soils in the plots (Al-Kaisi 2000). We acknowledge that this was a qualitative and relative measure of soil moisture but believe our broad categories of soil moisture adequately captured general conditions (i.e., whether eggs were in wet or dry soil). To estimate temperatures experienced by eggs in different treatments, we recorded temperature every 30 min from 23 April to 18 May using HOBO Dataloggers (HOBO 64K Pendant[®] Temperature/Alarm Waterproof Data Logger Part # UA-001-64) set on the ground at a random location in each plot.

Lizard Collection and Maintenance

We used *Anolis humilis* as a study organism because it is the most abundant lizard species at La Selva, with historical densities of at least 200 ha⁻¹ and has experienced declines associated with changes in litter depth (Whitfield et al. 2007, 2014). To obtain eggs for experimentation, we collected 34 gravid female *A. humilis* and held them in captivity until they deposited eggs, yielding 34 viable eggs. We housed gravid females at ambient temperatures in secure well-ventilated containers (61 × 91 × 91 cm) containing leaf litter, a perch, and a soil-filled nesting box. We fed females 3–4 field-collected invertebrates no larger than maximum anole head dimensions every other day and provided water *ad libitum*. We maintained females in captivity for no more than 45 days, and then released them at their capture location.

Beginning three weeks after litter manipulations (18 April 2017), we placed lizard eggs under the litter in each plot, mimicking known *Anolis* nest placement (Sexton et al. 1964, pers. obs.). We kept eggs in nylon mesh bags with 5 mm mesh size that allowed ants and other small invertebrates to enter but excluded most larger predators. We put eggs in the field plots within

three days of oviposition and in a randomized order, until all 30 plots had an egg. Then we repeated with a newly randomized plot order for the remaining eggs. However, one of the plots was stocked with an infertile egg that was not included in the final data analysis, such that five plots contained more than one egg and only 29 plots had eggs (34 total eggs). We checked each egg's status every other day, and then every day after 30 d, until either hatching or mortality occurred. Eggs were identified and recorded as “hatched” if they had smooth apical slits, “depredated” if they had irregular holes indicative of invertebrate predation or disappeared entirely (presumed vertebrate predation), or “dead” if they molded, hardened, and never developed.

Ant Surveys

Bait stations can be used to attract ants of certain groups using either proteinaceous or carbohydrate-rich baits (i.e., Hahn and Wheeler 2002). To measure predatory ant activity, we used bait stations with a protein-based food source. Bait stations were constructed out of 5-cm³ Ziploc® storage containers with 12 holes drilled around the bottom through which ants could enter. Holes were small (ca. 4 mm diameter) so larger invertebrate and vertebrate predators were excluded. Following completion of egg experiments (29 June 2017), we set out one bait station containing 2.5 g of tuna (made by mixing a drained can of Bumble Bee® Very Low Sodium Solid White Albacore in Water with ~60 mL extra virgin olive oil) in each plot for 12 h, recorded the remaining bait mass, and calculated the mass lost as a proxy for predatory ant activity. Based on preliminary trials, bait mass loss due to water loss in the field (no bait removal by ants) was less than the standard deviation of average bait mass loss with ants – thus, we use change in bait wet mass as a measure of predatory ant activity. We acknowledge that our measure of predatory ant activity may also be influenced by distances to ant nests and ant nest

densities but based on results of preliminary trials, we believe that 12 h was enough time for predatory ants in and around the plots to locate and recruit to the baits without giving enough time for the bait to be completely removed.

To assess terrestrial ant abundance, we used pitfall traps, sticky traps and Berlese litter samples in the field plots. For pitfall traps, we cut four 5×1.4 cm rectangular openings that were 2 cm apart along the rim of a 16-oz. collecting cup to limit access of larger organisms to the collection cup. We added enough soapy water to cover the bottom of the collection cup and floated a small (~ 25 cm²) piece of sponge on the liquid to prevent accidental vertebrate mortality. We then placed this cup, covered with a tight-fitting lid, inside a buried 16-oz. cup for a design that allowed easy removal of invertebrate samples and some protection from rainfall (similar design to the Nordlander trap described in Higgins and Lindgren 2012, but with removable inner cup). Prior to setting out eggs in the plots, we set one pitfall trap in the center of each plot with the openings flush to the ground level. We left the pitfall traps in the plots closed so that nothing could get in for at least 8 d to limit “digging-in” effects (high captures of ants immediately after placing the trap; Greenslade 1973), and then we opened the traps on 31 May, when 27 plots contained eggs. We collected pitfall traps 48 hours later and sorted and preserved all invertebrates in 70 percent ethanol. About four weeks later, on 15 June 2017, we set out one 158-cm² sticky insect trap (Trapper Monitor & Insect Glue Trap, TM2600, Bell Laboratories) per plot for 24 h. We placed sticky traps on the ground at a random location in each plot and processed them immediately after collection by photographing the traps and counting and identifying to genus all ants captured. Finally, due to the intrusive nature of the sampling process, we waited until the end of the experiment (20 June 2017; five eggs in plots) to take a 0.25-m² leaf litter sample from a random location in each plot. We used a litter sifter to concentrate the

invertebrates and reduce the amount of large detritus in the sample. Due to constraints in number of Berlese funnels, we collected ten litter samples in three randomized blocks of ten plots every four days (30 plots total, over 12 days). We processed samples under a 25-W bulb for 48 hours to collect ants and other arthropods in 70 percent ethanol. All ants collected from bait stations, pitfall traps and Berlese funnel samples were identified to genus and counted.

Data Analyses

We used a Type II One-way ANOVA and Tukey HSD post-hoc test of leaf litter depth to confirm that litter manipulation treatments differed significantly. To conform to assumptions of normality, we square-root transformed litter depth data before running it in the model. We checked assumptions of the ANOVA model using a Shapiro-Wilk test of the residuals and Levene's Homogeneity of Variance test (Levene 1960, Shapiro and Wilk 1965). We plotted other environmental measurements (canopy cover, number of litter layers, etc.) using Non-metric Multidimensional Scaling (NMDS) with a Bray-Curtis distance metric on two axes to visualize differences by litter treatment (Oksanen 2007). We incorporated relative soil moisture in the NMDS by exchanging qualitative metrics ("wet", "moist", "slightly moist", "dry") with a numbering system (1–4), where 4 represented drier soils. To determine whether plot temperatures differed by treatment, we used a linear mixed effect model with treatment as the fixed effect and time of day as a random effect. We also calculated daily temperature variability (maximum – minimum temperature) and tested whether it differed by treatment, with day as a random effect. When a significant effect of treatment on temperature was detected, we used a Tukey HSD test to determine which treatments significantly differed.

To determine whether probability of predation was influenced by litter treatment, we used a binomial logistic regression model and ran a Type II Wald chisquare post-hoc test. We

included plot as a random effect to account for non-independence of five cases where two eggs were placed within the same plot but chose to use egg as the statistical unit to increase sample size and robustness of our survival estimates. We also ran investigatory analyses where one of the two eggs in those five plots was removed at random and saw no difference in results. Two eggs that died during the experiment for unknown reasons (likely desiccation or fungal disease) were excluded from the binomial logistic regression model since our primary focus was probability of predation, leaving a sample size of 32 eggs.

To confirm that lizard egg survival probability was not affected by litter treatment, we used a survival analysis. The advantage of a survival analysis over the binomial logistic regression model is that survival analysis compares predation rates over time and mortality timing in addition to the final frequencies of predation. Time to hatching or mortality was known ± 1 d because eggs were checked every other day. Eggs that hatched during the experiment were treated as censored observations, since the time of death was unknown (Davis and McCaffrey 1986). Survival estimates were based on censored and uncensored (cases where mortality occurred) observations. Using the ‘survfit’ function from the “survival” package in R (Therneau and Grambsch 2000, Therneau 2015), we calculated survival curves for eggs in each treatment and compared the slopes using a log rank test. We also compared hazard ratios, representing the relative risk of death, between treatments using a Cox proportional hazards model.

With egg as the statistical unit and plot as a random effect, we used a binomial logistic regression model to test whether the probability of egg predation was related to mass loss from protein-based bait stations. To test whether bait mass loss differed by treatment, we square-root transformed bait mass loss to conform to normality assumptions and then used a Type II One-way ANOVA. We also used a linear regression model to test whether bait mass loss was

correlated with leaf litter depth. We checked model assumptions using visualizations of residual plots, Shapiro-Wilk tests of residuals for normality, and Levene's Homogeneity of Variance test.

To assess differences in ant density and abundance among treatments, we used a one-way MANOVA with litter treatment as the independent variable, after square-root transforming sticky and pitfall trap ant abundances and Berlese funnel ant densities to conform with normality assumptions. Using univariate ANOVAs of each measurement of ant abundance did not change the outcome. We used multivariate regression to compare ant density and abundance with bait mass loss. We checked model assumptions using the `mvn` function in the “mvn” package to check multivariate normality and plot Chi-square quantiles by Mahalanobis distance, Shapiro-Wilk tests of residuals for univariate normality, and plotted residuals for homogeneity of variance. Analyses were performed in R v.3.5.1 (R Core Team 2018) using the packages “car”, “vegan”, “mvn”, “lme4”, “multcomp” and “survival” (Therneau and Grambsch 2000, Hothorn et al. 2008, Fox and Weisberg 2011, Korkmaz et al. 2014, Bates et al. 2015, Therneau 2015, Oksanen et al. 2019).

Results

Environmental Characteristics

At the end of the experiment, leaf litter was significantly deeper in L+ and L0 plots than L- (One-way ANOVA: $F_{2,27} = 11.63$, $P < 0.01$), but L+ did not differ from the control ($P = 0.78$; Fig. 1). For other environmental measurements, points were oriented along two MDS axes: MDS1 primarily reflected number of litter layers, leaf litter cover, prop roots, and bare ground cover, while MDS2 reflected presence of buttresses, vines, fallen logs and understory stem density. Canopy cover and presence of palms were not well associated with either MDS. Litter control (L0) and L+ plots were not well-separated along MDS1, with both containing more litter

layers, leaf litter cover, prop roots, and fallen logs, while plots in litter removal treatments (L-) had relatively more bare ground and vines. Treatments were not separated along the MDS2 axis, indicating that treatment was not confounded with other habitat variables (Fig. 2). NMDS stress was 0.04, less than the preferred maximum of 0.1. Temperatures were significantly lower in L- plots (Linear Mixed Effect Model: $df = 2$, $\chi^2 = 54.67$, $P \ll 0.001$) and daily temperature variability was significantly higher in L- plots than other treatments ($df = 2$, $\chi^2 = 121.53$, $P \ll 0.001$). L+ and L0 plots did not significantly differ in plot temperature or daily temperature variability ($P > 0.05$).

Egg Predation

Of all lizard eggs in the experiment, 47 percent hatched successfully, 47 percent were depredated, and 6 percent died of unknown causes (most likely fungal infection or desiccation). Eggshells left over from invertebrate predation had one or more irregular holes in the exterior and sometimes dirt piled up around the egg, consistent with previous descriptions of ant predation on lizard eggs (Andrews 1982). A greater proportion of eggs were depredated in L0 plots than other treatments, and a higher proportion of eggs successfully hatched in L+ plots, while the only two “dead” eggs were in L- plots (Fig. 3). However, there were no significant differences by treatment and the null hypothesis that litter treatment and egg fate are independent was accepted (Type II Wald Chisquare Test: $df = 2$, $\chi^2 = 0.58$, $P = 0.75$). Likewise, based on the log-rank test of survival curves and Cox proportional hazards model, there was no significant difference in egg survival or relative risk of death between litter treatments ($df = 2$, $\chi^2 = 2.2$, $P > 0.3$). The survival curve for eggs in L0 treatments averaged the lowest of the treatments throughout incubation (Fig. 4).

The probability of lizard eggs being depredated in the experimental plots was correlated with bait mass loss (Binomial Logistic Regression: McFadden's $R^2 = 0.11$, $df = 31$, $z = 1.96$, $P = 0.05$; Fig. 5). Bait mass loss did not differ by treatment (One-way ANOVA: $F_{2,27} = 0.44$, $P = 0.65$), and was only weakly related to final leaf litter depth (Linear Regression: Adjusted $R^2 = 0.09$, $F_{1,28} = 3.72$, $P = 0.06$).

Ant Abundance

Ant abundance and density patterns varied across treatment by method. For Berlese samples, ant densities were highest in L+ plots (mean = 58.1 ± 13.2 SE ants/0.25-m²) and lowest in L- plots (14.9 ± 10.3 SE ants/0.25-m²). However, for all methods, ant abundance and density did not significantly differ by litter treatment (Fig. 6; MANOVA: $F_{2,26} = 1.00$, $P = 0.44$). All measurements of ant abundance and density were also unrelated to bait mass loss (Multivariate Regression: $F_{1,27} = 1.18$, $P = 0.34$). Focusing on ant genera that have been reported to depredate on lizards or their eggs (*Crematogaster*, *Pheidole*, *Solenopsis*, *Wasmannia*) yielded similar results to analyses conducted with total numbers of ants.

Discussion

We made three hypotheses at the start of the experiment: (1) egg survival would be highest in deep leaf litter; (2) predatory ant activity would be positively correlated with predation on lizard eggs; and (3) ant abundance would be highest in deep leaf litter. We found that predatory ant activity (measured by bait mass loss) was positively correlated with predation on lizard eggs, supporting hypothesis 2, and providing indirect evidence that ants are important predators of lizard eggs. However, lizard egg survival, predatory ant activity and ant abundance did not differ with litter depth (hypotheses 1 and 3). Even the survival analysis, which offered a more powerful analytical approach given our relatively small sample size of lizard eggs, failed to

detect any effect of litter treatment on survival rate or timing of mortality. Overall, our results suggest that ant predation is an important source of lizard egg mortality, but litter depth does not affect ant predation on lizard eggs within the timescale and habitats evaluated in this study.

Although lizard egg survival did not differ by litter depth in our study, patterns of egg survival and predation by invertebrates were similar to previous studies. Lizard egg survival in this study was 47 percent, comparable to 40 percent survival of *Anolis apletophallus* Köhler and Sunyer 2008 eggs in Panama (Andrews 1988). In a previous study, 69 percent of lizard egg mortality was attributed to predation by *Solenopsis* ants (Andrews 1988). In this study, 50 percent of lizard egg mortality could be attributed to invertebrate predators based on the presence of irregular holes in the eggshell. Because eggshell remains were consistent with previous descriptions of ant predation (Andrews 1982) and egg predation was significantly correlated with predatory ant activity, we believe that ants were the most likely source of invertebrate predation in this study. Future studies on tropical lizard eggs should continue to examine the relative importance of ants as egg predators.

Our finding that egg predation was correlated with bait mass loss suggests that predatory ant activity could predict egg predation risk. We acknowledge that our R^2 value of 0.11 was relatively low, suggesting there might be additional factors that affect egg predation. However, bait stations do attract many species of predatory or scavenging ants and because the protein-based bait station methodology used herein can be easily replicated without needing complicated equipment, this method could be useful for estimating lizard egg vulnerability to ant predation at broad spatial and temporal scales. However, using mass loss of protein-based bait stations does have a few drawbacks. In areas with lower humidity, we suggest taking moisture loss into account by determining an average dry mass of baits and calculating moisture loss of baits from

the field. Bait stations also provide only a snapshot of predatory ant activity, since ant activity and communities can differ over time. Additionally, some large predatory ants are excluded (i.e., *Paraponera*) and not all ants recruiting to the proteinaceous bait might be predators of lizard eggs, although the majority are at least generalist foragers or opportunistic predators. Frequent bait station visitors in this study included *Solenopsis* [subgenus *Diplorhoptrum*] (depredate *A. apletophallus* eggs in Panama; Chalcraft and Andrews 1999), *Solenopsis geminata* Fabricius 1804 (depredated *A. humilis* egg in preliminary lab trial), *Paratrechina* sp. (generalists), *Ectatomma* sp. (mostly generalist predators; Brown 1958), and *Pheidole* sp. (highly diverse genus with at least one species reported to depredate lizard eggs; Huang 2008). We hope to hone this technique for other habitats and ecosystems in future studies.

We observed no difference in ant abundance by litter depth, but patterns in ant abundance differed by sampling method. Sticky and pitfall traps are passive capture techniques that obtain whatever invertebrates happen to be moving through that surface (primarily terrestrial invertebrates for pitfalls; terrestrial, flying, and arboreal invertebrates for sticky traps). Berlese samples provide a more complete census of leaf litter arthropods and have both active and passive elements – the method involves actively collecting everything within a 0.25-m² square area at the time of sampling, and then passively encouraging invertebrates to move away from a light source into a vial of alcohol. Differences in patterns of ant abundance by sampling method suggest that although ant densities are greater on average in deeper litter, overall ant activity is not influenced by litter depth. As litter increases in depth, it also increases in complexity, so if ant densities were low and equal across litter treatments, deeper litter could enhance lizard egg survival by increasing the surface area for ants to traverse. However, if *Anolis* eggs are

frequently laid on the soil surface below the leaf litter, as current findings suggest (Sexton 1964), their distribution may be predictable regardless of litter depth and complexity.

Biotic interactions (i.e. ant predation on lizard eggs) are important for egg survival, but how the environment mediates these interactions is still unclear. Because leaf litter depth did not affect lizard egg mortality or ant abundance in our study, alternative mechanisms might explain correlation of reduced litter depth with lizard declines at La Selva. Reduced litter depth could also negatively affect lizard populations through lower prey availability, less refugia, more predators, or less favorable microclimate. Leaf litter buffers temperature, which could make litter depth important for species that have lower thermal optima (Uetz 1979, Huey et al. 2009). In our study, temperatures were significantly lower in L- plots than other treatments, L- plots experienced more daily temperature variability, and maximum temperature was highest on average in L- plots by up to 1.7 °C. Additionally, at least twice as many L- plots were rated as having “wet” relative soil moisture than L+ or L0 and drier soils were more associated with L+ and L0 plots in the NMDS. The observed trend toward more dead eggs in L- plots may have reflected suboptimal thermal and hydric conditions. Another possible explanation is that leaf litter promotes the formation of a beneficial microbiome on the eggshell, protecting the eggs from fungal infections.

The spatial and temporal scale of our experiment (i.e., plot size, study duration) may limit our inferences. Experimental plots may have been too small or the study too short to allow ant populations to respond to litter depth manipulations. Our study was only conducted for one dry to wet season transition in one habitat type at La Selva, and invertebrate responses to litter depth manipulations may vary by season and habitat.

Conclusion

Changes in food web interactions may have important effects on leaf litter reptile and amphibian populations. A mechanistic understanding of how habitat change, leaf litter depth, and food web interactions affect tropical reptiles is challenging due to the complexity of the system, but necessary for conservation of cryptic litter-dwelling species. New studies are reporting declines of neotropical lizard populations (Whitfield et al. 2007, Stapley et al. 2015, Lister and Garcia 2018), but more research is needed to determine the specific mechanisms responsible for these declines and how serious or widespread they are. Achieving this will require multiple studies and approaches. Our study provides some evidence against one mechanism (litter-depth-driven changes in egg survival) for tropical lizard declines but there are many more potential mechanisms to be tested and extrapolating our results to other tropical areas is not possible without landscape-scale replication.

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Figures

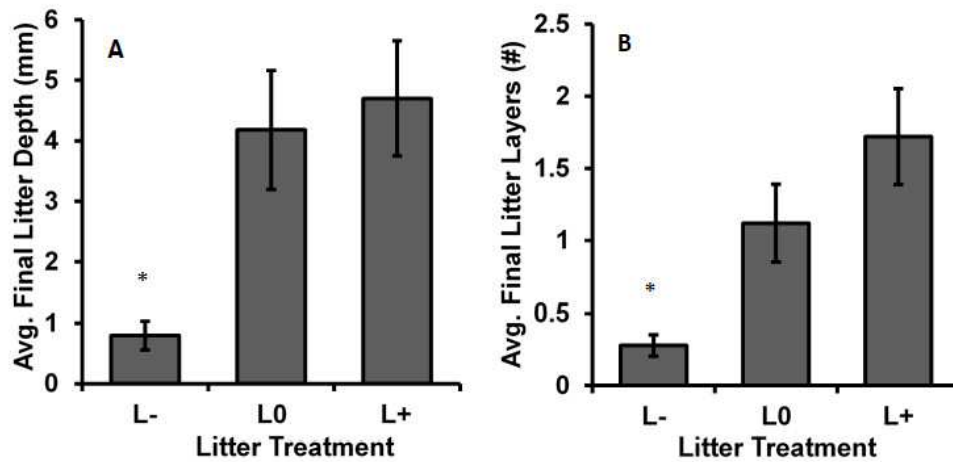


Figure 1. Leaf litter depth at the end of the experiment (A) in mm and (B) number of layers from litter removal (L-), control (L0), and litter addition (L+) plots. * = significantly different from other treatments ($P < 0.05$). Bars represent ± 1 SE.

