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**Sublethal Effects of Red Imported Fire Ant Envenomation on Hatchlings
of North American Oviparous Snakes**

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Environmental, Soil, and Water Science

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Abstract

Invasive species cause major ecological and economic damage. The Red Imported Fire Ant (RIFA; *Solenopsis invicta*) has successfully invaded much of the southeastern United States and has caused both widespread economic damage and is suspected to be the driver of enigmatic declines of several oviparous snake species. This study aimed to determine the sublethal effects of RIFA on hatchlings of six species of oviparous snakes (*Coluber constrictor*, *Lampropeltis calligaster*, *L. holbrooki*, *Opheodrys aestivus*, *Pantherophis emoryi*, and *P. obsoletus*) by exposing hatchlings to envenomation by RIFA and then measuring two performance metrics: righting response and swimming speed. We found a slight effect of envenomation on righting response that did not differ among species, but no significant effect of envenomation on swimming speed. However, we found a strongly significant effect of species on both performance metrics, suggesting that different species may have different responses when envenomated. This study provides as a baseline to further explore the impact of RIFA on native reptiles.

Introduction:

Invasive species pose immense risk to native wildlife around the world. Invasive species often outcompete native species and can cause irreversible ecological damage to native species and ecosystems through predation, competition, disease, parasite transmission, hybridization, and an array of indirect effects (Eiswerth and Johnson, 2002; Andersen et al., 2004; Dukes and Mooney, 2004). Additionally, invasive species are costly to the economy, public health, and nonmarket products, with invasive species causing damages that amount to almost \$120 billion a year in the United States (Andersen et al., 2004; Pimentel et al., 2005). The success of invasive species often results from their ability to tolerate a wide range of climatic conditions or aspects

of their behavior—such as aggression, migratory tendencies, or predator avoidance—that greatly improve the chance of establishment and spread (Weis, 2010).

Red Imported Fire Ants (*Solenopsis invicta*) have successfully invaded the southeastern United States with devastating consequences to both the economy and the ecosystem. Red Imported Fire Ants (henceforth RIFA) were introduced to the United States from South America through the port of Mobile, Alabama between 1933 and 1941 (Buren et al., 1974; Hoffman, 1995). RIFA have spread throughout much of the southeastern United States and have already caused widespread economic and ecological damage (Kemp et al., 2000). Across the United States, RIFA are calculated to have caused over \$5 billion in damages to agriculture, residential homes, and commercial and federal areas, with over \$3 billion in damage to residential homes alone (Lard et al., 2006). In terms of ecological impacts, RIFA are reported to have caused direct declines in populations of native species due to competition and predation on species that often lack appropriate defense behaviors in response to RIFA (Allen et al., 2004). RIFA display aggressive foraging behavior, lack of competitors or predators in their invasive range, and high reproductive potential, which aids in their invasive potential (Allen et al., 2004). They have particularly strong effects on vertebrates due to these factors and their venomous sting (Allen et al., 2004). RIFA venom mostly consists of water-insoluble 2,6- disubstituted piperidine alkaloids (Hoffman, 1995) and can cause cardiorespiratory failure and seizures in mammals (Howell et al., 2005).

Reptiles and amphibians are declining dramatically across the globe due to a combination of six factors: habitat loss and degradation, environmental pollution, disease, global climate change, unsustainable use, and invasive species (Gibbons et al., 2000; Collins, 2010; Todd et al., 2010). Several studies have speculated that some reptile declines, including ‘enigmatic’ declines

of several oviparous snake species in the southeastern US, such as members of the common kingsnake species complex (*Lampropeltis getula*, *L. holbrooki*, *L. nigra*), may be attributed to introduction of RIFA (Winne et al., 2007). Reptiles have displayed vulnerability to RIFA—there have been several studies on the direct effects of RIFA, such as predation on turtles and their nests (Landers et al., 1980; Buhlmann and Coffman, 2001; Allen et al., 2001; Parris et al., 2002). Indirect effects, however, are harder to quantify. For example, one study found that neonatal alligators had reduced body mass indices when in areas where RIFA are present (Allen et al., 1997). Another study also focusing on alligators found declining nest attendance when RIFA were present (Reagan et al., 2000). Turtles were also found to abandon nest excavation due to RIFA presence (Whiting, 1994). Both the juveniles and eggs of oviparous snake species were found to be vulnerable to predation by RIFA (Swartwout and Willson, in review). However, only a few of these studies have examined the foundational relationship between envenomation and performance in reptiles. Although one study examined sublethal effects of RIFA envenomation in fence lizards (*Sceloporus undulatus*; Boronow and Langkilde, 2009), no studies have evaluated the effects of RIFA venom on snakes or examined interspecific variation in sensitivity to envenomation that might mediate species' vulnerability to RIFA.