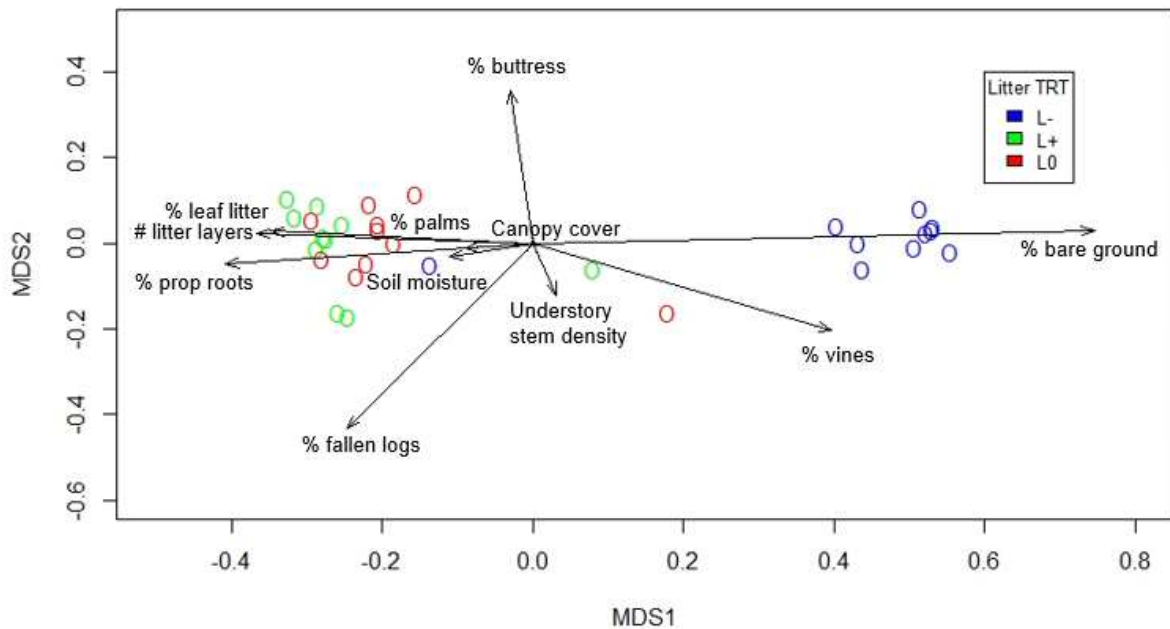


Figure 2. Non-metric Multidimensional Scaling (NMDS) biplot along 2 axes, where arrows represent loading by individual environmental characteristics. Each point represents a plot, and plots are coded with different symbols by litter treatment. L- = litter removal; L+ = litter addition; L0 = control.

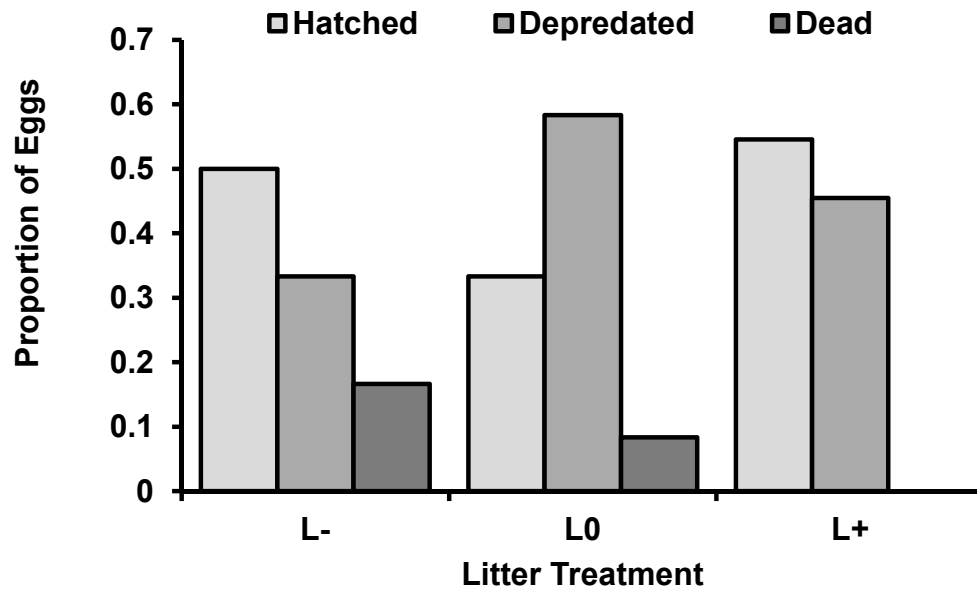


Figure 3. Proportions of lizard eggs hatched, depredated, or dead across litter treatments. Probability of lizard egg survival was not statistically different by treatment. L- = litter removal; L0 = control; L+ = litter addition.

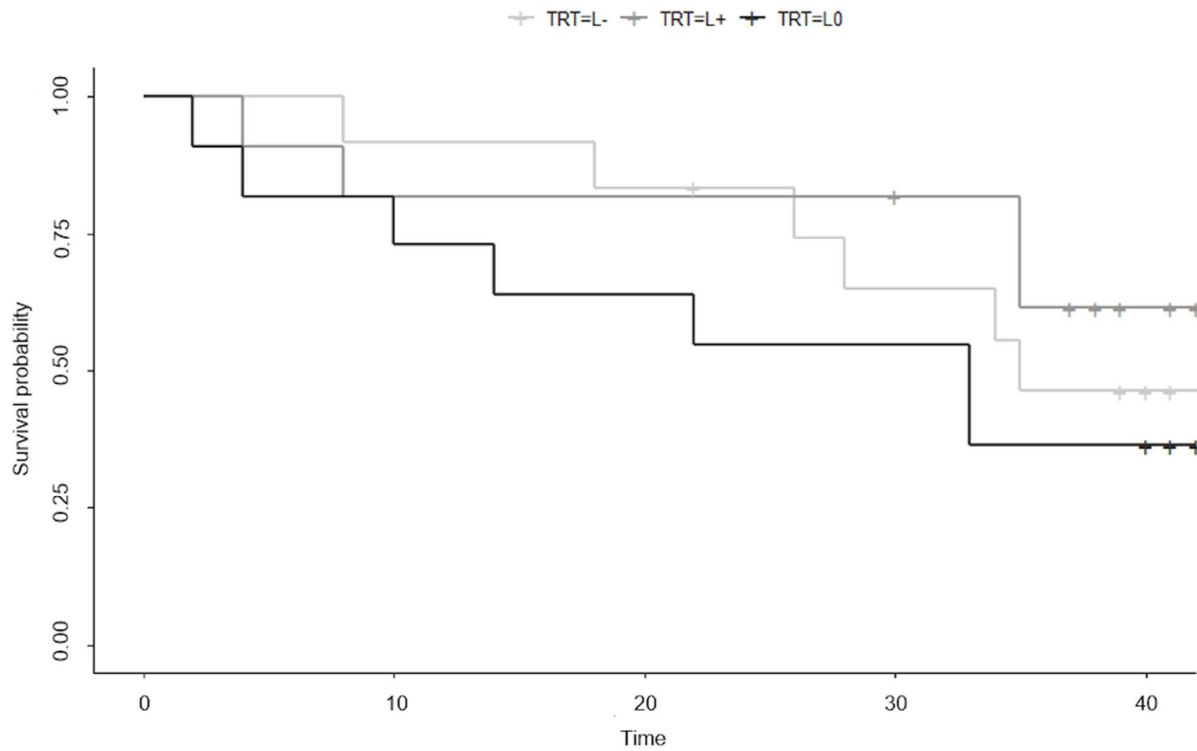
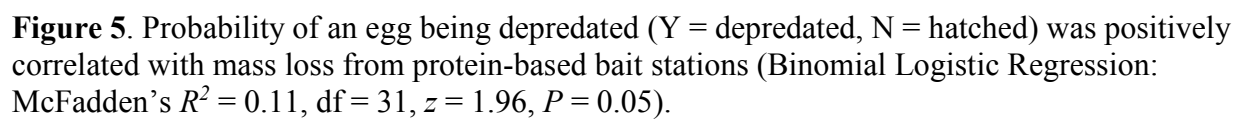


Figure 4. Survival curves for lizard eggs exposed to different leaf litter treatments did not statistically differ (Log-rank Test: $df = 2$, $\chi^2 = 2.2$, $P = 0.3$). Plus (+) symbols represent censored data from hatched eggs. L- (light grey) = litter removal; L0 (black) = control; L+ (grey) = litter addition.



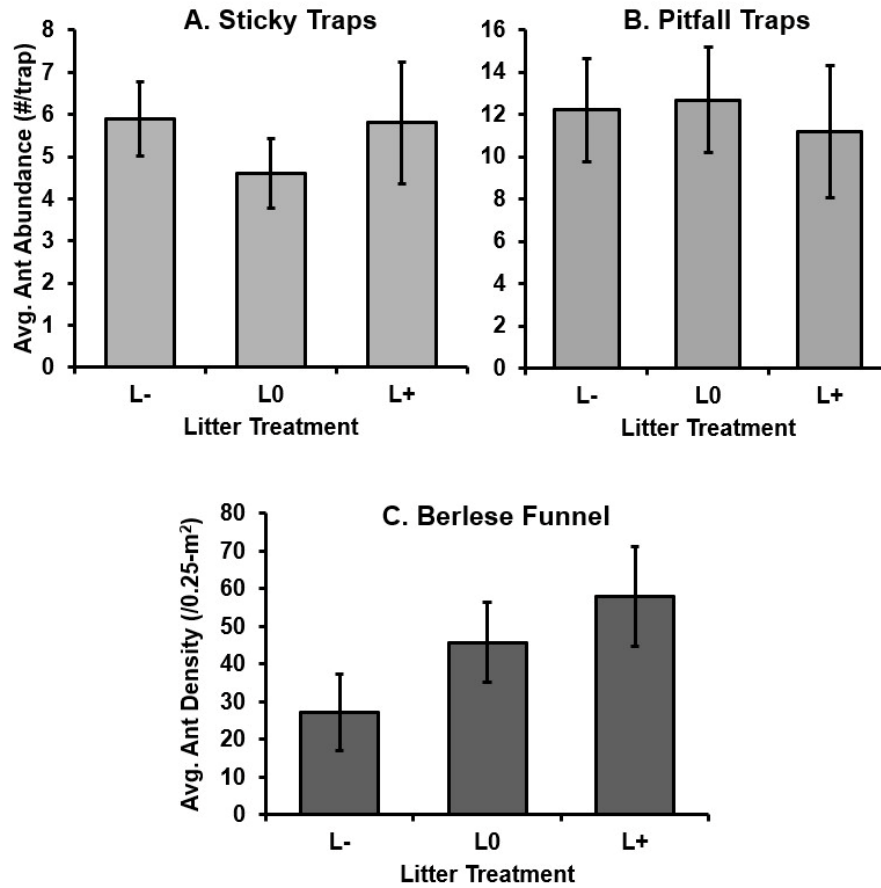


Figure 6. Ant density and abundance across litter treatments by sampling method: (A) sticky traps, (B) pitfall traps, and (C) Berlese samples. Ant density and abundance did not significantly differ by litter treatment ($P > 0.1$ for all). L- = litter removal; L0 = control; L+ = litter addition. Error bars represent ± 1 SE.

Appendix 1. IACUC Approval documents.



UNIVERSITY OF
ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: John David Willson
FROM: Craig N. Coon, Chairman
DATE: April 8, 2016
SUBJECT: IACUC Approval
Expiration Date: April 7, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 16058 "Disentangling mechanisms of tropical leaf litter lizard declines "

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond April 7, 2019 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

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Chapter 2:

Factors influencing tropical lizard reproduction vary by microhabitat but not forest type

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Abstract

Compared to temperate regions, species richness is higher in the tropics, resulting in complex community dynamics that vary across landscapes. Understanding spatial scale of community interactions is important for investigating mechanisms behind enigmatic declines of tropical reptiles and amphibians. In this study we examined variation in predation on lizard eggs, predatory ant activity, ant abundance, lizard prey availability and the number of lizards and lizard eggs encountered across four different forest types (abandoned agroforestry, abandoned plantation, secondary forest, primary forest) and three microhabitats (buttress, fallen log, leaf litter) in a lowland tropical rainforest in La Selva Biological Station. We found that none of the variables that were investigated varied by forest type, but leaf litter was deeper and we observed more lizards in buttress microhabitat, we discovered fewer lizard eggs in leaf litter plots, and predatory ant activity was lower in buttress than fallen log microhabitats. Our findings suggest that microhabitat may be important for tropical lizard population dynamics. Additionally, lizard observations were correlated with the number of prey invertebrates trapped, supporting the idea that prey availability is important for tropical anole lizard populations. Based on patterns observed in this study, we suggest that future studies investigating mechanisms that drive tropical lizard declines should consider habitat variables and food web interactions and focus on the microhabitat scale.

Introduction

Human-induced changes in climate and land use are driving global shifts in biodiversity, community structure and ecosystem function (Hansen et al. 2001, Hawkins et al. 2008, Blois et al. 2013). In conjunction with these changes, enigmatic declines (population declines with an unknown cause) have been reported for several tropical species of reptiles and amphibians. Some of these declines are correlated with changes in species interactions, such as introduction of invasive species (e.g., Fisher and Ineich 2012) and declines in prey availability (e.g., Lister and Garcia 2019). Predicting future changes in tropical food webs and community interactions is critical for making informed conservation and management decisions, but impossible without understanding current community dynamics.

Several populations of tropical anole lizards have experienced enigmatic long-term declines that are correlated with changes in climate, leaf litter depth, and prey availability (Whitfield et al. 2007, Stapley et al. 2014, Lister and Garcia 2019). Terrestrial anole lizards are widespread, typically occur in high abundances, and are a good model system for investigating relationships between climate, food webs and habitat characteristics. Because small-bodied tropical anole species are short-lived but reproduce frequently, egg survival is important for population persistence (Andrews 1982). Lizard egg survival varies by habitat and microhabitat and, when given a choice, female anoles prefer to oviposit in moist soil over dry or saturated soil, and areas with both soil and leaf litter over areas with just soil or just leaf litter (Schlaepfer 2003, Socci et al. 2005, DeSana et al. 2020). Thus, habitat, microhabitat, leaf litter depth and leaf litter cover likely influence lizard nesting, egg survival, and population dynamics.

It is critical to consider spatial scale when investigating ecological questions or seeking to understand mechanisms driving population declines. Leaf litter depth, chemistry, and structure

vary under different tree species in tropical ecosystems (Donoso et al. 2010). Additionally, tree buttresses accumulate greater leaf litter depth and have higher abundances of reptiles and amphibians than areas around trees without buttresses (Whitfield and Pierce 2005). Leaf litter herpetofaunal communities also differ underneath tree species that vary in their leaf litter structure and phenology (Folt and Reider 2013). Reptile and amphibian abundances at La Selva Biological Station, Costa Rica, are positively correlated with leaf litter depth and differences in litter depth across the forest floor could be important for structuring tropical reptile and amphibian communities (Lieberman 1986). Additionally, declines of a terrestrial anole lizard at La Selva were correlated with long-term reductions in leaf litter depth (Whitfield et al. 2007). Lizard population declines might be explained by changes in species interactions as a consequence of altered litter depth, such as increased predation or decreased prey availability. However, the intensity of species interactions can vary spatially (i.e., Feinsinger et al. 1988, Navarrete et al. 2005), making it difficult to detect food web effects without a large sample size or long-term data.

Previous research suggests that ants may be important predators of lizard eggs (Chalcraft and Andrews 1998, Huang 2008, Newman et al. 2014). Thief ants (*Solenopsis [Diplophoptrum]*) were the primary source of mortality for *Anolis apletophallus* Köhler and Sunyer 2008 eggs on Barro Colorado Island in Panama (Andrews 1982). At one site in La Selva, predation on *Anolis humilis* Peters 1863 eggs was correlated with predatory ant activity, as measured by mass loss from proteinaceous bait stations (Swartwout and Willson in review). Ant density and species composition varies spatially, even within La Selva, suggesting that predatory ant activity likely differs across microhabitats and forest types, as does leaf litter depth and other habitat characteristics that might influence species interactions (McGlynn et al. 2009). Therefore,

vulnerability of lizard eggs to ant predation likely also varies spatially depending on presence and activity of predatory ants in the environment.

To better understand complex interactions that may drive spatial variation in anole lizard population dynamics, we conducted surveys of lizard egg abundance and survival across various forest types (old-growth forest, abandoned agroforestry, abandoned plantation, and secondary forest) and microhabitats (buttress, fallen log, and leaf litter) in lowland rainforest at La Selva, Costa Rica. We also examined variation in abundance of prey resources and potential egg predators (predatory ants) of anole lizards. We hypothesized that (1) lizard egg abundance would be greater in abandoned plantation and buttresses because anole lizards have higher abundance there (Whitfield and Pierce 2005, Whitfield et al. 2007); (2) egg survival would differ across forest type and microhabitat; (3) predatory ant activity would be positively correlated with predation on lizard eggs across plots because this trend has been observed previously (Swartwout and Willson in review); and (4) predatory ant numbers would be negatively correlated with number of lizard eggs across all forest plots because ants are the primary predators of lizard eggs so female lizards may avoid nesting in areas with high densities of predatory ants.

Methods

Study Site

Our study was conducted at La Selva Biological Station, a 15-km² biological reserve in the lowland wet forest of Sarapiquí, Costa Rica (datum = WGS84; 10.431720, -84.007057; 35–137 m elev.) across four different forest types: abandoned agroforestry (AA), abandoned cacao and pejibaye plantations (AP), 18–24 yr old secondary forest (SF), and old-growth forest (OG). The AA, AP and OG areas were acquired by the station in 1968 and the SF in 1981. Plots were located on alluvial soil in AA, AP and SF areas, and volcanic soil for OG plots. The old-growth

forest site was dominated by *Pentaclethra* trees with *Capparis pittieri* Standl. and colonial palm *Bactris porschiana* Burret prominent in the forest understory, while secondary forest was dominated by *Cecropia insignis* Liebm., *C. obtusifolia* Bertol., *Laetia procera* (Poepp.) Eichler and *Rollinia microsepala* Standl. (Hartshorn and Hammel 1994). The abandoned plantation site had both native and introduced shade tree species as well as cacao, pejibaye, laurel and banana, and the abandoned agroforestry sites include riparian vegetation with *Ficus insipida* Willd., *chilamate* and *Pithecellobium longifolium* (Humb. & Bonpl. ex Willd.) Standl. common as well as *Piper* sp. and palms dominating the understory. We conducted the study from 11 April through 3 May 2018, during the transition from dry to wet season. Air temperature at the station during the study ranged between 20.01 and 34.96 °C (mean = 25.6 ± 3.16 SD). The average daily rainfall was 14.8 ± 23.1 SD mm and the maximum daily rainfall was 118 mm.

Field Methods

We established ten sites within each of the following forest types at La Selva Biological Station: abandoned agroforestry (AA), abandoned plantation (AP), secondary forest (SF), and old-growth forest (OG). At each site, a 3x3-m plot was demarcated in each of three microhabitats: leaf litter microhabitat (LL), at a fallen log (FL), and a buttress (BT), for a total of 120 plots across 40 sites. The edge of each plot was at least 5 m from the next adjacent one. In each plot, we measured habitat characteristics, number of ants, number of prey invertebrates, predatory ant activity, lizard egg abundance and survival, and number of lizard observations.

We sampled sites in four randomized blocks of ten sites, with two to three sites of each forest type represented in each block. We sampled one block per week between 11 April and 3 May 2018, until all sites were sampled. Surveys consisted of four consecutive days - the first day, we demarcated plot boundaries with flags, recorded habitat variables, and set out sticky

traps to collect data on ant abundance and prey availability; the second day, sticky traps were collected and analyzed in the lab, and tuna bait stations were set out and then collected after 12 h to measure predatory ant activity; and on the third and fourth days, we conducted 40-minute (0.67 person-hour) quadrat surveys for reptiles, amphibians, and reptile eggs.

For each plot, we recorded average litter depth ($N = 9$ measurements taken in the approximate center of nine 0.67-m^2 quadrants of the plot), average number of leaf litter layers (number of leaves pierced by a wire; $N = 5$ measurements from corners and center of each plot), canopy cover (as percent canopy overstory density) using a spherical densiometer (Lemmon 1956; Spherical Crown Densiometer, Concave Model C, Forestry Suppliers), and microhabitat availability (visually estimated percent ground cover of leaf litter, palms, vines, fallen log, buttress, and bare ground). We measured relative soil moisture in plots using a qualitative hand-feel method (as described in Al-Kaisi 2000), with 4 possible categories (“wet”, “moist”, “dry”, “very dry”). We acknowledge that this was a qualitative and relative measure of soil moisture, but we believe our broad categories of soil moisture adequately captured general conditions (i.e., whether eggs were in wet or dry soil).

To measure invertebrate abundance, we set one 158-cm^2 sticky insect trap (Trapper Monitor & Insect Glue Trap, TM2600, Bell Laboratories) in each plot for 24 h. We analyzed sticky traps in the laboratory immediately after collection, identifying all invertebrates to Order and ants to Genus. Invertebrates were classified as “prey” for *Anolis humilis* if they were members of taxa found in gut contents or personal observations of invertebrates eaten by *Anolis humilis* (Andrews 1979, Talbot 1979, Lieberman 1986). Thus, we calculated “prey availability” as the number of invertebrates collected from sticky traps that represent those taxa. We identified

“predatory ants” as ants from three genera reported to depredate lizards or lizard eggs:

Crematogaster, *Pheidole* and *Solenopsis* (Andrews 1982, Vogel 1983, Huang 2008).

Using the same method as in a previous study (Swartwout and Willson in review), we set one bait station with 2.5 grams of tuna bait in each plot for 12 h. We recorded bait mass lost after 12 h as a metric of predatory ant activity, which is correlated with predation of lizard eggs (Swartwout and Willson in review). To assess lizard and lizard egg abundance in plots, we conducted a 0.67 person-hour quadrat sampling for reptiles, amphibians, and lizard eggs in each plot, searching under and around logs, rocks, buttresses, and other microhabitat features, and sifting through all leaf litter and loose topsoil. Quadrat sampling has been used to discover lizard eggs and is more effective than visual encounter surveys for detecting terrestrial amphibians and cryptic leaf litter lizards in tropical rainforest (Andrews 1988, Doan 2003). When eggs were discovered, we recorded nest microhabitat, approximate egg dimensions, egg status (intact, hatched, depredated, or unknown), and photographed all lizard eggs. If eggs were intact, we left them in plots and monitored their status every 3–5 days until they were either hatched or depredated. Eggs were identified as “hatched” if they had smooth apical slits, “depredated” if they had irregular holes indicative of invertebrate predation, and “unknown” if eggs disappeared or the shells were too old to identify whether they were depredated or hatched.

Data Analyses

To characterize plots, we plotted habitat measurements (canopy cover, relative soil moisture, etc.) using Non-metric Multidimensional Scaling (NMDS) with a Bray-Curtis distance metric on two axes to visualize differences across plots and colored points by forest type and microhabitat (Oksanen 2007). We incorporated relative soil moisture in the NMDS by exchanging qualitative metrics (“wet”, “moist”, “dry”, “very dry”) with a numbering system (1–

4), where 4 represented drier soils. We visually confirmed that the percent ground cover of buttress, fallen log and leaf litter were highly correlated with their respective microhabitats (BT, FL, LL), and then removed those three variables from the NMDS. We then used a Goodness-of-fit test to test for significance. We also used a Type II two-way ANOVA to test whether leaf litter depth differed by forest and microhabitat type.

For egg fate analyses, we took the proportion of depredated eggs by plot and used plot ($N = 45$ containing eggs) as the statistical unit. Anole lizards have clutch sizes of 1 to 2 eggs (Andrews and Rand 1974) and out of all eggs analyzed, only two appeared to be from the same clutch based on their proximity. For this reason, we did not include clutch as a random effect. To test whether probability of predation for lizard eggs differed across forest type or microhabitats, we used a two-way ANOVA with proportion of depredated eggs as the dependent variable and checked assumptions of normality and homogeneity of variance by plotting the residuals.

To test whether bait mass loss was correlated with egg predation across plots, we used a binomial logistic regression with egg as the statistical unit for maximum sample size ($N = 69$, excluding nine eggs with unknown fate) and plot as a random effect. Adding microhabitat and forest type as random effects did not change the results.

We used Type II two-way ANOVAs to test whether bait mass loss, number of lizard eggs, number of lizard observations, and number of predatory ants captured in sticky traps differed by forest or microhabitat type. For significant ANOVA models, we used Tukey HSD post-hoc tests to determine which factors were significantly different. We checked assumptions of the ANOVA model using a Shapiro-Wilk test and visualization of the residuals and plots of homogeneity of variance (Shapiro and Wilk 1965). When testing whether the dependent variables “number of lizard eggs” and “number of lizards” varied with microhabitat and forest

type, the ANOVA assumption of homogeneity of variance was violated, so for those models, we used the White-adjusted ANOVA with a heteroscedasticity-consistent coefficient variance matrix (in R, using “Anova()” function with argument “white.adjust = TRUE”; White 1980). For factors that were significant, we used a post-hoc Games-Howell test to determine which levels significantly differed (package “userfriendlyscience”; Toothaker 1993). We also log-transformed number of lizard eggs and number of lizards to improve conformation with assumptions of normality, although ANOVAs are robust to some departure from normality.

We used linear regressions to test whether predatory ant abundance (*Crematogaster*, *Pheidole* and *Solenopsis*) was correlated with bait mass loss (predatory ant activity) or lizard egg abundance. We tested whether prey availability (number of prey insects captured in sticky traps) differed by microhabitat and forest type using a two-way ANOVA and ran a linear regression model to determine whether prey availability was positively correlated with lizard observations.

All analyses were conducted in R v. 3.6.1 with packages “car”, “ggplot2”, “performance”, “see”, “userfriendlyscience” and “vegan” (Wickham 2016, Peters 2018, Fox and Weisberg 2019, Oksanen et al. 2019, R Core Team 2019, Lüdtke et al. 2020a,b).

Results

For habitat measurements, points were oriented along two MDS axes: MDS1 primarily reflected area covered by bare ground and palms, leaf litter depth and layers, and canopy cover (percent overstory density) and MDS2 primarily reflected visually estimated percent cover by vines. Plots were not well-separated by forest type, indicating that the habitat variables measured did not differ significantly among the forest types examined (Goodness-of-fit Test: $P > 0.2$). However, plots were well separated by microhabitat, with BT plots having wetter soil, more vines, leaf litter depth and layers, LL plots with more palms and bare ground cover, and FL plots

with relatively more litter depth and layers, canopy cover and soil moisture ($P = 0.001$; Fig. 1). NMDS stress was 0.09, which was less than the preferred value of 0.1. Leaf litter depth did not differ significantly by forest type but was greater in buttress (BT) microhabitats than LL or FL (Two-way Type II ANOVA: Forest type – $F_{11,108} = 1.50$, $P = 0.22$; Microhabitat – $F_{11,108} = 7.68$, $P < 0.01$; Forest*Microhabitat – $F_{11,108} = 0.82$, $P = 0.56$).

During quadrat sampling, we discovered 81 reptile eggs, all but 3 of which could be easily identified as *Anolis* eggs by small size (<12 mm long), elongate shape and clutch size (1 or 2). The number of lizard eggs varied significantly by microhabitat but not forest type (White-adjusted ANOVA: Forest type – $F_{3,108} = 1.71$, $P = 0.17$; Microhabitat – $F_{2,108} = 16.5$, $P < 0.01$; Forest*Microhabitat – $F_{6,108} = 1.37$, $P = 0.23$; Fig. 2). Using a post-hoc Games-Howell test, we found that the number of lizard eggs was significantly lower in leaf litter (LL) microhabitats than other microhabitats ($P < 0.01$).

Probability of predation for lizard eggs did not differ by microhabitat or forest type (Two-way ANOVA: Forest type – $F_{3,33} = 1.93$, $P = 0.15$; Microhabitat – $F_{2,33} = 2.39$, $P = 0.11$; Forest*Microhabitat – $F_{4,33} = 1.32$, $P = 0.29$). Bait mass loss (used as a measure of predatory ant activity) was not significantly correlated with egg predation (Binomial Logistic Regression: $df = 66$, $z = 0.22$, $P = 0.8$, McFadden's $R^2 = 0.01$; Fig. 3). Bait mass loss varied significantly by microhabitat but not forest type (Two-way Type II ANOVA: Forest – $F_{3,108} = 0.43$, $P = 0.73$; Microhabitat – $F_{2,108} = 3.98$, $P = 0.02$; Forest *Microhabitat – $F_{6,108} = 0.56$, $P = 0.76$). Bait mass loss was significantly lower in buttress (BT) than fallen log (FL) plots (Tukey HSD: $P = 0.02$).

Predatory ant abundance was greater on average in old-growth forest but did not significantly differ by microhabitat, forest type or an interaction of the two (Type II ANOVA: Forest – $F_{3,108} = 0.31$, $P = 0.82$; Microhabitat – $F_{2,108} = 0.36$, $P = 0.70$; Forest*Microhabitat –

$F_{6,108} = 1.23$, $P = 0.30$). Predatory ant abundance was not correlated with number of lizard eggs or bait mass loss across plots (Linear Regression: Lizard egg abundance – $F_{1,118} = 0.02$, $P = 0.88$, $R^2 \ll 0.01$; Bait mass loss – $F_{1,118} = 1.32$, $P = 0.25$, $R^2 = 0.01$).

Number of lizards observed was significantly higher in BT microhabitat than FL or LL but did not differ by forest type (Type II ANOVA: Forest – $F_{3,108} = 0.32$, $P = 0.81$; Microhabitat – $F_{2,108} = 3.41$, $P = 0.04$; Forest*Microhabitat – $F_{6,108} = 0.53$, $P = 0.78$; Fig. 4). Number of prey invertebrates did not differ by microhabitat, forest type or an interaction of the two (Type II ANOVA: Forest – $F_{3,108} = 0.82$, $P = 0.48$; Microhabitat – $F_{2,108} = 0.21$, $P = 0.81$; Forest*Microhabitat – $F_{6,108} = 1.42$, $P = 0.21$). The number of lizards observed was positively related to the number of prey invertebrates, though not significantly correlated (Linear Regression: $F_{1,118} = 3.64$, $P = 0.06$, $R^2 = 0.03$; Fig. 5).

Discussion

Our hypotheses for this study were that (H1) lizard egg abundance would be greater in abandoned plantation and buttresses; (H2) egg survival would differ across forest types and microhabitats; (H3) predatory ant activity would be positively correlated with predation on lizard eggs across plots; and (H4) predatory ant abundance would be negatively correlated with number of lizard eggs. Our results provided partial support for H1 and no support for the other three hypotheses. There was no statistical difference in the number of lizard eggs discovered by forest type, though we found more eggs in abandoned plantation than old-growth forest. However, we did observe significantly more lizard eggs in buttress and fallen log microhabitats than leaf litter, with the most eggs found in fallen log microhabitats. The habitat variables measured by plot (i.e., litter depth, relative soil moisture, canopy cover, etc.) differed significantly by microhabitat in a NMDS biplot but were not differentiated by forest type. We likely observed statistically fewer

lizard eggs in LL plots because nesting substrates were not as ideal since litter depth was lower, and the plots tended toward greater bare ground cover. Overall, factors potentially influencing lizard population dynamics varied more by microhabitat than forest type. The greater variation in factors among microhabitat types highlights the importance of fine-scale differences in habitat conditions and microclimate in tropical rainforests.

Despite differences in number of lizard eggs among microhabitats, we did not observe a statistical difference in egg fate by forest type or microhabitat, leading us to reject H2. A previous study found that lizard egg survival was not influenced by leaf litter depth, one of the habitat variables that differed among microhabitats in this study (Swartwout and Willson in review). We may have seen no differences between forest types and microhabitats because there is too much variation in species interactions between individual plots to allow for generalization in lizard egg fate by forest type and microhabitat. Our finding that egg fate did not differ, coupled with no correlation of egg predation with predatory ant activity and no negative correlation of predatory ant abundance with lizard eggs across plots, suggests that egg predation likely is not a primary driver of relationships between habitat variables and lizard populations in this system.

Our finding that predatory ant activity was not correlated with egg predation across the study area, even with microhabitat and forest type included as random effects in the model, differed from a previous study conducted in one forest type (abandoned agroforestry) that found predatory ant activity and egg predation were statistically correlated (Swartwout and Willson in review). However, our ability to detect correlations between predatory ant activity and egg predation within forest types and microhabitats in this study was limited by uneven lizard egg sample sizes (i.e., we only recovered 5 eggs from LL plots and 16 from old-growth forest,

compared to 48 in FL plots and 26 in abandoned agroforestry). Additionally, there likely is a seasonal and/or temporal component to predatory ant activity (e.g., Argentine ant peak foraging activity in June to October in California, U.S., Rust et al. 2000). Bait mass loss trials in the previous study were conducted over 1 day in the wet season (late June) and in this study we conducted trials over multiple days during the transitional period (April/May).

Our measure of predatory ant activity, bait mass loss, was significantly lower in BT than FL plots. However, predatory ant observations on sticky traps did not differ by microhabitat or forest type. Sticky traps are a passive trapping method and likely picked up randomly foraging predatory ant workers with the measure primarily influenced by predatory ant abundance and general activity, while bait mass loss measures are influenced by predatory ant abundance, activity, and recruitment ability or species dominance (Bestelmeyer et al. 2000). Ant species that were able to rapidly recruit to baits may have been more abundant in fallen log microhabitats, whereas sticky traps or bait stations placed in buttresses may have been more difficult for ants to access and recruit to because they would need to climb over the buttress wings. Ants recruiting to baits in the buttress microhabitat may also potentially have been exposed to arboreal predators such as birds, lizards and frogs. Moisture may also influence ant detection of baits since BT plots had drier soils and in a previous study, greater soil moisture was correlated with increased ant predation on fruit fly puparia in the soil (Cao et al. 2012). We also acknowledge that, in drier conditions, moisture loss of the baits must be considered when assessing bait mass loss. We suggest that future studies determine the average dry mass of baits and then calculate moisture loss of baits from the field based on that average.

Overall, none of the variables that we measured differed statistically by forest type, but many differed by microhabitat. Statistical differences in habitat variables at microhabitat level

could explain why number of lizard eggs, lizard observations, and bait mass loss differed by microhabitat. The lizards that we observed in this study were primarily small leaf litter species that have relatively small home ranges (i.e., ca. 150-m² for *Anolis humilis*, Guyer 1988b). Due to their small size, finer scale differences in environmental conditions found in microhabitats may be more important to lizard populations than large-scale variation in forest type. Perhaps the relative availability of microhabitats such as fallen logs and buttresses should be considered in evaluating suitability of tropical habitats for litter-dwelling herpetofauna, and coarse woody debris and litter depth could be augmented in some cases for reptile conservation (e.g., added CWD enhanced reptile abundance in recovering Australian woodlands; Manning et al. 2013).

The number of lizards observed in quadrat samples was statistically higher in BT plots than other microhabitats, matching previous evidence that lizard abundance is higher around trees with buttresses (Whitfield and Pierce 2005). The number of lizard eggs discovered tended to be highest in fallen logs, rather than buttresses, although this was not statistically significant. Differences in the pattern of lizard observations and numbers of lizard eggs in this study could be explained by adult lizards using slightly different microhabitats than those selected by females for oviposition. Lizards may prefer buttress microhabitats because the leaf litter is deeper, and the buttress wings provide cover and a vantage point to survey the forest floor for prey. We acknowledge that our study only measured the number of lizards observed during a short visual encounter survey and did not take detection probability into account, so it is also possible that lizards were simply easier to detect in BT plots than FL or LL. To fully understand microhabitat use by tropical lizards, studies that use repeated surveys or CMR to account for differences in detection are needed to determine variation in lizard abundance by microhabitats.

In our study, the number of potential prey invertebrates caught on sticky traps was weakly positively correlated with the number of lizards observed. A previous study in Puerto Rico found that long-term declines in invertebrate abundance were correlated with declines in anole lizard populations (Lister and Garcia 2019). Population dynamics of the leaf litter anole (*Anolis humilis*) at La Selva also are influenced by prey availability, as demonstrated through a food supplementation experiment (Guyer 1988a). Proportionally, the most common invertebrates in 26 *A. humilis* stomachs were ants, spiders, isopods, and Hemiptera (Lieberman 1986). Earlier studies reported Orthopterans, Dipterans, Coleopterans, and Lepidopteran larvae as additional major dietary components for *A. humilis* (Andrews 1979, Talbot 1979). All prey groups were well-represented in sticky trap samples, except for isopods and Lepidopterans. Prey availability or invertebrate abundance may be a driver of relationships between habitat variables (i.e., leaf litter depth) and tropical lizard populations, and future food web studies focusing on importance of invertebrate prey for tropical lizards are warranted.

Conclusion

Spatial scale is important to consider in tropical ecology since rainforests are the most diverse terrestrial ecosystem and exhibit tremendous variation in some variables (i.e., species composition, diversity, abundance, etc.) at smaller or larger scales (Hill and Hamer 2004). We found that egg fate did not differ by microhabitat or forest type, but leaf litter depth, the number of lizard eggs, lizard observations and bait mass loss differed among microhabitats. Conversely, none of the variables measured differed by forest type. Species interactions such as lizard predation on invertebrates and predation on adult lizards may vary across microhabitats and forest types, mediated by habitat variables, but our results suggest that ant predation on lizard eggs does not. Incorporating studies at spatial scales relevant to the organism of interest (i.e.,

microhabitat level for small leaf litter organisms) and investigating food web interactions will help us to better understand drivers of enigmatic tropical reptile and amphibian declines.

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Figures

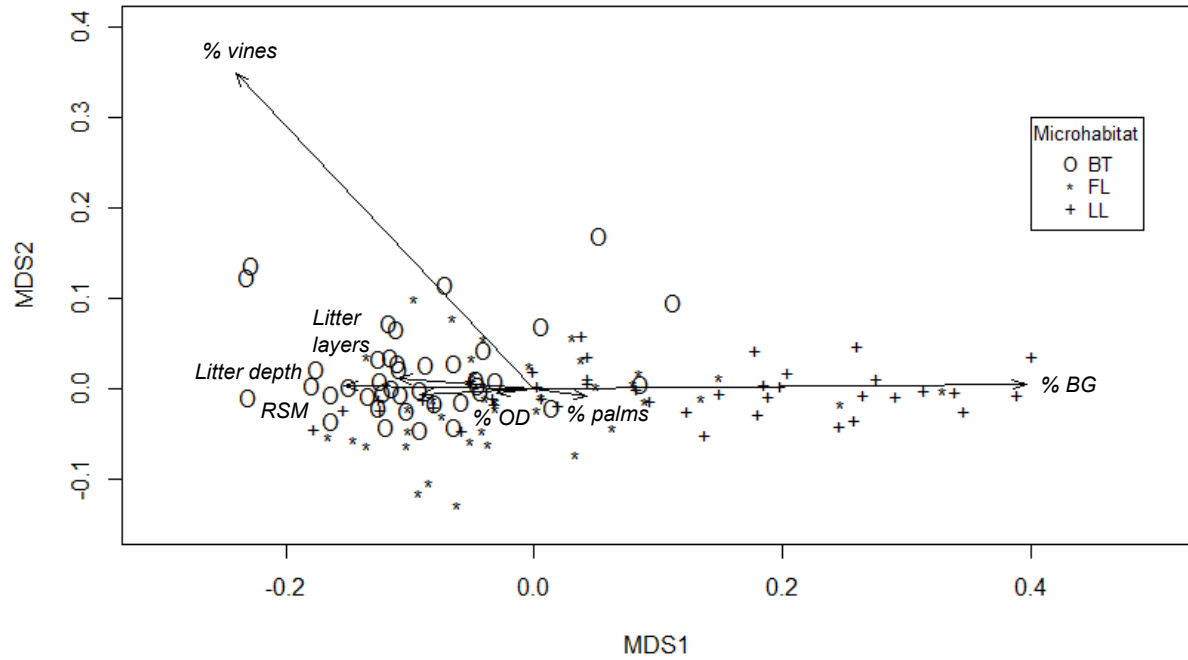


Figure 1. Two-axis NMDS ordination of sampling plots organized by microhabitat. MDS1 and MDS2 represent direction cosines (regression weights) for habitat measurements. Goodness-of-fit tests show that habitat measurement values formed discrete groups by microhabitat ($r^2 = 0.318$, $P = 0.001$), but not forest type ($r^2 = 0.033$, $P = 0.280$). NMDS stress was 0.09. Abbreviations: RSM = relative soil moisture, OD = overstory density, BG = bare ground.