In this study, we aimed to test the threat posed by RIFA to oviparous snakes in the southeastern United States and determine if hatchlings of native snake species differ in their behavioral and physiological responses to RIFA. Specifically, we exposed juveniles of six oviparous snake species (*Coluber constrictor*, *Lampropeltis calligaster*, *L. holbrooki*, *Ophedryx aestivus*, *Pantherophis emoryi*, and *P. obsoletus*) to RIFA envenomation and measured righting response and swimming performance. Our study provides baseline data on the risk posed by RIFA to native snake species that can be used to assess the current and future effects of RIFA

invasions. We expected to find a negative relationship between RIFA exposure and performance that varied across species. We also expected species that have experienced enigmatic declines in parts of their range (e.g., *Lampropeltis holbrooki*) to be most strongly affected.

Materials and Methods:

We incubated and hatched eggs (N=125) of six terrestrial snake species native to Arkansas in an environmental chamber (Model# 141VLC9, Percival Scientific) at 25°C and 80% relative humidity (for details of collection and incubation see Swartwout and Willson, in review). Species included: Eastern Racer (*Coluber constrictor*; N=22 from 5 clutches), Prairie Kingsnake (*Lampropeltis calligaster*; N=19 from 3 clutches), Speckled Kingsnake (*Lampropeltis holbrooki*; N=42 from 8 clutches), Rough Green Snake (*Ophedryx aestivus*; N=17 from 3 clutches), Great Plains Rat Snakes (*Pantherophis emoryi*; N=12 from 1 clutch), and Black Rat Snake (*Pantherophis obsoletus*; N=13 from 2 clutches). Within 24 hours after hatching, we weighed each hatching (to the nearest 0.01 g), sexed them, and took snout-vent-length (SVL) and tail length (TL) measurements. We housed hatchlings by clutch in 10-20 gallon aquariums with 40-watt lightbulbs or heat tape positioned at one end to provide a thermal gradient. Each aquarium was outfitted with aspen bedding and at least one hide. Water was available ad libitum.

Prior to experiments, we separated hatchlings into 17.55 x 17.25 x 12.19 cm tupperware containers and placed them in an environmental chamber set to 25°C and 80% relative humidity for 24 hours in order to acclimate them to the tupperware and environmental conditions.

Acclimation—In order to limit confounding variables, ca. 24 h prior to baseline and trial performances, we acclimated hatchlings to a swim track constructed out of a single 304.8 x 15.24 cm aluminum rain gutter with 5 cm deep water. The track was marked at 1 cm increments along its length and water temperature was maintained at 25°C using two submersible aquarium

heaters. Prior to trials, we verified water temperature using an infrared digital thermometer. Each hatchling was prompted to swim the full length of the swim track once. If a hatchling was stationary while in the swim track, we encouraged it to swim via gentle prodding. After the acclimation trials were completed, we placed hatchlings back in their individual tupperware containers and returned them to the environmental chamber.

Baseline Trials—We measured baseline performance to provide a control for treatment effects. We ran baseline trials ca. 24 hours after acclimation trials and all trials were recorded using a digital video camera (Sony Handycam HDR-CX190; 29.97 fps). Baseline righting trials took place in a 41.91 x 27.43 x 16.51 cm plastic container lined with a paper towel for traction. We suspended the video camera from a tripod and centered it over the tupperware. Following the procedures of Boronow and Langkilde (2009), we held each hatchling underneath the jaw and at the vent, then flipped it on its back so both the head and vent were bottom up. Hatchlings were then released and allowed to return to their stomach. Immediately upon righting, we flipped the hatchling on its back again until three consecutive flips were achieved.

Swimming performance procedures followed the general methods of Winne and Hopkins (2006). We placed each hatchling in the water at the start of the track and allowed it to swim forward. One experimenter followed the hatchling and provided encouragement via gentle prodding if the snake stopped. A second experimenter held the video camera and recorded the runs. Hatchlings swam a total of 152.4 cm three consecutive times. We analyzed videos and quantified them frame-by-frame.