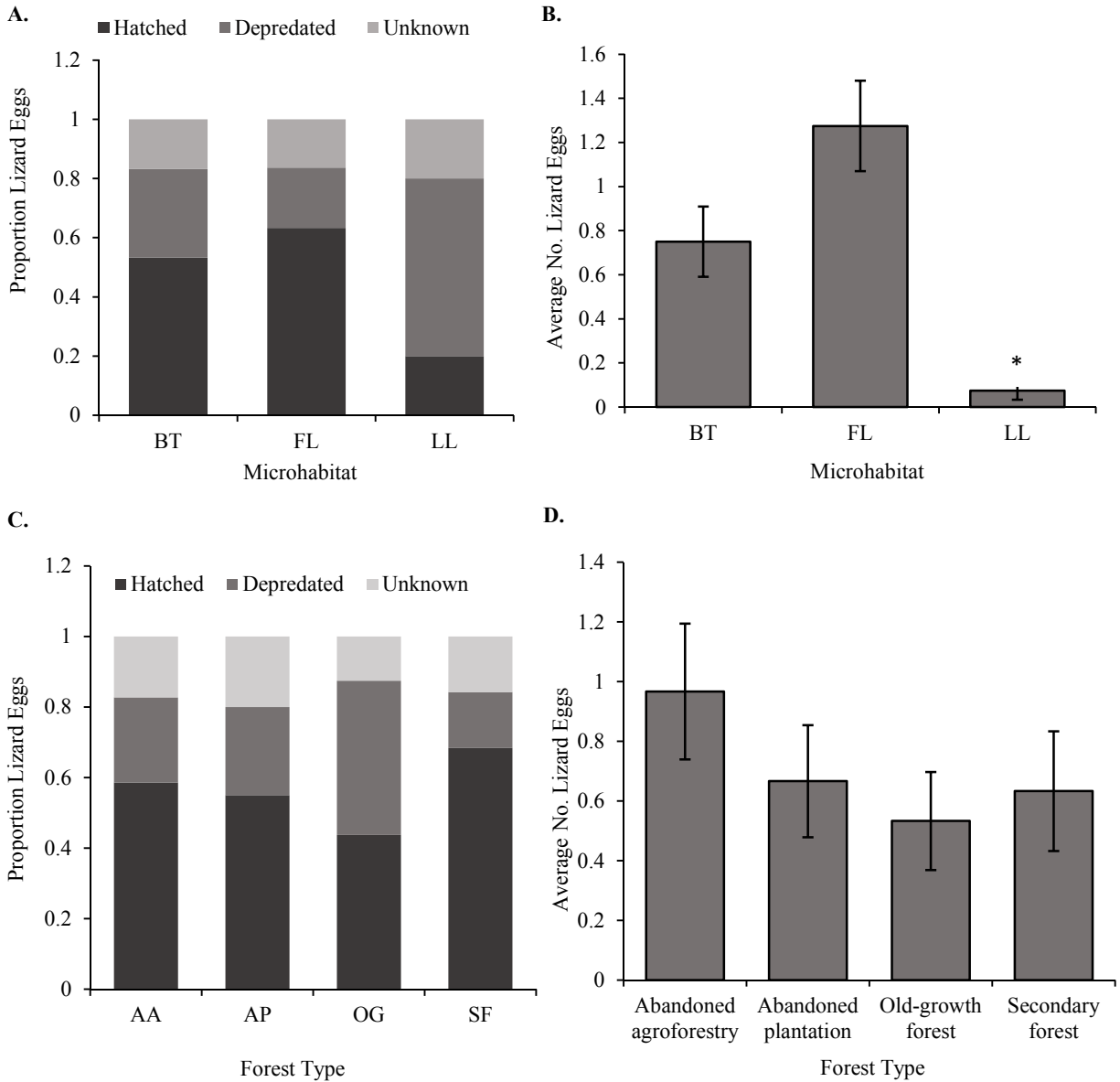


Figure 2. (A) Proportion of lizard eggs hatched, depredated or unknown by microhabitat; (B) average number of lizard eggs discovered by microhabitat; (C) Proportion of lizard eggs hatched, depredated or unknown by forest type; (D) average number of lizard eggs discovered by forest type. Error bars represent ± 1 SE. * = significantly different. BT = buttress, FL = fallen log, LL = leaf litter. AA = abandoned agroforestry, AP = abandoned plantation, OG = old-growth forest, SF = secondary forest.

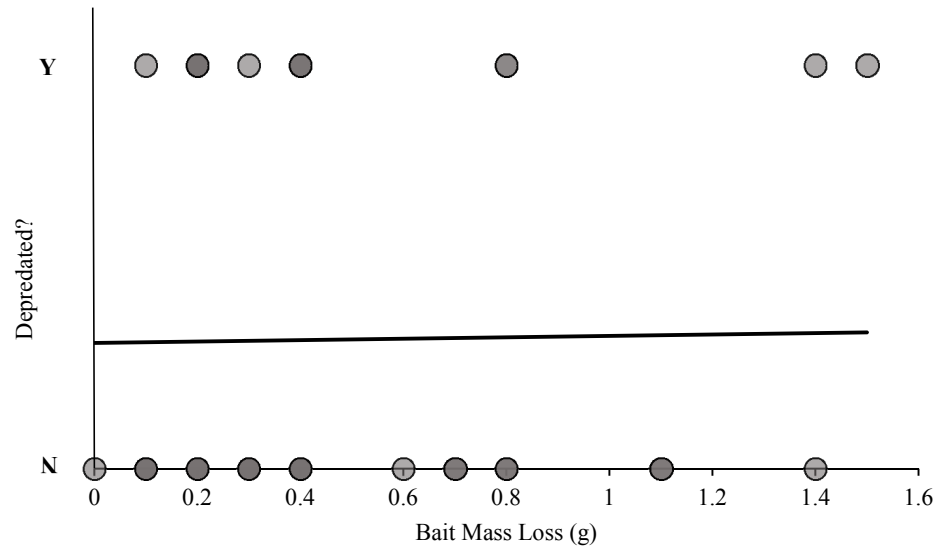


Figure 3. Binomial logistic regression of probability of lizard eggs being depredated by predatory ant activity, measured by bait mass loss in grams. Bait mass loss was not significantly correlated with probability of predation ($P > 0.50$; $R^2 = 0.01$).

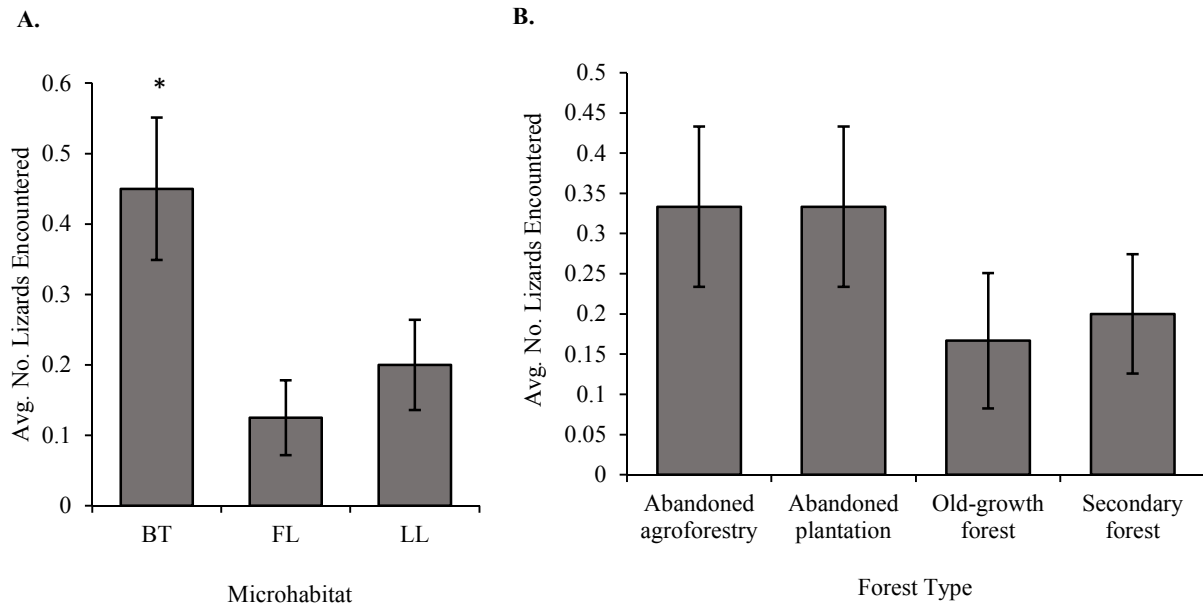


Figure 4. (A) Average number of lizard observations by microhabitat; (B) average number of lizard observations by forest type. Error bars represent ± 1 SE. * = significantly different. BT = buttress, FL = fallen log, LL = leaf litter.

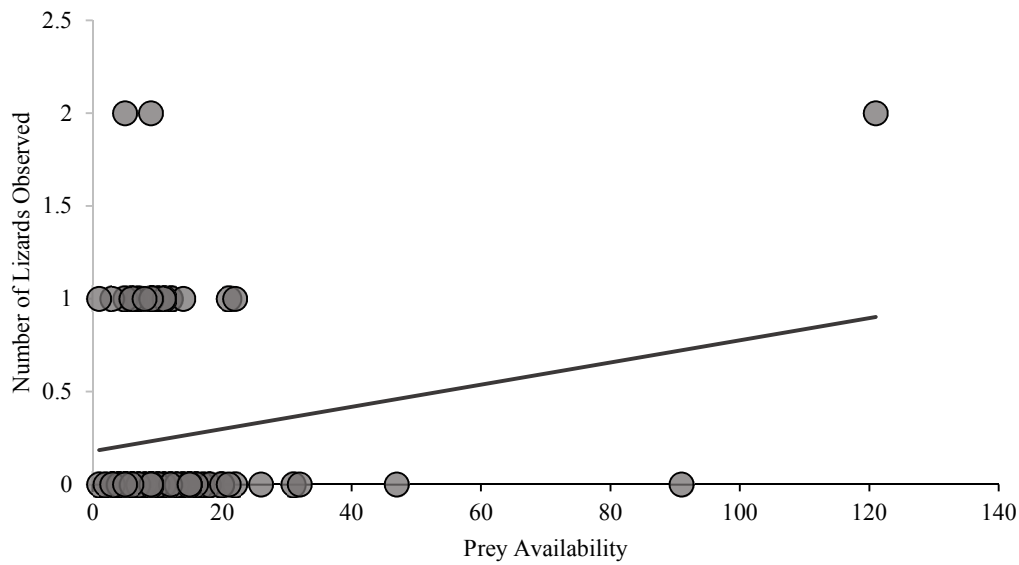


Figure 5. Linear regression of number of lizard observations by prey availability (number of prey invertebrates captured on sticky traps). Number of lizards observed was positively related to prey availability (Linear Regression: $F_{1,118} = 3.64$, $P = 0.06$, $R^2 = 0.03$).

Chapter 3:

Southeastern U.S. snake species are vulnerable to egg predation by red imported fire ants

(Solenopsis invicta)

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Abstract

Invasion and spread of red imported fire ants (RIFA; *Solenopsis invicta*) is cited as a possible cause for enigmatic reptile declines in the southeastern United States. RIFA negatively affect reptiles through predation of eggs, hatchlings, and adults. We used short term (12 h) field trials early in incubation to evaluate whether RIFA could successfully depredate intact eggs from six species of native terrestrial oviparous snakes: *Coluber constrictor*, *Lampropeltis holbrooki*, *L. calligaster*, *Opheodrys aestivus*, *Pantherophis obsoletus*, and *P. emoryi*. Then we used an artificial nest field experiment at the end of incubation to test whether RIFA predation differed between a species that has apparently declined in areas of its range where RIFA has invaded (*L. holbrooki*) and a species that has apparently not declined (*C. constrictor*). We measured pip–hatch and incubation time for each species in the laboratory and hypothesized that differences in time between pipping and hatching (pip–hatch time) could result in inter-specific differences in RIFA predation on eggs. *Coluber constrictor* had significantly shorter pip–hatch times than other species, but probability of predation by RIFA did not differ for *C. constrictor* and *L. holbrooki*. However, RIFA predation rates on snake eggs were high overall for all species (25–67% during early trials, 50–100% at end of incubation), although *P. obsoletus* was only depredated after hatching in the field. Our study provides novel observations of RIFA predation and suggests that time spent in nest, eggshell characteristics and nest microhabitat may be more important than pip–hatch time in mediating vulnerability of snake eggs to RIFA predation.

Introduction

Red imported fire ants (RIFA; *Solenopsis invicta*) are native to South America but have been introduced globally and are ranked among the most damaging invasive species in the world (Lowe et al. 2000). The first potential report of RIFA in the United States was in Mobile, AL in 1918; by 1955 RIFA had spread throughout the southeastern U.S. (Callcott and Collins 1996). RIFA continue to expand their invasive range northward and westward. RIFA are generalist predators that form large colonies and aggressively defend their colony mound using venomous stings. Their venom makes them a health risk to humans as well as native wildlife (Kemp et al. 2000). Known ecological effects of RIFA include predation of endangered cave invertebrates in Texas (Elliott 1993), predation on live-trapped small mammals (Masser and Grant 1986, Flickinger 1989), reduced loggerhead shrike and native insect abundance (Allen et al. 2001) and predation on bobwhite quail chicks (Allen et al. 1995). RIFA are also highly problematic for reptiles and amphibians, since they affect their food sources (invertebrates), depredate eggs, and occasionally depredate adults (Wojcik et al. 2001; Todd et al. 2008). Additionally, there is some evidence that presence of RIFA results in population declines, based on an experimental reduction of RIFA density that led to increased abundance and diversity of a South Carolina reptile and amphibian community (Allen et al. 2017).

Predation by RIFA on reptile eggs has been documented in the laboratory for diverse turtle and snake species (Diffie et al. 2010) and observed in field conditions for *Sceloporus undulatus* (Newman et al. 2014, Thawley and Langkilde 2016), *Cnemidophorus* (= *Aspidozelis*) *sexlineatus* (Mount et al. 1981), *Pseudemys* spp. (Allen et al. 2001, Aresco 2004), *Trachemys scripta* (Buhlmann and Coffman 2001), *Opheodrys aestivus* (Connors 1998a) and *Coluber constrictor* (Thawley 2014) in the U.S. During laboratory observations, species with more

durable eggshells (i.e., musk turtle, bobwhite quail) were not depredated by RIFA but these species are still vulnerable to RIFA predation during the pipping stage or right after hatchling emergence (Diffie et al. 2010). The amount of time a hatchling reptile spends pipped in the egg or escaping the nest chamber could strongly influence their probability of being depredated by RIFA. Species may differ in pip–hatch times, where slower pip–hatch times increase probability of RIFA predation. Increased ant predation on reptile eggs likely has important population-level consequences, as is the case for tropical anole lizards that have relatively high adult mortality (Andrews 1982). Although evidence shows that RIFA are problematic for native oviparous reptiles in the U.S., there are still relatively few studies on vulnerability of reptile species, especially snakes, to RIFA predation.

RIFA are one of the proposed causes for enigmatic reptile declines in the southeastern U.S. Enigmatic reptile declines are being reported worldwide and are defined as declines for which there is not currently a known cause (Todd et al. 2010). For example, the common kingsnake (*Lampropeltis getula* complex) has declined in southeastern portions of its range, including a protected area where habitats have been relatively undisturbed by humans (Krysko and Smith 2005, Winne et al. 2007). Additionally, dramatic local population declines of *L. getula* complex on Paynes Prairie State Preserve in Florida coincided with invasion by polygynous RIFA colonies (Kauffeld 1957, Bartlett 1997). Curiously, other snake species occupying the same range as *L. getula* complex are reported as having stable populations, and *Agkistrodon contortrix* has even increased in abundance, potentially due to release from predation by kingsnakes (Steen et al. 2014). Even other oviparous snake species such as North American racers (*Coluber constrictor*) and ratsnakes (*Pantherophis* spp.) remain common in areas where kingsnakes have

declined and RIFA are present. To date, no studies have tested whether southeastern snake species differ in their vulnerability to egg predation by RIFA under field conditions.

Our study was designed to evaluate interspecific differences in egg predation risk that might contribute to population trends observed for southeastern snake species. Possible factors that might influence predation risk of snake eggs include incubation time, time between pipping and hatching, eggshell durability, and nesting location. We chose to focus on incubation and pip–hatch timing in this study. We examined predation on eggs of six terrestrial snake species but particularly focused on comparisons between *Lampropeltis holbrooki* (the local member of the *L. getula* complex at our study area in Arkansas) and *C. constrictor*, a common sympatric terrestrial and oviparous species that has not experienced widespread population declines. Our study consisted of three components – a short field trial early in incubation, an end of incubation field experiment that incorporated hatching behavior, and laboratory observations of hatching behavior – to test (1) whether RIFA can depredate eggs of various snake species; (2) whether an apparently declining species (*L. holbrooki*) and stable species (*C. constrictor*) differ in egg predation risk; and (3) whether snake species differ in pip–hatch and incubation times. We hypothesized that *C. constrictor* would experience less predation by RIFA than *L. holbrooki* and have shorter pip–hatch times.

Methods

Female Collection and Maintenance

From April to June 2020, we collected gravid female snakes of six terrestrial oviparous species (*Coluber constrictor*, *Lampropeltis holbrooki* [= *getula holbrooki*], *L. calligaster*, *Opheodrys aestivus*, *Pantherophis obsoletus*, and *P. emoryi*) in Benton, Washington, Sebastian and Franklin Counties in Northwest Arkansas. Snakes were housed in 38–76 L aquaria furnished

with aspen shavings, a water bowl, and a hidebox and kept in a partially shaded, screened outbuilding in Fayetteville, Arkansas, U.S.A. that matched ambient temperatures (mean = 13.4–25.8°C) that snakes would experience in the wild. We provided each female with a rectangular Tupperware half-filled with moist coconut fiber as a nesting box for egg deposition and offered water *ad libitum*.

Starting 1 June 2020, we checked cages for eggs at least once per day. Oviposition began on 6 June and finished on 10 July 2020. Following oviposition, we took postpartum mass of each female snake and then released her at the site of capture. In this way, we obtained 78 *C. constrictor*, 88 *L. holbrooki*, 26 *L. calligaster*, 18 *O. aestivus*, 25 *P. obsoletus* and 12 *P. emoryi* eggs from 5, 8, 3, 3, 2 and 1 clutches, respectively. We processed eggs from each clutch by marking each egg with an identifying number, recording mass to the nearest 0.01 g, and length and width to the nearest mm. To avoid imparting human scent on eggs and limit potential contact with cutaneous bacteria and fungi, we wore nitrile gloves when handling eggs. We kept clutches together in tupperwares half-filled with a 1:1 mass ratio of vermiculite:water and incubated eggs at 25°C and 80% relative humidity in an environmental chamber (Model # I41VLC9, Percival Scientific). To maintain a consistent moisture level in egg containers, we recorded mass of all containers and eggs weekly and replenished any evaporated water.

Field Methods

We conducted field experiments at Kessler Mountain Regional Park (Washington County, Fayetteville, Arkansas, U.S.A.; 36.026146°N, 94.204868°W; datum = WGS84). Our field site was on the edge of RIFA invasive range, and RIFA are still rare and very localized in Northwest Arkansas, with few colonies persisting through the winter. The primary area we used was a ~20 x 8 m rectangular area on a SW-facing grassy slope bordering a forest dominated by

eastern red cedar (*Juniperus virginiana*) trees. A preliminary survey revealed at least 40 active RIFA mounds on the grassy slope and along the top of the incline by the forest edge. For each RIFA mound surveyed, we recorded the widest diameter of the mound to the nearest cm using a measuring tape, percent overstory canopy density using a spherical crown densiometer (Lemmon 1956; Concave Model C, Forestry Suppliers), whether the mound was in the open field or on the forest edge, distance from closest adjacent RIFA mound, and the distance of the mound from the forest edge to the nearest cm. We did not determine whether colonies were polygynous.

To test whether RIFA could depredate intact snake eggs early in incubation (0–17 days post-oviposition), we set up a small-scale field experiment using eggs from *C. constrictor* ($N = 20$), *L. holbrooki* ($N = 20$), *L. calligaster* ($N = 3$), *O. aestivus* ($N = 3$), *P. obsoletus* ($N = 3$) and *P. emoryi* ($N = 2$). We set individual eggs in 4-mm deep coconut fiber in small square tupperwares (7.77 x 5.49 x 8.26 cm) with twelve 3-mm holes drilled around the edge. We placed a single container 0.5-m from a RIFA mound. Eggs were set out on eight different occasions between 17 June and 15 July 2020 (ambient temperature range = 16.1–31.1°C). No mounds were re-used. We left eggs for 12 h, typically between ~1900 and 700 h, and then recorded whether eggs had (1) been depredated and (2) experienced mortality. Depredated eggs had RIFA swarming the egg, one or more chew marks or holes in the eggshell, and often leaked fluid.