Experimental Trials—We conducted trials with RIFA ca. 24 hours after baseline trials were completed. We collected several hundred RIFA workers and larvae using a shovel from naturalized colonies around the University of Arkansas campus, Fayetteville, Arkansas, and

placed them in a 2 L plastic container with Insect-a-Slip (PTFE Fluoropolymer DISP30) around the rim to prevent escape. In the lab, the container was kept under a heat lamp to keep the ants active while trials were running.

All tests were conducted in a lab environment to control variables when hatchlings were exposed to RIFA venom. A digital video camera (Sony Handycam HDR-CX190) was suspended from a tripod and centered over the plastic container to quantify exposure to stinging RIFA. We placed each hatchling individually into the RIFA container for 10 seconds, then immediately removed the hatchling (with clinging ants) and placed it in a 17.55 x 17.25 x 12.192 cm tupperware container covered with a clear acrylic lid, allowing the RIFA to continue to sting. After 180 seconds in the tupperware container, we removed all RIFA from hatchlings. We counted each stinging ant to quantify potential venom exposure, while the hatchling was placed in a clean tupperware and allowed to rest in the environmental chamber for 10 minutes prior to performance trials. We further quantified the number of ant stings based on video footage. Each video was played using the slow speed setting. We counted each ant that crawled on the hatchling's body during the 180 second exposure using a tally counter.

Following the 10 minute rest, we tested and recorded righting performance (3 successive trials) and swimming performance (3 successive trials) in the same manner as during baseline trials. After performance measures, we placed hatchlings in the environmental chamber for another ca. 12 hours before returning them to their original cages.

Video Analysis— We completed video analysis for righting response using Windows Media Player. We analyzed each video by counting each video frame from the frame the hatchling was released to the frame where both the head and vent of the hatchling had touched the ground. Frames were counted using a tally counter. This was done for all three righting

ability trials. We excluded any righting trials that did not meet the criteria for a successful righting ability—where the snake was released fully on its back with the neck and base of the tail touching the ground—from analyses.

We completed video analysis on swimming speed by analyzing the videos from 10 hatchlings per species using Tracker: Video Analysis and Modeling Tool v. 5.1.5 (Open Source Physics). An anchor point was placed on each 10 cm increment. We counted the individual frames from the time the tip of the snake's nose crossed the first marked increment to the time the snake's nose crossed the last increment. We recorded the amount of frame required to cover each 10 cm interval. We defined maximum swim speed (maxS) as the fastest velocity achieved across a 10 cm interval and converted the measure from video frames to velocity in centimeters per second using the formula: $(10 \text{ cm} / \text{no. FRAMES}) * (29.97 \text{ frames} / 1 \text{ sec})$.

Statistical Analysis— We quantified RIFA venom exposure by counting the number of ants on the hatchling's body in the video and then dividing that number by the hatchling snake's mass to get ants/g. Any individual ants that attached themselves throughout the video were counted, so there was the possibility of double-counting if an ant fell off and then reattached and stung again, meaning that we may have overestimated the number of ants. We used a One-way ANOVA to determine whether snake species statistically differed in exposure to fire ants, with a Tukey HSD post-hoc test for significance among snake species.

For hatchling righting ability, we used the minimum number of frames taken for a snake to right itself after exposure to stinging RIFA across the three righting trials as our outcome (minR). After removing individuals where minR or ants/g could not be determined, we had 117 observations of righting ability. We used a linear mixed effects model with minR as the dependent variable, species and ants/g exposure as fixed effects, and trial date as a random

effect. We decided not to include clutch as a random effect because its ICC2 value was less than 0.7, where values > 0.7 are considered highly reliable at differentiating levels within the dependent variable (Kensler et al., 2009). We ran a model with an interaction between fixed effects and found that there was no significant interaction between species and ants/g (LRT: $df = 5$, $P = 0.89$) so we used a model with species and ants/g as non-interactive fixed effects.

For swim speed analyses, we used the minimum number of frames for an individual snake to swim 10-cm during the first 152-cm long trial run (maximum swim speed; maxS) as our response variable. Due to constraints on time, we had only 10 observations per species (60 observations total). We used a linear mixed effects model with maximum swim speed as the dependent variable, species and ants/g as fixed effects and clutch as a random effect. We did not include trial date in this model as a random effect because its ICC values were very low and the model fit was improved without it. As with righting ability analyses, we ran a model with an interaction between fixed effects and found no significant interaction between species and ants/g (LMM: $df = 5$, $P = 0.34$), so we used a model with species and ants/g as non-interactive fixed effects.