To test for interspecific differences in RIFA predation near the end of incubation, we set up *C. constrictor* ($N = 30$), *L. holbrooki* ($N = 33$), and *P. obsoletus* ($N = 9$) eggs in artificial field nests of three eggs per nest after ca. 50 d of incubation (~15 d before hatching). We created artificial nests using 4-mm deep coconut fiber in a 7.5-cm diameter circular Tupperware with twelve 3-mm diameter holes around the perimeter and a window on the lid made of 3-mm diameter hardware cloth that allowed RIFA to pass through. We buried artificial nests so that the

eggs were at least 10 cm below the soil surface with a plastic bag of soil covering the lid of the artificial nest and a 30.5 x 30.5 cm plywood board on top to limit interference by vertebrate predators and prevent flooding by rain events (artificial nest setup modified from Thawley and Langkilde 2016). We buried artificial nests 1 m from a RIFA mound and monitored them daily to check for predation or successful hatching. We also set up three control nests each for *C. constrictor* and *L. holbrooki*, located at least 300 m away from all RIFA mounds in similar habitat (open field bordered by cedar trees) to establish background predation rates on snake eggs in the absence of RIFA predation. Our artificial nest setup allowed experimenters to visually check eggs for ant activity and predation by removing the bag of soil and lid. We recorded eggs as “depredated before pipping” when RIFA were swarming in and out of multiple holes in the eggshell, “depredated after hatching” when hatchlings were killed after emerging from the egg, and “hatched” when hatchlings were recovered from artificial nests without being swarmed by RIFA. Once eggs in a nest had pipped, the artificial nests were checked twice a day for hatchlings. If an egg was “pipped”, the hatchling was still present inside the egg but had slit open the eggshell and might have its head or snout poking out. Once the egg “hatched”, the hatchling was fully emerged from the eggshell.

Laboratory Incubation

After ca. 50 days of incubation (35 days for *O. aestivus*), we placed the remaining viable snake eggs not used in field experiments (43 *C. constrictor*, 42 *L. holbrooki*, 19 *L. calligaster*, 18 *O. aestivus*, 13 *P. obsoletus* and 12 *P. emoryi* eggs) in plastic shoeboxes with clear acrylic lids that allowed video monitoring of the eggs. We set a Trophy Cam HD Brown wildlife camera (Model 119874, Bushnell Corporation) to record 30 second videos every 30 min and positioned it above the eggs to record time between when eggs pipped and hatched (hereafter, pip–hatch

time) to the nearest 30 min. Once one egg in a clutch had pipped, we visually checked eggs in that clutch twice daily.

Statistical Analyses

To test whether racer (*C. constrictor*) and kingsnake (*L. holbrooki*) eggs differed in predation risk early in incubation we used a binomial logistic regression model with egg as the statistical unit and clutch as a random effect. We fit the mixed model using Gauss-Hermite quadrature, which is more accurate than Laplace approximation, and used a likelihood ratio test to determine the p-value for the fixed effect, “species” (Bolker et al. 2009). To validate the model, we used the `dispersion_glm` function in the “blmeco” package to calculate a dispersion factor and check for overdispersion (Korner-Nievergelt et al. 2015) and plotted residuals. Small sample sizes precluded statistical analysis of survival and predation risk for other snake species (*L. calligaster*, *O. aestivus*, *P. obsoletus*, and *P. emoryi*). Thus, we evaluated predation risk qualitatively as proportion depredated (Fig. 1).

For artificial nest field experiments at the end of incubation, we used a binomial logistic regression model with egg as the statistical unit and artificial nest ID as a random effect to test whether *C. constrictor* and *L. holbrooki* eggs placed near RIFA mounds differed in probability of predation. We tested whether the random effects “clutch” and “nest ID” were important to include in the model by comparing AICc values for models with both random effects, one of the two random effects, and no random effects (Burnham and Anderson 2002). Based on AICc values, the model with only nest ID as a random effect was ranked above models with clutch as a random effect or no random effects. The original model would not converge, so we standardized the model using the function “standardize” in the R package “arm”, which rescales binomial variables to have a mean of 0 and difference of 1 between the two categories (Gelman 2008).

The mixed model was fit using Gauss-Hermite quadrature and we used a likelihood ratio test to determine the p-value for “species” (Bolker et al. 2009). We used a linear mixed effect model with a Type II Wald chisquare test to determine whether species differed in time to predation. Two *C. constrictor* eggs in this experiment were excluded from analyses because the hatchlings had pipped within nests that were being depredated by RIFA and were removed from artificial nests early due to an experimenter error. We also did not include two artificial nests (one *P. obsoletus* and one *L. holbrooki*) that were depredated by mammals (identified by nests dug up and eggshells destroyed and empty) the day after they were placed in the field. Sample sizes for *P. obsoletus* were too low for statistical comparisons so we qualitatively reported proportion depredated (Fig. 2).

We used binomial logistic regressions with artificial nest as the statistical unit to test whether probability of predation in the artificial nest experiment was correlated with RIFA mound diameter, average distance to forest edge or canopy cover. Eggs in artificial nests were either all depredated or all hatched, so probability of predation fit a binomial distribution. Since we did not detect a species effect on predation in earlier analyses, all three snake species were pooled in this analysis.

In 2019 we did a preliminary study and recorded pip-hatch and incubation times for eggs from one clutch of *C. constrictor* ($N = 3$), three clutches of *L. holbrooki* ($N = 23$), one clutch of *L. calligaster* ($N = 3$), and one clutch of *O. aestivus* ($N = 3$). Because there was no statistical difference between years that might indicate a bias (Welch Two Sample t-test: Pip-hatch Time – $t_{20,69} = 0.15$, $P = 0.88$; Incubation Time – $t_{37,150} = -1.73$, $P = 0.09$), we pooled data from 2019 and 2020 to increase sample size. To determine whether snakes differed in pip-hatch or incubation time we used one-way ANOVAs with species as the fixed effect and clutch as a random effect.

After testing for significance of the random effect using a LRT to compare models with and without it, we determined that clutch did not need to be included as a random effect for pip–hatch time ($P > 0.99$), but it was important for incubation time ($P < 0.001$). Therefore, we used a simple linear model to test whether pip–hatch time differed by species. When the fixed effect was significant, we used a post-hoc Tukey HSD test to examine species differences in pip–hatch times and a Tukey contrast multiple comparison of means for incubation times. We checked assumptions of models with visualizations of residual plots. All analyses were conducted in R version 3.6.1 (R Core Team 2019) using “car”, “MASS” and “lme4” packages (Venables and Ripley 2002, Bates et al. 2015, Fox and Weisberg 2019).

Results

In the early incubation field experiment, RIFA successfully pierced the eggshells and depredated 5 *Coluber constrictor* and 6 *Lampropeltis holbrooki* eggs (out of 20 each), 2 *L. calligaster* and 2 *Opheodrys aestivus* eggs (out of 3 each) and 1 out of 2 *Pantherophis emoryi* eggs. None of the three *P. obsoletus* eggs set out were depredated after 12 hours (Fig. 1). Egg sample sizes for *L. calligaster*, *O. aestivus*, *P. obsoletus* and *P. emoryi* were too small for statistical comparisons, but *C. constrictor* and *L. holbrooki* did not differ in predation probability during 12 hour field trials early in incubation (Binomial Logistic Regression: $df = 1$, $\chi^2 = 0.29$, $P = 0.59$). The dispersion factor of the model was 0.9 which is less than 1.4, indicating our model was not overdispersed.

In the artificial nest field experiment near the end of incubation, all but three *C. constrictor* eggs were depredated (18 depredated before pipping and 9 depredated after hatching), all *L. holbrooki* eggs were depredated (22 before pipping and 8 after hatching) and 50% of the *P. obsoletus* eggs were depredated after hatching (the other three eggs hatched in the

field). No eggs in control nests (9 eggs each for *C. constrictor* and *L. holbrooki*) located away from RIFA mounds were depredated; all control eggs successfully hatched in the field without mortality (Fig. 2). Egg sample sizes for *P. obsoletus* were too small for statistical comparisons, but *C. constrictor* and *L. holbrooki* did not differ in predation probability during field nest experiments (Binomial Logistic Regression: $df = 1$, $\chi^2 = 1.20$, $P = 0.27$). The dispersion factor of the model was 0.18, which was less than 1.4, indicating that our model was not overdispersed. Number of days to predation did not differ for *C. constrictor* (8.60 ± 0.81 SE d) and *L. holbrooki* (9.55 ± 1.82 SE d) eggs (Type II Wald chisquare test: $df = 1$, $\chi^2 = 0.41$, $P = 0.52$).

Probability of predation was not significantly correlated with overstory canopy density or RIFA mound size (Binomial Logistic Regression: Overstory Canopy Density – McFadden's $R^2 = 0.20$, $df = 21$, $\chi^2 = 2.27$, $P = 0.10$; RIFA Mound Size – McFadden's $R^2 = 0.08$, $df = 21$, $\chi^2 = 1.10$, $P = 0.30$) but was significantly positively correlated with distance from forest edge (McFadden's $R^2 = 0.33$, $df = 21$, $\chi^2 = 4.41$, $P = 0.036$).

After pipping, *C. constrictor* emerged from their eggs faster (mean pip–hatch time = 11.14 ± 1.06 SE h) than all other snake species that we tested. *Coluber constrictor* had a significantly shorter pip–hatch time than all species (One-way ANOVA: $F_{5,83} = 6.89$, $P < 0.001$), except for *L. calligaster* ($P = 0.13$; Fig. 3A). *Lampropeltis holbrooki* averaged 7.8 h (70%) longer pip–hatch times, *L. calligaster* 5.5 h (49%) longer, *O. aestivus* 10 h (92%) longer, *P. emoryi* 14 h (129%) longer and *P. obsoletus* 11 h (103%) longer than *C. constrictor*. Species differed significantly in their incubation period lengths (Linear Mixed Effect Model: $F_{5,179} = 16.8$, $\chi^2 = 83.8$, $P < 0.001$). *Opheodrys aestivus* had shorter incubation times than any other snake ($P < 0.005$), *C. constrictor* had shorter incubation times than *L. calligaster* and the

Pantherophis species ($P < 0.02$), and *P. obsoletus* had longer incubation times than *L. holbrooki* ($P < 0.001$; Fig. 3B).

Discussion

Eggs of all snake species tested in this study except *Pantherophis obsoletus* were depredated during 12 h early incubation trials, suggesting that leathery snake eggshells (and even eggs with a granular surface such as *Coluber constrictor*) can be readily punctured by RIFA. Lack of predation on control nests during the end of incubation experiment indicates that proximity to RIFA colonies substantially increases predation risk for snake eggs, providing additional support to the idea that in areas where they have invaded, RIFA are potentially important egg predators. Although *C. constrictor* had significantly shorter pip–hatch times, *L. holbrooki* and *C. constrictor* eggs did not differ in probability of predation by RIFA in either early incubation trials or the end of incubation experiment. Additionally, we found that placement of the artificial nest relative to the forest edge influenced probability of predation, suggesting that the role of nest microhabitat warrants further investigation. Thus, our results suggest predation on eggs by RIFA is a threat to southeastern snake species but did not provide direct support for increased egg vulnerability to RIFA predation as a possible mechanism for enigmatic declines of *L. getula* complex.

Contrary to our predictions, pip–hatch time was not an important determinant of species' vulnerability to predation by RIFA. Overall, snake species significantly differed in pip–hatch and incubation times, with *C. constrictor* emerging from their eggs faster than most other snake species, and *O. aestivus* incubating faster than other snake species. However, some caution should be taken in interpreting our results because we incubated all species at the same temperature, while their optimal incubation temperatures likely differed. *Coluber constrictor* had

a significantly shorter pip–hatch time than *L. holbrooki* but did not differ in probability of predation. The time that hatchlings spend in the nest after pipping may be a more important driver of predation risk than pip–hatch time. We observed that pipped eggs in field nests were filled with fluid and ants typically did not enter the eggs at that time, but hatchlings would be attacked after emerging. Our observation of hatchling mortality primarily after emergence matches previous studies (Connors 1998, Buhlmann and Coffman 2001), although one study suggested that turtles were most vulnerable while still in the egg after pipping (Allen et al. 2001). Our experimental design did not test how fast hatchlings were able to escape the nest and disperse, and a more natural setup would be needed to evaluate the role time spent in the nest after hatching might play on hatchling vulnerability.

Excluding *P. obsoletus*, predation rates in our study were high, with 25–67% of eggs depredated during the 12 h field trials and 90–100% during the end of incubation field experiment. Studies using artificial field nests found rates of RIFA predation on eggs to be 24% for *Sceloporus undulatus* over only 15–20 d of incubation (Thawley and Langkilde 2016) and 60%, 50% and 27% for *Trachemys scripta*, depending on distance from RIFA colony (Buhlmann and Coffman 2001). Week-long laboratory observations of RIFA predation on various snake, turtle and bird eggs found predation rates of 100% for *Malaclemys terrapin*, *Trachemys scripta scripta*, *Chrysemys picta picta*, and *Elaphe* (= *Pantherophis*) *obsoleta quadrivittata*, 92% for sea turtles (*Caretta caretta*), 75% for Burmese pythons (*Python molurus bivittatus*), and 0% for bobwhite quail (*Colinus virginianus*), *Sternotherus odoratus* and *Apalone ferox* (Diffie et al. 2010). Previous data and results of this study suggest that although some eggs might resist RIFA predation in short term trials, over the entire course of incubation, nests in proximity to RIFA mounds are likely to experience relatively high rates of predation (60–100%).

Although sample sizes were small, *P. obsoletus* eggs were not depredated by RIFA in the 12 h early incubation trials and were not depredated until after hatching in end of incubation field experiments. Eggs of all other snake species were depredated during the early incubation trials, suggesting that some aspect of eggshell structure protected *P. obsoletus* eggs from being penetrated by RIFA. *Pantherophis obsoletus* eggs were about twice the volume and mass of other species, on average. Large egg size results in greater surface area, possibly making it more difficult for RIFA to aggregate in one location and penetrate the eggshell. In the presence of predatory rats, larger seabird eggs had more durable shells and higher survival probability than smaller eggs and took longer for rats to depredate (Latorre et al. 2013). In birds, eggshell thickness and durability typically scales with mass (Ar et al. 1979, Rahn and Paganelli 1989), so *P. obsoletus* eggs likely also have thicker eggshells, but there are relatively few studies on reptile eggshell thickness. Although we did not measure eggshell thickness or durability in this study, it has been suggested that reptile eggshell durability can influence vulnerability to RIFA predation (Diffie et al. 2010), perhaps affording species with large eggs such as *P. obsoletus* some degree of protection from RIFA.

Nest site selection by female snakes could also mediate vulnerability of snake eggs to RIFA predation. Our study found that distance of artificial nest from forest edge was positively correlated with probability of predation, likely because all nests in the end of incubation field experiment except for two *C. constrictor* nests on the forest edge were depredated. Higher probability of predation away from the forest edge can be explained by RIFA preference for sunny, open areas and increased foraging activity correlated with lower canopy cover (Brown et al. 2012). Female snakes may protect their eggs from RIFA by nesting in alternative locations and could actively avoid nesting near RIFA mounds, though a study on habitat use by a lizard,

Sceloporus undulatus, observed that they did not avoid RIFA mounds (Langkilde 2009). Nest depth and substrate could also be important factors influencing snake egg vulnerability to RIFA predation. *Pantherophis obsoletus* and *O. aestivus* often nest in tree hollows or rotten wood, both of which are microhabitats less commonly used by RIFA (Ernst and Ernst 2003, Trauth et al. 2004, Gibbons 2017). A lack of basic natural history information on snake nesting behavior limits our ability to understand the role that nest microhabitat could play in vulnerability of snake eggs to predation. Given the secretive nature of snakes, we encourage naturalists to continue to record and publish observations of snake nesting behavior to contribute to our growing knowledge of the role that reproductive behavior may play in snake population declines.

Our study is among the first to provide direct data indicating that RIFA predation may be a critical source of mortality for oviparous snakes, yet many questions remain. We still do not know how RIFA locate reptile eggs or exactly which factors allow RIFA to pierce some reptile eggshells but not others. Future studies should examine additional species traits that might contribute to egg vulnerability to RIFA predation, such as eggshell thickness and durability, nest microhabitat and nesting behavior. Additionally, while this study does provide a potential mechanism through which RIFA might cause widespread oviparous snake declines, the link between RIFA introductions and enigmatic reptile declines is still tenuous. Additional mechanistic field studies of vulnerable oviparous snakes and long-term data on snake populations are needed to determine whether invasion by RIFA is responsible for population declines. The results of this study contribute to the growing body of research into the role RIFA invasions might play in reptile declines.

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Figures

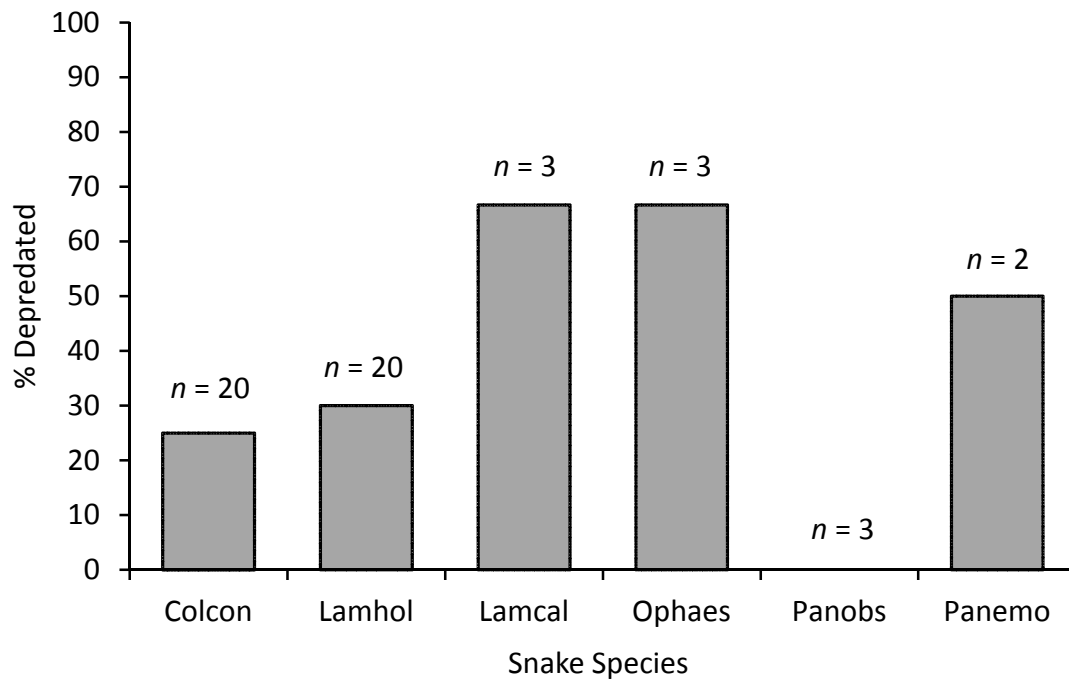


Figure 1. Percent of eggs depredated for each species in early incubation field experiment. Sample size (number of eggs) is given for each species. Abbreviations: Colcon = *Coluber constrictor*, Lamhol = *Lampropeltis holbrooki*, Lamcal = *L. calligaster*, Ophaes = *Opheodrys aestivus*, Panobs = *Pantherophis obsoletus*, Panemo = *P. emoryi*.

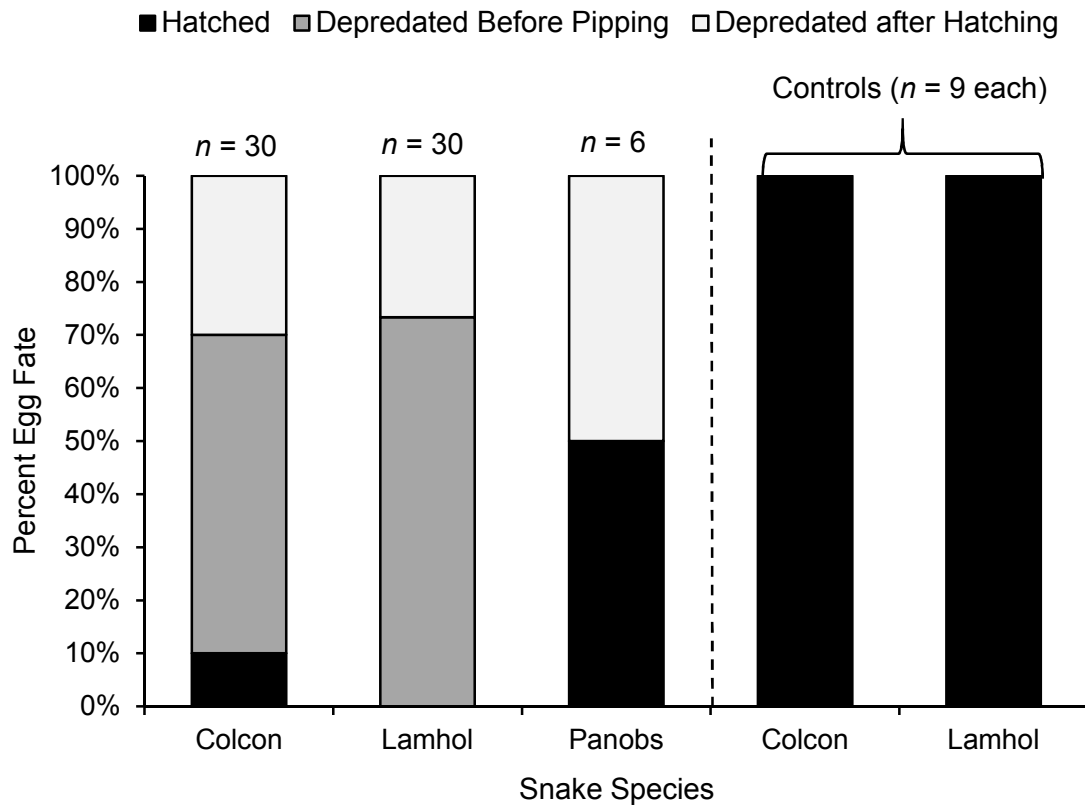


Figure 2. Percent of eggs hatched, depredated before pipping, or depredated after hatching for each species in the artificial nest field experiment at the end of incubation. All eggs in control nests hatched. Abbreviations: Colcon = *Coluber constrictor*, Lamhol = *Lampropeltis holbrooki*, Panobs = *Pantherophis obsoletus*.

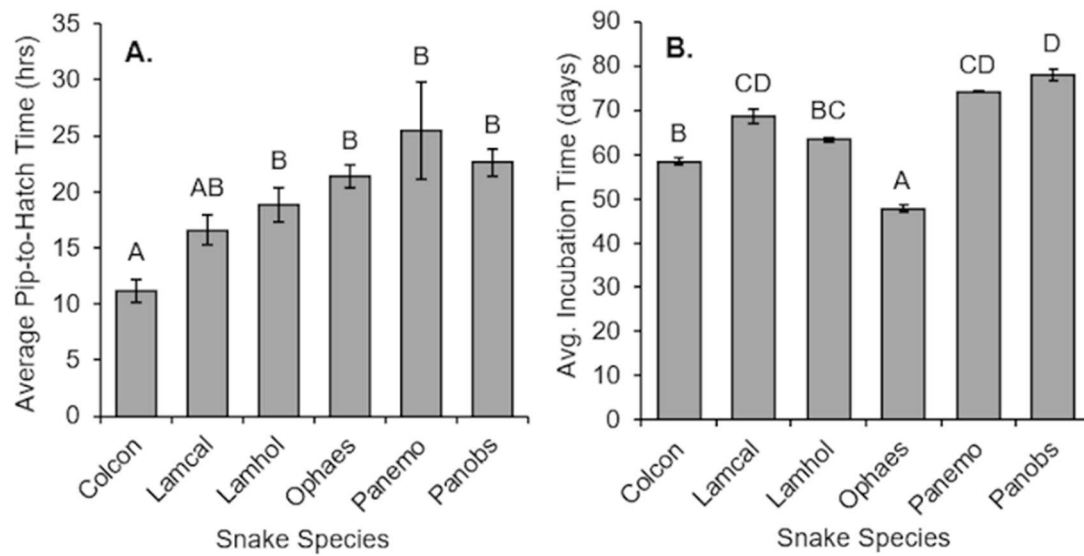
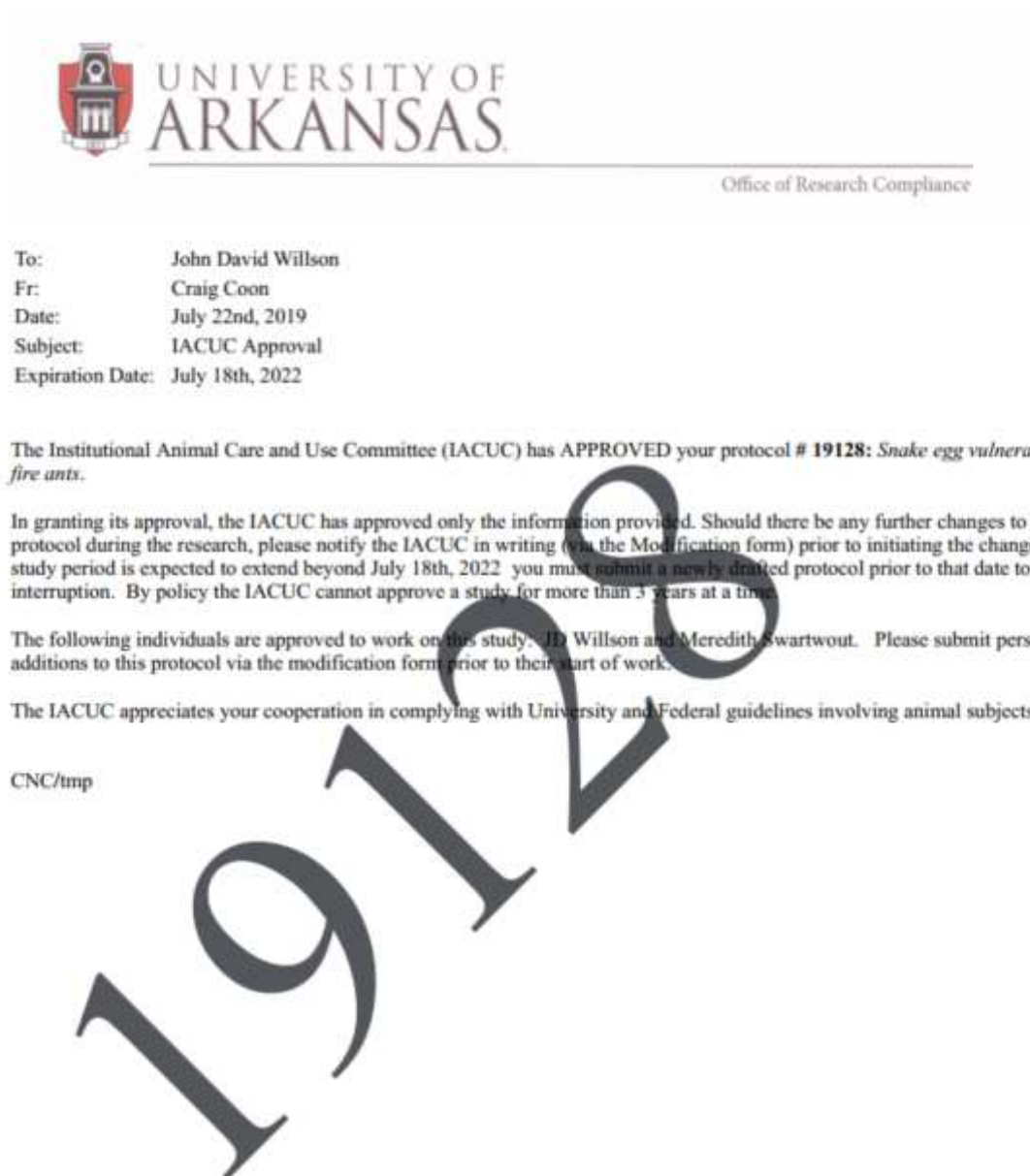


Figure 3. Average time between pipping and hatching (A) and average incubation time (B) for eggs of six snake species. Letters above bars (i.e., A, B, C, D) indicate significant differences between species. Error bars are ± 1 SE. Abbreviations: Colcon = *Coluber constrictor*, Lamcal = *Lampropeltis calligaster*, Lamhol = *L. holbrooki*, Ophaes = *Opheodrys aestivus*, Panemo = *Pantherophis emoryi*, Panobs = *P. obsoletus*.

Appendix 1. IACUC Approval documents



Chapter 4:

Life history and ecological variables associated with fire ant (*Solenopsis invicta*) invasion predict declining status in southeastern U.S. snakes

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Abstract

Reptile populations are declining globally, and the proximate causes of many declines are unclear. Invasion by red imported fire ants (RIFA; *Solenopsis invicta*) has been suggested as a contributing factor to enigmatic snake declines in the southeastern U.S. Previous studies have found that slow life history traits (i.e., greater age at maturity, smaller clutch size), habitat or prey preferences, and small geographic range size were important correlates of imperilment in snakes, but our study is the first to specifically examine variables relating to vulnerability to RIFA as correlates of decline in snakes of the southeastern U.S. We reviewed existing ecological and life history data for 53 snake species to generate a series of variables testing well-known life history correlates of decline and factors expected to mediate vulnerability to RIFA. We used a linear discriminant analysis (LDA) and MANOVA to assess the ability of our variables to predict a qualitative assessment of snake species status as ‘Declining’, ‘Stable’ or ‘Unknown’. We found that declining species had smaller geographic range sizes, larger percent overlap of range with RIFA, nested in underground burrows, were more associated with xeric and upland pine habitats, and were less tolerant of urbanized habitat. Overall, our results support RIFA invasion and habitat loss as drivers of snake population declines in the southeastern U.S. Our analyses also supported well documented associations between slow life history characteristics and declining status.

Introduction

Reptile populations are declining worldwide due to factors such as habitat loss, climate change, pollution, overexploitation, disease, and invasive species (Todd et al. 2010). While these are the ultimate mechanisms behind declines, in many cases the proximate mechanisms driving population declines remain enigmatic. Regional declines and local extirpations of native snake populations are particularly prevalent in the Coastal Plain of the southeastern U.S. For example, population declines and possible local extirpation of the common kingsnake (*Lampropeltis getula*) were reported from a large, protected area where other snake species remained abundant (Winne et al. 2007). Road surveys revealed significant declines in abundance of rough green snakes (*Opheodrys aestivus*) in South Florida (Croshaw et al. 2019), and *L. getula* have apparently declined precipitously throughout Florida (Krysko and Smith 2005, Godley et al. 2017) and in the Red Hills region of southern Georgia and the Florida panhandle (Stapleton et al. 2008). Additionally, Southern hognose snakes (*Heterodon simus*) have been extirpated from about 67.5% of their historical range along the Gulf Coast (Tuberville et al. 2000), a region that has also documented declines in timber rattlesnakes (*Crotalus horridus*) and Louisiana pine snakes (*Pituophis ruthveni*; Rudolph and Burgdorf 1997).

Although the causes of most snake population declines in the southeastern U.S. remain unproven, one possible contributing factor is the invasion and spread of the red imported fire ant (RIFA; *Solenopsis invicta*). RIFA were introduced at the port of Mobile, Alabama, in the 1930's or 1940's and have spread throughout the southeastern United States, including areas where most enigmatic snake declines have been reported (Callcott and Collins 1996). They are a highly aggressive generalist predator ant species with a venomous sting that enables them to overwhelm and consume small vertebrates, resulting in substantial negative effects on biodiversity (Wojcik

et al. 2001). RIFA affect reptiles directly by preying on eggs, hatchlings, and occasionally even adults (Montgomery 1996, Allen et al. 2004, Thawley and Langkilde 2016). They also may negatively affect reptiles by reducing prey availability, altering suitability of habitats, and through sublethal effects of their venom (Allen et al. 2004, Boronow and Langkilde 2010). RIFA have been proposed as a potential factor contributing to declines of *L. getula* at Paynes Prairie and range wide declines of the southern hognose snake, *Heterodon simus* (Kauffeld 1957, Tuberville et al. 2000, Krysko 2001). However, throughout these same regions, other snake species remain relatively common with stable or increasing populations (e.g., Steen et al. 2014), suggesting that interspecific differences in ecology or life history might play a role in vulnerability of snakes to RIFA.

Previous studies have examined the influence of life history variables on extinction risk of snake species. In the southeastern U.S., snake species that primarily consumed vertebrates, were more aquatic, and had smaller range sizes had higher sensitivity to anthropogenic land use change (Todd et al. 2017). Range size was also the most important predictor of extinction risk based on IUCN criteria for all squamate reptiles worldwide (Böhm et al. 2016). In Mediterranean island reptile assemblages, extinction rates were most strongly correlated with low population abundance and high habitat specialization (Foufopoulos and Ives 1999). Species with slow life histories, maturing later in life and with high adult survival but relatively low fecundity, are generally more vulnerable to extinction (i.e., Webb et al. 2002). In Australian elapids, ambush foraging and lack of male-male combat associated with larger female body size were correlated with threatened status (Reed and Shine 2002). However, previous studies did not consider variables that specifically relate to risk from invasive ant predators like RIFA, such as reproductive traits, habitat preference, and extent of range overlap with RIFA.

Recent studies have revealed that RIFA can depredate eggs from a variety of terrestrial snake species, including *L. getula* complex (Swartwout and Willson in review, Diffie et al. 2010). Variation in reproductive behavior might contribute to species' resilience to RIFA invasion and explain species status. Maternal choice in nest microhabitat affects predation and desiccation risk for reptile eggs (Reedy et al. 2013, DeSana et al. 2020). Eggs are a relatively vulnerable life stage since they are immobile and confined to their nest site, so the longer they need to incubate the greater chance for them to be depredated, desiccated, or flooded. Although likely to be important, relatively few reproductive variables have been used in previous investigations of traits that predict species' status (clutch size and reproductive frequency in Webb et al. 2002; male combat, reproductive mode, female size at maturity, and clutch size in Reed and Shine 2002; reproductive mode and clutch size in Todd et al. 2017). In general, reproduction is relatively understudied for reptiles and many species are data deficient, even within the southeastern United States.

The objective of this study was to evaluate whether ecological variables predicted to enhance vulnerability to RIFA invasion were strong predictors of declining status in southeastern U.S. snake species, as well as to test factors that are well known to associate with extinction risk, such as slow life history and small geographic range size. We reviewed existing ecological and life history data for all 53 southeastern snake taxa that occupy the Coastal Plain region of the U.S., spanning a wide variety of habitats, modes of reproduction, and range sizes. Additionally, we included nest microhabitat, incubation time, and percent overlap of species range with RIFA invasive range as potential predictors. We predicted that declining species status would be strongly associated with greater range overlap with RIFA, oviparity, nesting terrestrially or

underground, smaller clutch sizes, longer incubation times, and open canopy xeric/pine habitats, all of which would increase the vulnerability of a snake species to egg predation by RIFA.

Methods

Data Collection

We compiled life history and reproductive data for the 53 snake taxa inhabiting the southeastern region of the United States, focusing on the Coastal Plain and Piedmont regions from Virginia to eastern Texas. Qualitative and quantitative data were obtained from reference books and scientific papers (see Appendix 1) and then summarized within species. For wide-ranging species or species-complexes, we used life history values from Piedmont or Coastal Plain regions, when possible.

There has been a widespread movement to split wide-ranging U.S. snake species into separate taxa based on large-scale patterns of genetic structure, often despite contiguous distributions, large ‘hybrid’ zones, and similar life history information (e.g., *Pantherophis obsoletus* complex – Burbrink 2001, *Lampropeltis getula* complex – Pyron and Burbrink 2009a, *Agkistrodon piscivorus/conanti* and *A. contortrix/laticinctus* – Burbrink and Guiher 2014, *Lampropeltis calligaster* complex – McKelvy and Burbrink 2017). Based in part on scarcity of ecological data for population subunits, we generally followed traditional taxonomy that considered these wide-ranging species-complexes as single species. The *Pantherophis obsoletus* complex, *Agkistrodon piscivorus* complex and *A. contortrix* complex were treated as single species based on the presence of large intergrade/hybrid zones, *Lampropeltis calligaster* complex were grouped together based on similar life histories and lack of data for some populations, and the *L. getula* complex was lumped together based on similar life histories and adjacent ranges. We separated pine snakes (*Pituophis ruthveni* and *P. melanoleucus*), milk snakes (*L. triangulum*

and *L. elapsoides*), and corn snakes (*Pantherophis emoryi* and *P. guttatus*) based on non-overlapping ranges and/or differences in life history (Rodríguez-Robles and De Jesús-Escobar 2000, Burbrink 2002, Pyron and Burbrink 2009b). Due to a lack of ecological information on some populations, we lumped *Pantherophis slowinskii* with *P. guttatus* and *Pituophis lodingii* with *P. melanoleucus*.

The ecological variables that we compiled for each species included: species status, habitat type, urbanized habitat use, geographic range size, percent overlap of range with the range of RIFA, maximum adult size, age and size at maturity, parity, maximum clutch size, average hatchling mass, nest microhabitat, and maximum incubation time. We qualitatively assessed “Status” for species based on reports of extirpations and declines as well as anecdotes about population status taken from the references, using three categories: Declining, Unknown, or Stable. Twelve taxa were reported as “declining”, including *Opheodrys aestivus* (Croshaw et al. 2019), *Heterodon simus* (Tuberville et al. 2000), *Nerodia clarkii*, *Farancia erythrogramma*, *Pituophis ruthveni* (Rudolph et al. 2006), *P. melanoleucus*, *Lampropeltis getula* complex (Krysko and Smith 2005, Winne et al. 2007), *Lampropeltis extenuata*, *Crotalus adamanteus*, *C. horridus* (Rudolph and Burgdorf 1997), *Tantilla oolitica*, and *Drymarchon couperi*.

Parity was coded as a 1 for oviparous and 0 for viviparous. Habitat type was coded using a qualitative scoring system with 1 = aquatic, 2 = habitat generalist/mesic forest, 3 = xeric/pine-associated, and 4 = xeric/pine restricted. We coded our habitat variable based on species dependence on upland xeric/pine habitats because of significant loss of that habitat (Van Lear et al. 2005) and concomitant wildlife population declines, including snakes (e.g., *Heterodon simus* – Tuberville et al. 2000, *Pituophis ruthveni* – Rudolph et al. 2006). Secondly, use of upland xeric/pine habitats may put snakes at risk of RIFA predation due to the relatively open canopy

that favors invasion by RIFA (Langkilde 2009). If species were reported by sources to persist in urbanized habitats (i.e., residential lots, gardens, drainage ditches, urbanized streams, etc.) they were coded binomially as a 1 for urbanized habitat use. Range size was calculated for snakes as the number of states in which the species had research grade observations from a citizen scientist program, iNaturalist (<https://www.inaturalist.org>; Accessed 26 February 2021). Maximum adult size and size at maturity were recorded as the total body length in cm. Age at maturity was reported as the youngest age females were observed to reproduce in years. Average hatchling mass was calculated from all averages reported for a species and was recorded in grams. To test whether declining species nested in terrestrial microhabitats vulnerable to RIFA predation, we qualitatively scored nest microhabitat from 1 to 4, where 1 = burrows (completely underground), 2 = shallow burrow or mix of burrows and under debris, 3 = under debris, and 4 = inside debris, in a tree hollow or on something completely aboveground. Maximum incubation time was the longest time that eggs were reported to incubate in days and was coded as 86 (the average maximum incubation time for all oviparous species) for viviparous species to avoid strongly biasing the factor toward oviparous species in statistical analyses.

To calculate the percent overlap of species' range with the extent of RIFA observations in the southeastern U.S., we pulled coordinates from research grade iNaturalist observations using the Global Biodiversity Information Facility (GBIF) database. We obtained occurrence data as .csv files for each snake species and for RIFA, and DOIs are available to access all the occurrence data incorporated in this study (Appendix 2). We plotted points for each species using the WGS84 coordinate system in QGIS v. 3.16 (QGIS.org 2021) and drew minimal polygons by hand around the extent of the points as an estimate of the species range. Then we

used the Overlap Analysis tool in the QGIS' Toolbox to calculate the percent overlap of a snake species' range with the RIFA's range.

Statistical Analyses

Reproductive life history traits such as clutch size, maximum adult size, and age at maturity are known to covary in squamate reptile species (Dunham and Miles 1985). Therefore, we used a principal components analysis (PCA) to condense our reproductive variables (parity, maximum incubation time, average hatchling mass, size and age at maturity, maximum adult size, maximum clutch size) into uncorrelated component dimensions. First, because the variables had different measurement scales, we standardized them by converting to z-scores, which are calculated by subtracting the arithmetic mean and dividing by the standard deviation of a variable for each data point within that variable (Jain et al. 2005). We tested assumptions of normality and homogeneity of variance of the data using Shapiro-Wilk and Levene Tests (Levene 1960, Shapiro and Wilk 1965). Since observations were missing for some of the variables, we used the `estim_ncpPCA` and `imputePCA` functions in the R package “missMDA” to predict missing values using cross-validation (Josse and Husson 2012). We found that four dimensions were needed for the iterative PCA algorithm to impute data, and these four dimensions cumulatively explained 90.7% of the variation in the model.

We used a Linear Discriminant Analysis (LDA) to predict species status based on ecological variables (PC values, habitat type, urbanized habitat use, range size and percent range overlap with RIFA). We first normalized and standardized the predictors using z-score transformation in a standalone method, with no training dataset to avoid loss of sample size (“center” and “scale” method in `preProcess()` function of “caret” package in R; Kuhn 2020). Then we analyzed predictors by species status in a linear discriminant analysis using the `lda()`

function in the R package “MASS” (Ripley 1996, Venables and Ripley 2002). We assessed model accuracy by viewing the LDA class results in comparison to the original data and calculating the percent of species misclassified.

To determine whether our variables of interest differed significantly by species status, we performed a Type II MANOVA test with Pillai trace on the PC dimensions representing reproductive traits and the continuous variables range size and percent range overlap with RIFA. Before performing statistical analyses, we tested for multicollinearity among our variables of interest using a Pearson correlation test and found no values above 0.9. The homogeneity of variance assumption was not met for PC1, so when significance was detected, we used Welch Anova tests (Delacre et al. 2019) and Games-Howell post-hoc tests.