We used likelihood ratio tests (LRT) with single term deletions to determine p-values of fixed effects in our models. For significant species effects, we used a Tukey Contrast post-hoc test to determine which species differed by the response variable (minR or maxS). We tested assumptions of linearity, homogeneity of variance, and normality of residuals for linear mixed effect models using plots of residuals and Levene tests (Levene, 1960). All analyses were conducted in R v. 3.6.1 using packages “car”, “MASS”, “lme4”, “multcomp” and “multilevel” (Venables and Ripley, 2002; Hothorn et al., 2008; Bates et al., 2015; Bliese, 2016; Fox and Weisberg, 2019; R Core Team, 2019).

Results:

Hatchling snakes were exposed to between 1 and 136 stinging ants (0.22 to 36.8 ants/g; mean = 5.56 ± 0.54 SE ants/g). *Opheodrys aestivus* were exposed to significantly more ants/g than all other species (One-way ANOVA: $F_{5,111} = 10.2$, $P < 0.01$), but no other snake species differed in exposure (all $P > 0.3$; Figure 1).

We found a significant effect of species on minR (LMM: $df = 5$, $P < 0.01$) and a marginal effect of ants/g ($df = 1$, $P = 0.06$; Figure 2). Across species, individuals exposed to the highest rates of RIFA envenomation righted 0-13 seconds slower than those with the lowest exposure. *Coluber constrictor*, *Lampropeltis calligaster* and *Pantherophis emoryi* were significantly faster at righting than *O. aestivus* (all $P < 0.02$; Figure 3). Based on relatively high ICC values (ICC1 = 0.22, ICC2 = 0.79), the random factor (Trial Date) could be reliably differentiated by the response variable, minR (Zuur et al., 2009, Kensler et al., 2009).

We observed a significant effect of species on maxS (LMM: $df = 5$, $P = 0.02$), but no effect of ants/g treatment ($df = 1$, $P = 0.18$; Figure 4). *Coluber constrictor* swam significantly slower than *L. holbrooki* ($P < 0.01$; Figure 5). *C. constrictor* were the slowest swimmers overall (14.4 ± 1.86 SE frames) and *L. holbrooki* were the fastest (8.81 ± 1.01 SE frames). The random factor in the model (Clutch) explained much of the variance in the outcome and could be reliably differentiated by the response variable, maximum swim speed (ICC1 = 0.61, ICC2 = 0.82; Zuur et al., 2009).

Discussion:

The objectives of this study were to investigate sublethal effects of RIFA envenomation on hatchling snake performance, and variation in those effects among snake species. We also investigated species effects during performance trials. We found subtle negative effects of

envenomation on righting ability that were consistent across species, providing some support for our predictions, but no effects of envenomation on swim speed, despite snake species being exposed to widely varying levels of envenomation.

The lack of substantial sublethal effects seen during our trials, especially swimming performance, mirrors previous findings of Boronow & Langkilde (2009). Similar to our study, Boronow & Langkilde (2009) focused on envenomation effects utilizing sprint speed, righting ability, and bite force to measure performance in *Sceloporus undulatus* (Eastern Fence Lizard), but found no significant effects between these performance measures and RIFA envenomation. One explanation for the lack of effects in our study could have been limitations on our ability to achieve levels of envenomation sufficient to induce locomotor impairment. However, we were successful in achieving a wide range of RIFA exposure, ranging from 0.2 to 36.8 ants/g. Further, we saw behavioral and physical responses that indicated successful venom delivery, including discoloration on the body and bumps underneath the snakes' scales after venom injection. As this was the first study on snake tolerance to RIFA envenomation, we had no other reference of their response or resilience; thus future studies should explore higher exposure rates to explore the possibility of stronger effects at higher rates of envenomation.

Our study was also limited by the fact that we were only able to quantify the number of stinging ants, not the exact amount of venom that each snake was exposed to. We used Ants/g instead of number of total stinging ants as our independent variable in this study in order to compensate for size differences among snake species, thereby simplifying our statistical analyses. However, there are constraints to using ants/g as well. Ants/g is likely an imprecise measure of the amount of venom each snake received and by using ants/g, we may have reduced our ability to detect relationships between RIFA exposure and performance. We did not measure

the amount of time each ant stayed attached while stinging and it is likely that the amount of venom injected varied among individuals. Quantifying the exact venom exposure could improve our ability to detect sublethal effects that were not found in this study, either by more tightly controlling how ants inject venom, or by injecting RIFA venom artificially.

Finally, high variability in performance measures may have weakened our power to detect significant effects of RIFA envenomation. We were able to account for several sources of intrinsic variation in performance by including species, clutch, and trial date, in our statistical model. Our experimental design aimed to account for additional variation in performance by taking baseline performance measurements prior to RIFA exposure. Having a baseline as a covariate would potentially allow us to account for some intrinsic variation in performance among individuals and thereby give greater power to detect RIFA effects. Unfortunately due to time constraints, we were not able to include baseline measures at this point, but baseline trials will be analyzed in the future, along with the remainder of the completed swim speed trials. These additional data should improve our ability to detect treatment effects of RIFA on swimming performance in our final analysis.

In contrast to Boronow & Langkilde's (2009) study, we did find evidence of subtle sublethal effects of RIFA venom on righting ability of oviparous snake hatchlings that were consistent across species. Specifically, we found that across species, individual hatchlings that experienced high rates of envenomation righted themselves approximately 0-13 seconds more slowly than those that experienced low rates of envenomation. This effect could have important consequences for hatchling survival and fitness. For example, slower righting response may be the difference between a hatchling snake successfully escaping from the nest chamber or being overwhelmed and predated by ants. Even in the event of escaping, envenomation may hinder a

hatchling's ability to escape other predators, find food, find shelter, or perform other behaviors that mediate fitness. Previous studies have found that RIFA have delayed effects on survival but not growth of *S. undulatus* and the same could be true for snakes (Langkilde & Freidenfelds, 2010).

Curiously, we found a stronger effect of envenomation on righting ability than on swimming performance. This suggests that swimming speed may not be as sensitive an indicator of sublethal physical and physiological impairment as righting ability. It would be interesting to further study the physiological effects of envenomation by utilizing other performance measurements. Other, more integrative performance measures, such as foraging ability, could be used to further quantify the full effects of envenomation. For example, Chin et al. (2013) completed a study on the sublethal effects of mercury contamination in neonatal northern watersnakes (*Nerodia sipedon*) and found that foraging performance and learning were affected, while simpler performance measures such as swimming speed were not.

We found strongly significant effects of species in both performance trials, suggesting that the variation in performance measures among species generally outweighs the sublethal effects of RIFA envenomation. Three of the snake species (*C. constrictor*, *L. calligaster* and *P. emoryi*) had significantly slower righting abilities than *O. aestivus*. Swimming performance varied differently; in order of fastest to slowest, the snake species ranked: *L. holbrooki*, *P. emoryi*, *P. obsoletus*, *L. calligaster*, *O. aestivus*, and *C. constrictor*. Factors that may drive the variation among species include body morphology, activity levels, or antipredator behavior (Jayne, 1985; Scribner and Weatherhead, 1995; Jayne, 1998; Baxter-Gilbert et al., 2018). These results suggest that different species could have different responses to RIFA venom. Our study

included limited sample sizes of several species; thus, future studies aimed at determining sublethal effects on snakes would benefit from larger sample sizes of more snake species.

Interestingly, we also found that clutch was an important predictor of swimming performance. While we did have a single *C. constrictor* outlier, clutch differences explained some variance we found in our swimming performance model. In snakes, clutches often vary in body size and other morphological traits (Ford & Seigel, 1989), which in turn can influence swimming performance (Hopkins & Winne, 2009). This result could also suggest that swimming performance is genetically inheritable and thus subject to selection. It is important to note that this finding suggests that clutch is an important factor to consider in future studies and it is essential to incorporate multiple clutches in experimental evaluations of hatchling performance.

The introduction of invasive species has been linked to declines of several reptile and amphibian species. RIFA is one such species that is rapidly expanding its introduced range and coming into contact with more reptile and amphibian species. Already, studies have found both indirect and direct effects of RIFA on reptiles and amphibians (Landers et al., 1980; Reagan et al., 2000; Allen et al., 1997; Whiting, 1994; Boronow and Langkilde, 2009). This study found subtle sublethal effects on performance when snake hatchlings were exposed to RIFA venom. RIFA have been found previously to prey on hatchling snakes, but this study supports the idea that RIFA have other adverse effects on hatchling snakes that may not have been detected previously (Swartwout and Willson, in review). However, we did not see patterns of interspecific variation in this effect that support the suspicion that enigmatic declines of some oviparous species (e.g., *Lampropeltis* sp.) are the result of RIFA predation. Although this study provides a baseline exploration of the sublethal effects of RIFA on hatchling snakes, future studies are

needed to fully characterize these effects and further explore the idea that RIFA may contribute to enigmatic reptile and amphibian declines.