To determine whether status was independent of categorical predictor variables that could not be included in the MANOVA (habitat type, urbanized habitat use, and nest microhabitat), we conducted three Fisher’s Exact Tests. We used a Bonferroni correction to adjust for multiple tests. Nest microhabitat only contained data from oviparous species, so we did not include it in any additional statistical analyses to avoid skewing results.

We used R version 3.6.1 with packages “car”, “tidyverse”, “mvnrmtest”, “missMDA”, “FactoMineR”, “rstatix”, “MASS” and “caret” for all statistical analyses (Venables and Ripley 2002, Le et al. 2008, Slawomir 2012, Josse and Husson 2016, Fox and Weisberg 2019, R Core Team 2019, Wickham et al. 2019, Kassambara 2020_a, Kuhn 2020). We used the R package “ggpubr” to generate publication quality graphs (Kassambara 2020_b).

Results

For reproductive variables, points were primarily oriented along four PC dimensions (Fig. 1). We focused on the first two that explained 66.87% of the variation. Maximum adult

size, age and size at maturity, average hatchling mass, and maximum incubation time increased with positive values of PC1 (explaining 46.13% of model variation). Parity tended toward oviparity, maximum clutch size decreased, maximum incubation time increased, and age at maturity decreased with positive values of PC2 (20.74% of variation). Species with Declining status were positively related to PC1 and those with Unknown status were negatively related to PC1 (ANOVA model in `dimdesc()` function: $R^2 = 0.17$, $P = 0.01$; Fig. 2). Species with Stable status were significantly negatively correlated with PC2 ($R^2 = 0.13$, $P = 0.02$) and Declining species were positively correlated with PC2 ($P = 0.05$).

Our linear discriminant analysis (LDA) used two linear discriminants, with species range size and percent range overlap with RIFA having the largest coefficients and thus greatest influence on species status (Table 1, Fig. 3). LD1 achieved 85.5% separation of species by status and LD2 achieved 14.5% separation. Declining and stable species separated best along LD1 (Fig. 4). The LDA did not achieve perfect separation of groups and the model only classified 63% of species correctly (7 declining, 18 stable, 8 unknown out of 53 total species).

We found that our combined life history variables (excluding the categorical variables habitat type, nest microhabitat, and urbanized habitat use) significantly differed by species status (MANOVA: approx. $F_{2,96} = 2.74$, $P < 0.01$). Follow-up univariate ANOVAs with Bonferroni adjusted alpha level of 0.013 did not reveal any statistically significant differences by status (all $P > 0.02$). However, snakes with stable status had greater range sizes than declining species (Games-Howell Test: $P = 0.02$) and lower range overlap with RIFA ($P = 0.06$).

Habitat type, persistence in urbanized habitats, and nest microhabitat use were not independent from species status, even at the Bonferroni-adjusted p-value (Fig. 4; Fisher's Exact Tests: all $P < 0.017$). Only declining species were classified in habitat index 4 (upland xeric/pine

specialist) and nest microhabitat index 1 (underground burrows). Declining species were also less likely to persist in urbanized habitats than stable species.

Discussion

Our study on correlates of snake declines explicitly considered factors that enhance species' vulnerability to RIFA, along with more well-established drivers of imperilment such as species life history and geographic range size. We found that species' range size and percent overlap with RIFA range were the most important predictors of species status, where stable species had larger ranges and less range overlap with RIFA. Nest microhabitat, habitat type, and tolerance of urbanized habitats were related to species status, with declining species nesting in burrows, utilizing more xeric habitats, and being less tolerant of urbanized habitats than stable species. Our results support previous findings that declining species have some life history factors in common and suggest that RIFA invasion and habitat loss are the primary drivers of snake declines in the southeastern U.S.

Our results supported invasion by RIFA being associated with snake declines in the southeastern US. We found that geographic range overlap with RIFA was one of the most important predictors of species status and was higher in declining species. However, overall geographic range size was also a very important predictor of status and many species with small range sizes occurred within the area RIFA have invaded in the southeastern U.S (i.e., *Pituophis ruthveni*, *Lampropeltis extenuata*). Our assessment of spatial alignment between RIFA and declines was extremely coarse since we did not examine whether the pattern of declines within species coincided with RIFA invasion or account for the timeline of species declines relative to RIFA invasion. Most well-documented snake population extirpations in the southeastern U.S. are along the Gulf Coast (e.g., east Texas, southern LA, MS, AL, FL panhandle) which is

coincidentally close to the region of RIFA introduction in the mid-1900's (Callcott and Collins 1996). Patterns of declines and extirpations of *Heterodon simus* in the region were spatio-temporally consistent with RIFA invasion (Tuberville et al. 2000), and indigo snakes (*Drymarchon couperi*) were extirpated from their range along the Gulf Coast (AL, MS, and parts of the FL panhandle), though they are now being reintroduced in AL (Enge et al. 2013, Steen et al. 2016). *Pituophis ruthveni* have likely been extirpated from 7 out of 11 counties in TX and 2 out of 5 parishes in LA since 1929, after the period of RIFA introduction (Rudolph et al. 2018). Extant populations are limited to upland xeric/pine habitat patches in east TX and west LA, though between 1992 and 2015 only two animals were found during trapping efforts in TX, raising serious concerns about their species status (Rudolph et al. 2006, Rudolph et al. 2018). Species in the Alabama Coastal Plain that tended to inhabit relatively open habitats that RIFA favor (i.e., *Pituophis melanoleucus*, *Heterodon simus*, *Cemophora coccinea*) appeared to experience the most population declines relative to livebearers and species occupying habitat types that are generally damp and shady (i.e., *Pantherophis obsoletus*, *Heterodon platyrhinos*; Mount 1981). The synchrony of Gulf Coast snake population declines and RIFA invasion status warrants further investigation by examining species declines at finer spatial scales (i.e., county level) in comparison to the historical timeline of RIFA invasion and spread.

The association of declining species with upland xeric/pine habitats and underground burrows provides additional support to the idea that RIFA invasion is a driver of declines. Upland pine and scrub habitats in the southeastern U.S. are typically characterized by open canopies and clay-influenced sandy soils that favor invasion by RIFA colonies (Ali et al. 1986, Langkilde 2009). Additionally, presence of RIFA affected arthropod communities in upland pine habitat in Mississippi, indicating that RIFA abundance in that habitat can be high enough to

cause ecological damage (Epperson and Allen 2010). Eggs and hatchlings of racers (*Coluber constrictor*), green snakes (*Opheodrys aestivus*), rat snakes (*Pantherophis* spp.) and kingsnakes (*Lampropeltis* spp.) are vulnerable to predation by RIFA (Diffie et al. 2010, Thawley 2014, Swartwout and Willson in review). Nest microhabitat can affect probability of predation and eggs are likely to be more vulnerable to predation by RIFA when laid underground or terrestrially, since RIFA forage using underground tunnels (Tschinkel 2011). Our data supported that expectation, since all species that used underground burrows were reported as declining. We also expected that oviparity and longer incubation times would be associated with declining species status based on increased vulnerability to RIFA predation, and found limited support for that prediction based on declining species being grouped with longer incubation times and oviparity in the PCA. To conclusively correlate RIFA invasion with snake population declines, further research is needed, along with a better understanding of geographic variation in snake reproductive ecology, behavior, and life history.

The association of declining species with upland xeric/pine habitats is consistent with RIFA as a driver of snake declines, but that habitat type has also been widely degraded by anthropogenic activities, such as pine silviculture and urban development (Mitchell et al. 2006). Open-canopy pine habitat (i.e., pine savannah) is one of the most threatened habitats in the U.S. and is estimated to have been lost from 97% of its original extent (Van Lear et al. 2005). Other species associated with pine habitats that appear less likely to be directly affected by RIFA, such as the red-cockaded woodpecker (*Leuconotopicus borealis*), have also experienced significant population declines (Walters 1991). Additionally, stable snake species were more likely to use urbanized habitats than declining species, indicating that threatened snakes may be sensitive to anthropogenic development. A previous study found that aquatic snakes were more sensitive to

human land use in the southeastern U.S. (Todd et al. 2017). Our data do not support this finding. More aquatic and generalist species tolerated urbanized habitats than xeric/pine specialists (Fisher's Exact Test: $P = 0.03$). Additionally, we found more aquatic species classified as "stable" than "declining" or "unknown," with only two out of 16 aquatic species listed as declining – the saltmarsh watersnake (*Nerodia clarkii*) and the rainbow snake (*Farancia erytrogramma*). However, our methodology differed substantially from that used in the previous study since we used a binomial measure of urbanized habitat use and a qualitative score of species status encompassing the southeastern U.S., rather than a quantitative ranking of sensitivity to human land use based on species occurrences around natural landscapes in North and South Carolina and IUCN and NatureServe species assessments (Todd et al. 2016). Nevertheless, our studies agree that species habitat use and sensitivity to anthropogenic development are important correlates of decline (Todd et al. 2017). Our results also supported previous findings that species with smaller geographic range size have higher sensitivity to land use change or greater extinction risk (Böhm et al. 2016, Todd et al. 2017).

Although we did not find any significant differences in life history factors between declining and stable species in our MANOVA, PC1 was an important predictor of species status in our LDA (Fig. 4). Based on the PCA, Declining species tended to be larger, have greater size at maturity, be oviparous, have longer incubation times, and have smaller clutches, Stable species tended to be viviparous, have larger clutches, lower incubation times, and greater age at maturity, and Unknown species tended to be viviparous, smaller, mature faster at smaller sizes, and have faster incubation times. Reproductive effort and body size are well-established as important life history factors that determine species risk across taxa (Kunin and Gaston 1993, Gaston and Blackburn 1995). Our results match previous findings that large body size and slow life histories

are associated with imperilment in snakes (Reed and Shine 2002, Webb et al. 2002). Although reproductive variables (except nest microhabitat) were not a significant predictor of declining status in this study, the groups established by our PCA of life history variables intuitively make sense – Unknown species tended to be smaller fossorial species (i.e., *Carphophis amoenus* and *Tantilla* spp.), Stable species tend to be viviparous species with larger clutch sizes (i.e., *Thamnophis* and *Nerodia* spp.), and Declining species tended to be large oviparous species with small clutches of large eggs (i.e., *Drymarchon couperi* and *Pituophis* spp.).

While our findings largely matched those of other studies, our methodology did have some drawbacks. We condensed our reproductive variables in a PCA into dimensions that explained variance in the dataset rather than using them independently, limiting our power to draw conclusions about each predictor (Song et al. 2013). Our variables nest microhabitat and maximum incubation time were only applicable to oviparous species, though we handled missing viviparous data for incubation time by standardizing all viviparous incubation times to the average to avoid strong biases towards oviparous species in the PCA. Most previous studies evaluating correlates of decline used phylogenetic generalized least squares (pGLS), or similar cladogram analyses, to account for similarities in life history based on phylogeny (Reed and Shine 2002, Böhm et al. 2016, Todd et al. 2017). We did not statistically account for phylogeny in our study; however, declining species in our study were broadly spread throughout the phylogeny of North American snakes (Pyron et al. 2011; Fig. 5). We also used a qualitative assessment of species status rather than a quantitative one, which allowed us to incorporate species with localized declines as “declining” but limited our power to correlate species status with predictor variables. Evaluations of species status are complicated by a high number of unknown and data deficient snake species as well as anecdotal reports of declines, highlighting a

need for more population assessments of southeastern snake species. We found that cryptic fossorial snake species were particularly deficient in ecological and reproductive data (e.g., *Rhadinaea flavilata*, *Tantilla* spp., *Carphophis amoenus*, *Lampropeltis extenuata*). Species with Unknown status had low PC1 values representing small maximum adult size, lower age and size at maturity, and shorter incubation times. The secretive behavior and low detectability of many snakes complicates population studies and makes assessment of species status challenging (Steen 2010, Durso et al. 2011).

Overall, we found that declining snake species in the southeastern US were more likely to be xeric/pine specialists, have smaller range sizes with more overlap with RIFA range, nest in burrows, and use less urbanized habitats. Our results suggest that invasion by RIFA and habitat loss are major drivers of southeastern snake declines. Spatial patterns of declines and extirpations relative to RIFA invasion suggest that further snake declines could be expected in more recently invaded areas such as the Atlantic Coastal Plain. Management of RIFA within the range of sensitive species and preservation of xeric/pine habitats are important strategies for conservation of threatened snake species, as well as protection of gopher tortoises and small mammals such as pocket gophers that create burrows used by large upland snakes for sheltering and nesting (e.g., Rudolph and Burgdorf 1997). RIFA suppression increases herpetofaunal diversity and abundance (Allen et al. 2017) and is being considered as part of active management for some threatened herpetofauna, such as gopher tortoises (*Gopherus polyphemus*; Epperson et al. 2021). Population assessments for cryptic snake species are needed to evaluate whether additional species are at risk due to RIFA invasion and habitat loss. Additionally, we found that nest microhabitat was significantly related to snake species status, but field data on nests and how nest microhabitat relates to RIFA predation are still lacking. More controlled studies and

experiments are needed to evaluate what factors increase species vulnerability to RIFA predation and how to mitigate those negative effects (e.g., Swartwout and Willson in review).

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Tables and Figures

Table 1. Coefficients of linear discriminants from the Linear Discriminant Analysis (LDA).

Predictor	LD1	LD2
PC1	-0.5073	-0.7041
PC2	0.3268	0.2885
Habitat	-0.5447	-0.0643
Urbanized habitat use	0.3773	-0.7304
Range size (no. U.S. States)*	0.8819	-0.6952
% range overlap with RIFA*	0.5923	-1.134

* = differed between stable and declining species in MANOVA ($P < 0.07$).

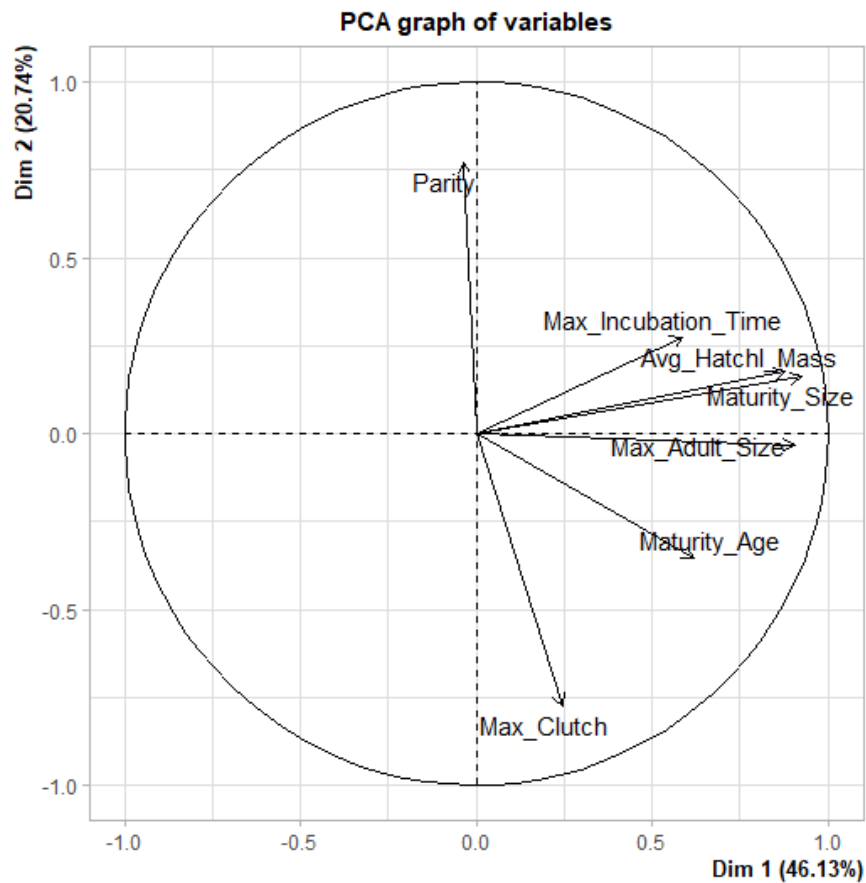


Figure 1. Variable loadings for the first two dimensions used in a principal component analysis (PCA) to condense reproductive variables.

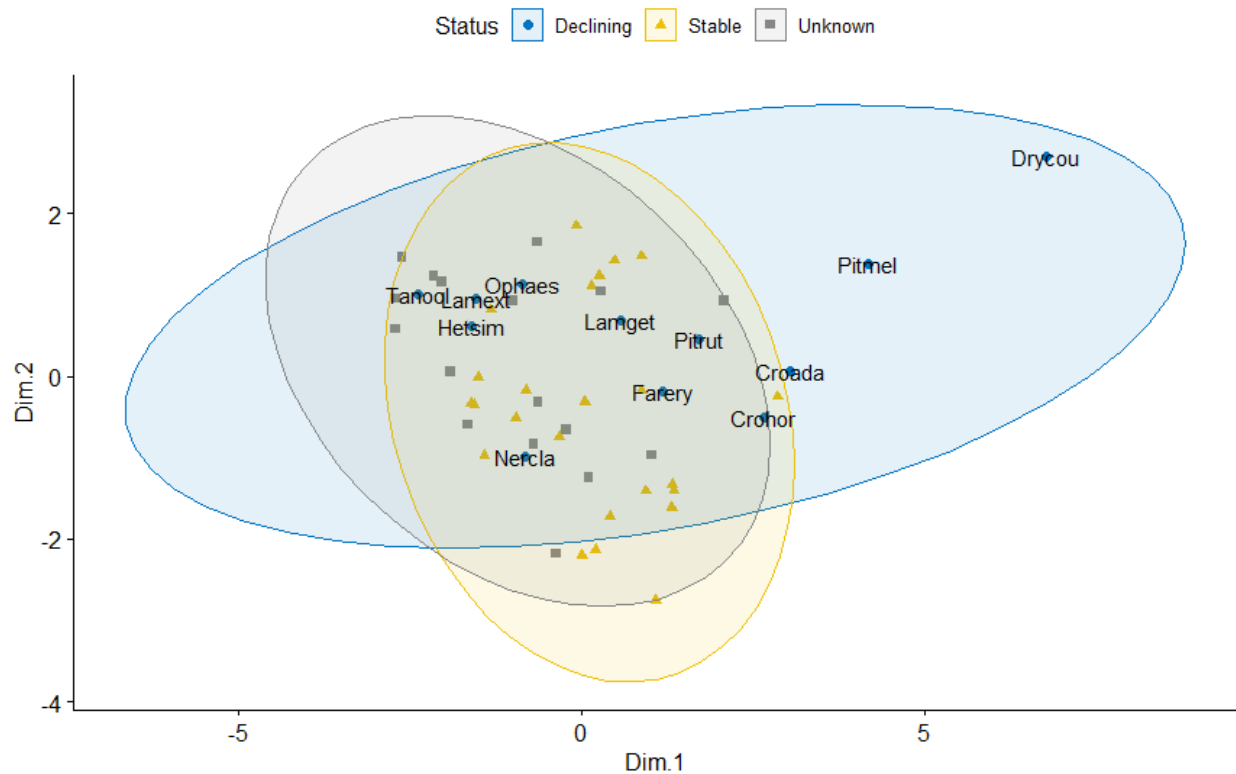


Figure 2. Separation of species along the first two dimensions of principal component analysis (PCA), with species status color-coded. Declining species are labelled with species abbreviations. Drycou = *Drymarchon couperi*, Pitmel = *Pituophis melanoleucus*, Pitrut = *P. ruthveni*, Croada = *Crotalus adamanteus*, Crohor = *C. horridus*, Farery = *Farancia erythrogramma*, Lamget = *Lampropeltis getula* complex, Lamext = *L. extenuata*, Ophaes = *Opheodrys aestivus*, Hetsim = *Heterodon simus*, Tanool = *Tantilla oolitica*, Nercla = *Nerodia clarkii*.

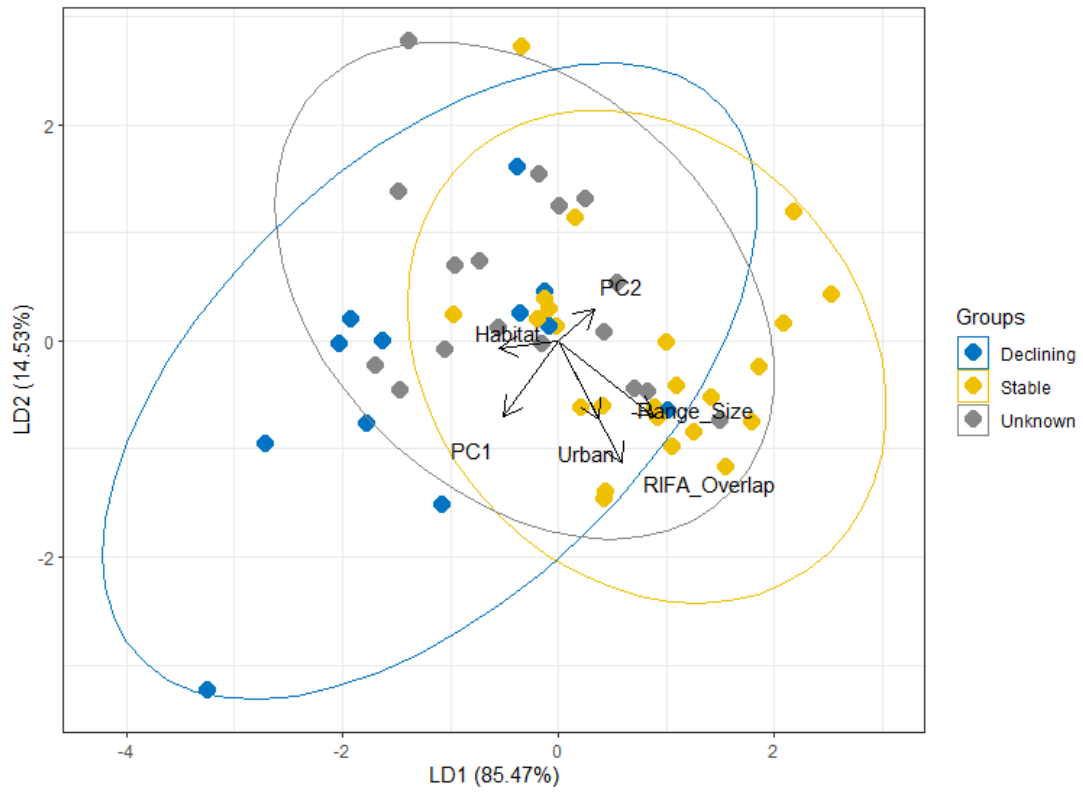


Figure 3. Color-coded species status groups as predicted by linear discriminant analysis (LDA). Coefficients of the linear discriminants are plotted as vectors.

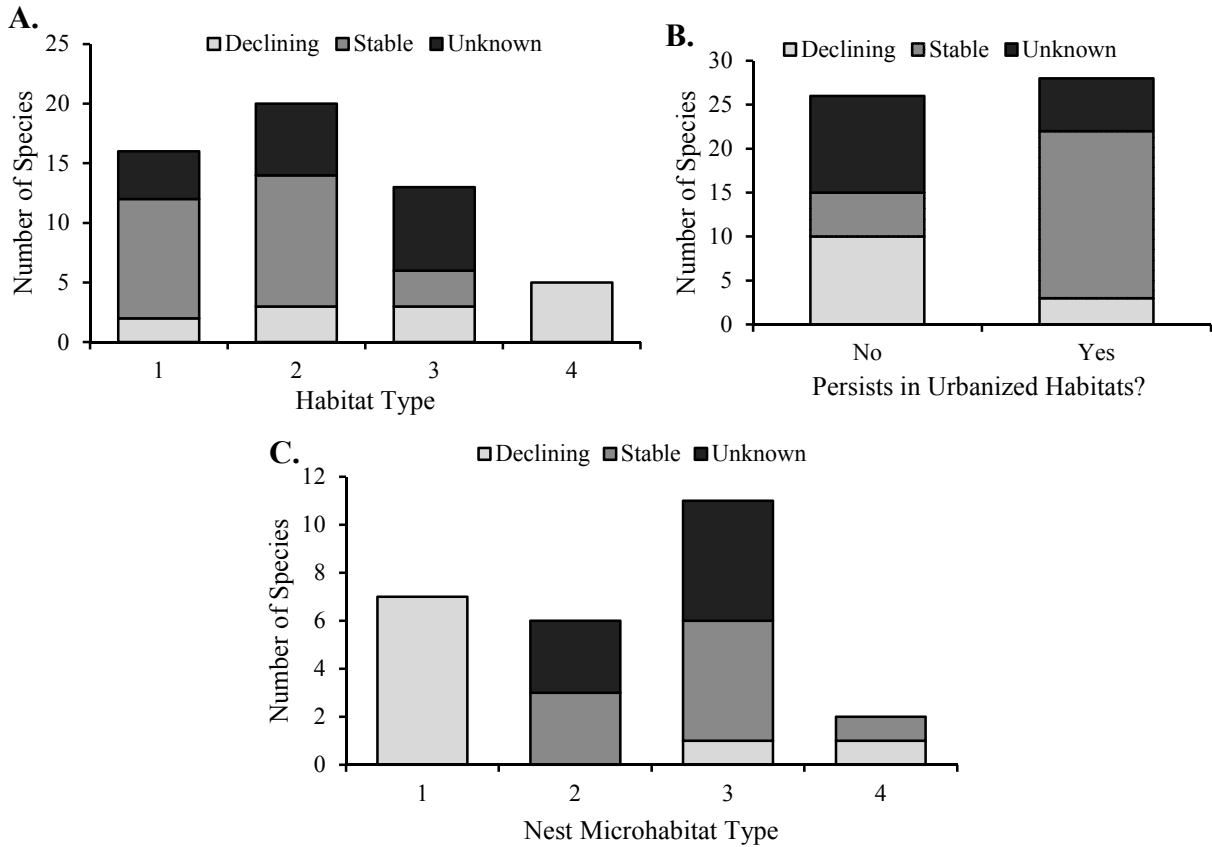


Figure 4. Number of species with declining, stable, and unknown status by habitat type (A), urbanized habitat use (B), and nest microhabitat (C). Habitat type: 1 = aquatic, 2 = habitat generalist/mesic forest, 3 = xeric/pine-associated, 4 = upland pine restricted. Nest microhabitat type: 1 = burrows/underground, 2 = shallow burrow or under debris, 3 = under debris, 4 = inside debris/aboveground.

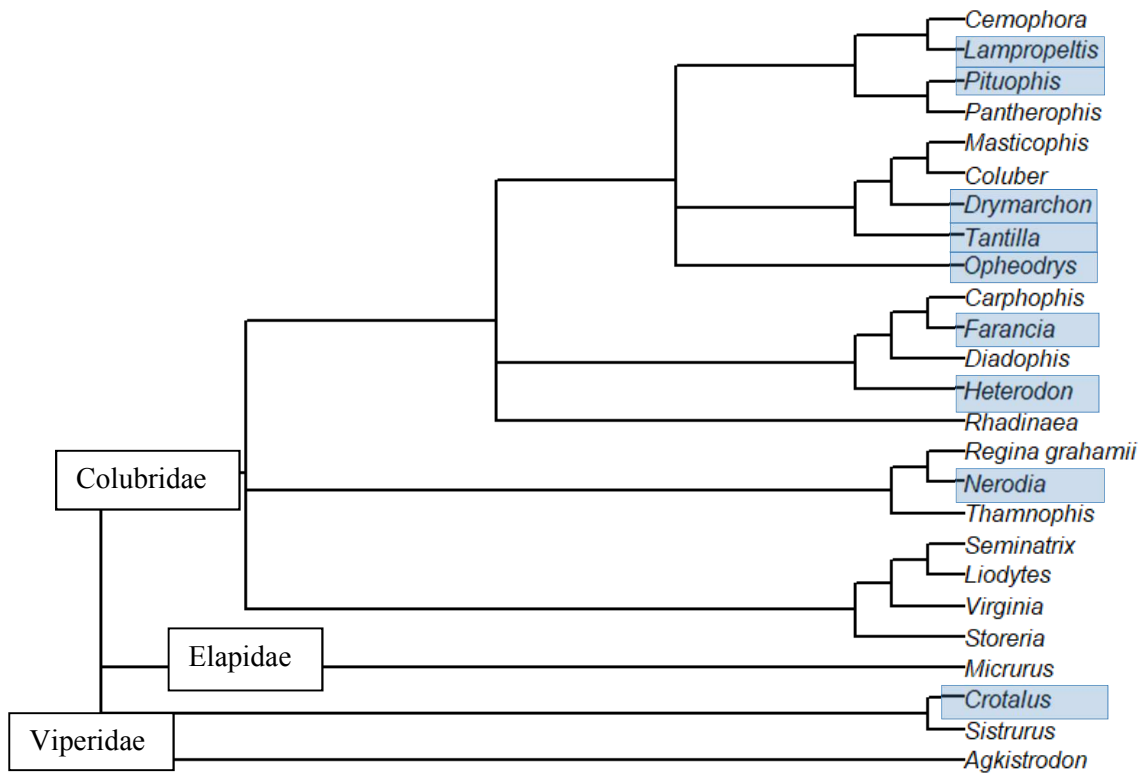


Figure 5. General cladogram of North American snake genera based on phylogeny of Pyron et al. 2011. Genera containing species considered “Declining” in this study are highlighted in blue.

Appendix 1 cont.

<i>Pantherophis emoryi</i>		X		X					X	X	
<i>Pantherophis guttatus</i>	X	X		X	X	X	X	X	X		X
<i>Pantherophis obsoletus</i>	X	X		X	X	X	X	X	X	X	X
<i>Pituophis lodingi</i>									X		
<i>Pituophis melanoleucus</i>	X	X		X	X	X	X	X	X		X
<i>Pituophis ruthveni</i>		X		X					X		X
<i>Regina grahamii</i>		X		X					X	X	X
<i>Regina septemvittata</i>		X		X	X	X		X	X	X	
<i>Rhadinaea flavilata</i>	X	X		X	X				X	X	
<i>Sistrurus miliarius</i>		X		X	X	X		X	X	X	X
<i>Storeria dekayii</i>		X			X			X	X		X
<i>Storeria occipitomaculata</i>		X			X			X	X		X
<i>Tantilla coronata</i>	X	X		X	X		X	X	X		
<i>Tantilla gracilis</i>		X							X		X
<i>Tantilla oolitica</i>		X		X					X		
<i>Tantilla relicta</i>		X		X	X				X		
<i>Thamnophis proximus</i>		X		X					X	X	X
<i>Thamnophis sauritus</i>	X	X		X	X	X	X	X	X		X
<i>Thamnophis sirtalis</i>	X	X	X	X	X	X	X	X	X	X	X
<i>Virginia valeriae</i>		X			X			X	X		X

* also, Wines, M. P., V. M. Johnson, B. Lock, F. Antonio, J. C. Godwin, E. M. Rush and C. Guyer. 2015. Optimal husbandry of hatchling eastern indigo snakes (*Drymarchon couperi*) during a captive head-start program. *Zoo Biology* 34: 230-238; Wines, M. P. 2014. Optimal egg incubation temperature, and the effects of diet on growth of hatchling Eastern Indigo Snakes (*Drymarchon couperi*) during a captive head-start program. MSc. Dissertation. Auburn University.

+ also Beane, J. C., M. D. Martin, D. C. Smith and S. J. Horton. 2021. *Heterodon simus* (Southern Hog-nosed Snake). Reproduction/Nesting. *Herpetological Review*

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Appendix 1 cont.

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Appendix 2. List of 53 snake taxa and RIFA (denoted by +) occurrence data used to calculate percent range overlap with RIFA with GBIF DOI accession numbers for databases.

Species	DOI
<i>Agkistrodon contortrix</i>	10.15468/dl.ywwafh
<i>Agkistrodon piscivorus</i>	10.15468/dl.jhub9r
<i>Carphophis amoenus</i>	10.15468/dl.aucpaz
<i>Cemophora coccinea</i>	10.15468/dl.dyuvxs
<i>Coluber constrictor</i>	10.15468/dl.w427ay
<i>Coluber flagellum</i>	10.15468/dl.r7sw97
<i>Crotalus adamanteus</i>	10.15468/dl.ud3699
<i>Crotalus horridus</i>	10.15468/dl.vphhy9
<i>Diadophis punctatus</i>	10.15468/dl.y7ybs
<i>Drymarchon couperi</i>	10.15468/dl.583sp6
<i>Farancia abacura</i>	10.15468/dl.76kakw
<i>Farancia erythrogramma</i>	10.15468/dl.kmghe5
<i>Haldea striatula</i>	10.15468/dl.svvnct
<i>Heterodon platirhinos</i>	10.15468/dl.jg6rt3
<i>Heterodon simus</i>	10.15468/dl.s78yg9
<i>Lampropeltis calligaster</i>	10.15468/dl.athqgw
<i>Lampropeltis elapsoides</i>	10.15468/dl.k467qe
<i>Lampropeltis extenuata</i>	10.15468/dl.8qz7ez
<i>Lampropeltis getula</i>	10.15468/dl.5yq6zk
<i>Lampropeltis triangulum</i>	10.15468/dl.vgxnr8
<i>Liodytes alleni</i>	10.15468/dl.r2q7ps
<i>Liodytes pygaea</i>	10.15468/dl.6584fu
<i>Liodytes rigida</i>	10.15468/dl.8jac47
<i>Micrurus fulvius</i>	10.15468/dl.dg6gfu
<i>Micrurus tener</i>	10.15468/dl.pqzdaa
<i>Nerodia clarkii</i>	10.15468/dl.afdwgc
<i>Nerodia cyclopion</i>	10.15468/dl.86j6gd
<i>Nerodia erythrogaster</i>	10.15468/dl.yvvn8v
<i>Nerodia fasciata</i>	10.15468/dl.cq8bx9
<i>Nerodia floridana</i>	10.15468/dl.ny3k46
<i>Nerodia rhombifer</i>	10.15468/dl.fk4j9a
<i>Nerodia sipedon</i>	10.15468/dl.bckzhh
<i>Nerodia taxispilota</i>	10.15468/dl.432f48
<i>Opheodrys aestivus</i>	10.15468/dl.4rxcm3
<i>Pantherophis emoryi</i>	10.15468/dl.9t32k5
<i>Pantherophis guttatus</i>	10.15468/dl.e9atcf
<i>Pantherophis obsoletus</i>	10.15468/dl.akuzjp
<i>Pituophis melanoleucus</i>	10.15468/dl.28sfxe
<i>Pituophis ruthveni</i>	10.15468/dl.q6aapr
<i>Regina grahamii</i>	10.15468/dl.rfdanm
<i>Regina septemvittata</i>	10.15468/dl.gvzrft
<i>Rhadinaea flavilata</i>	10.15468/dl.9m7wcx
<i>Sistrurus miliarius</i>	10.15468/dl.8uj99g
<i>Storeria dekayi</i>	10.15468/dl.vnuaw8
<i>Storeria occipitomaculata</i>	10.15468/dl.7eptag
<i>Tantilla coronata</i>	10.15468/dl.ms5f7u
<i>Tantilla gracilis</i>	10.15468/dl.ad3yu6
<i>Tantilla oolitica</i>	10.15468/dl.8c2ds8
<i>Tantilla relicta</i>	10.15468/dl.n4pj7d
<i>Thamnophis proximus</i>	10.15468/dl.xekf6d
<i>Thamnophis sauritus</i>	10.15468/dl.mexzj8
<i>Thamnophis sirtalis</i>	10.15468/dl.gx7yjt
<i>Virginia valeriae</i>	10.15468/dl.e82532
<i>Solenopsis invicta</i> +	10.15468/dl.t3ydu2

Conclusions

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The increase in numbers of enigmatic reptile population declines being reported worldwide is alarming. There are relatively few studies investigating food web interactions as possible drivers of decline in squamate reptiles, especially in the context of egg predation. In this dissertation, I have provided a foundation for future studies examining ant predation on squamate reptile eggs, continued research on mechanisms of decline for terrestrial lizards in Costa Rica, and evaluated vulnerability of snake species to RIFA invasion in the southeastern U.S.

In Chapter 1, I reported results of a field experiment at La Selva Biological Station, Costa Rica, that tested whether leaf litter depth influenced predation rates on leaf litter anole (*Anolis humilis*) eggs. I had three hypotheses: (1) that egg survival would be highest in deep leaf litter; (2) that predatory ant activity would be positively correlated with predation on lizard eggs; and (3) that ant abundance would be highest in deep leaf litter. I used 30 leaf litter plots, with three treatments: litter removal (L-), litter addition (L+), and a control (L0) that was sham manipulated to control for effects of litter disturbance. I set out 34 *Anolis humilis* eggs collected from gravid females in captivity and monitored survival. I assessed predatory ant activity using mass lost from tuna bait stations. The results supported hypothesis 2, but lizard egg survival, predatory ant activity, and ant abundance did not differ with litter depth. I found 50% of lizard egg mortality could be attributed to invertebrate predators, which mirrored previous findings of 69% of egg mortality caused by *Solenopsis* ants in Panama (Andrews 1988). The results signify that invertebrate predators are an important source of egg mortality for tropical lizards. However, I did not find any evidence of a negative correlation between litter depth and egg survival that could contribute to lizard declines. Our results indicate that further investigation of leaf litter community dynamics is needed for this system.

In Chapter 2, I tested whether nest microhabitat or forest type influenced predation on lizard eggs, predatory ant activity, ant abundance, lizard prey availability and the number of lizards and lizard eggs. I conducted quadrat surveys of 120 plots across four forest types (old-growth forest, abandoned agroforestry, abandoned plantation, and secondary forest) and three microhabitat types (buttress, fallen log, and leaf litter) at La Selva Biological Station, Costa Rica. I hypothesized that (1) lizard egg abundance would be greater in abandoned plantation habitat and buttress microhabitat; (2) egg survival would differ across forest type and microhabitat; (3) predatory ant activity would positively correlate with predation on lizard eggs; and (4) predatory ant numbers would be negatively correlated with number of lizard eggs. I observed significantly more lizard eggs in buttress and fallen log microhabitats than leaf litter, providing partial support for hypothesis 1, but I found no support for the other three hypotheses. I found that lizard observations were positively correlated with the number of prey invertebrates trapped, supporting previous findings that prey availability is important for tropical anole lizard populations (Guyer 1988, Lister and Garcia 2018). The results of this study suggest that microhabitat and prey availability should be considered in future studies of tropical lizard populations.

In Chapter 3, I used short term field trials, an artificial nest field experiment, and laboratory incubation, respectively, to evaluate whether (1) RIFA could successfully depredate intact eggs from six species of native terrestrial snakes, (2) RIFA predation at the end of incubation differed between a species that has apparently declined in the Southeast (*Lampropeltis holbrooki*) and a species that has remained common (*Coluber constrictor*), and (3) snake species differed in pip–hatch and incubation times. I hypothesized that *C. constrictor* would experience less predation by RIFA than *L. holbrooki* and have shorter pip–hatch times.

For short term field trials, between 2 and 20 eggs from each of six snake species - *C. constrictor*, *L. holbrooki*, *L. calligaster*, *Opheodrys aestivus*, *Pantherophis obsoletus*, and *P. emoryi* - were set next to RIFA mounds for 12 hours early in incubation. In artificial nest field experiments, I buried 30 *C. constrictor*, 30 *L. holbrooki* and 9 *P. obsoletus* eggs in groups of three near RIFA mounds late in incubation and monitored through hatching. I monitored a subset of eggs from each clutch in the lab to obtain pip–hatch and incubation times. I found that *C. constrictor* had significantly shorter pip–hatch times than any other species, but probability of predation did not differ between species. However, RIFA predation rates on snake eggs were high overall for all species (25-67% during field trials, 50-100% at end of incubation field experiment), signifying that RIFA could be a substantial source of mortality for snake eggs. This study revealed that RIFA are important predators of snake eggs in general, but more in-depth studies incorporating nest microhabitat, time spent in the nest, and eggshell characteristics might be needed to fully explore interspecific variation in vulnerability to egg predation.

In Chapter 4, I evaluated whether ecological variables predicted to enhance vulnerability to RIFA invasion were strong predictors of declining status in southeastern U.S. snake species, along with factors known to associate with extinction risk (i.e., slow life history, small geographic range size). I predicted that declining status would be associated with greater geographic range overlap with RIFA, oviparity, nesting terrestrially or underground, smaller clutches, longer incubation times, and use of open canopy xeric/pine habitats, all of which would increase species' vulnerability to egg predation by RIFA. I reviewed existing life history and ecological data for 53 snake taxa inhabiting the Coastal Plain of the southeastern U.S. to generate a series of predictor variables for snake species status (Declining, Stable, or Unknown). I found that declining species had smaller geographic range sizes, large percent range overlap with

RIFA, nested in underground burrows, were more associated with upland xeric/pine habitats and were less tolerant of urbanized habitats. The results also supported well documented associations between slow life history characteristics (i.e., low clutch size, greater age at maturity, etc.) and declining status. Overall, results supported my predictions and provided evidence that habitat loss and RIFA invasion are drivers of snake population declines in the southeastern U.S.

In summary, our combined results reveal that ants are an important source of egg mortality for lizards and snakes in both a tropical and temperate site. However, although I found evidence for RIFA as a driver of snake declines in the southeastern U.S., my results did not support the idea that changes in ant predation have caused lizard declines in Costa Rica. One reason for our lack of evidence may be the complexity of tropical systems, limitations of our study designs, or there could be other mechanisms driving declines in anole lizards, such as reduced prey availability or loss of favorable microclimates. Future research and conservation efforts in the southeastern U.S. should focus on reducing RIFA abundance in habitats of threatened lizard and snake species, and in Costa Rica, future research should investigate whether there have been long-term declines in prey availability associated with declines in litter depth that might explain reduced lizard populations.

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