Figures:

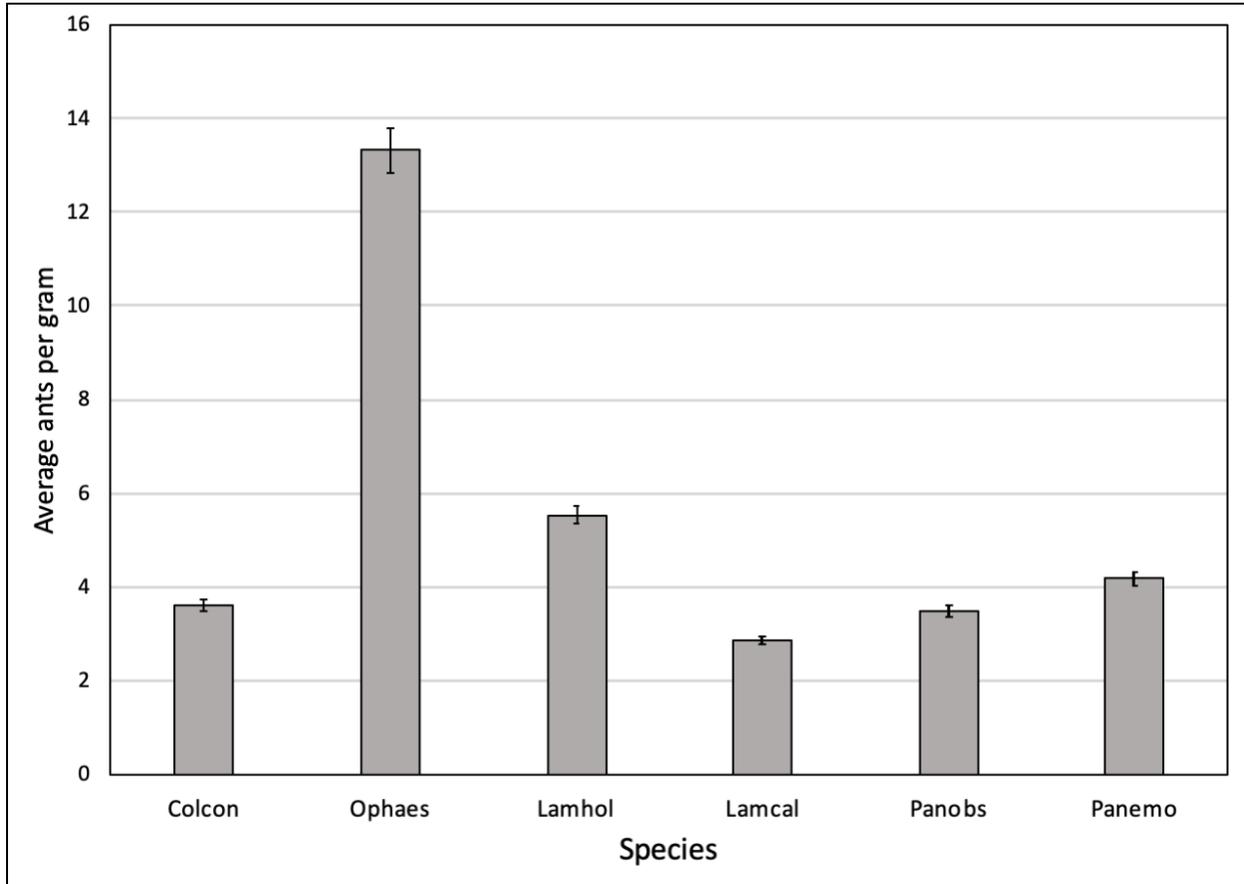


Figure 1: The average number of ants/gram by species. Species abbreviations: Colcon = *Coluber constrictor*, Lamhol = *Lampropeltis holbrooki*, Panemo = *Pantherophis emoryi*, Lamcal = *L. calligaster*, Ophaes = *Opheodrys aestivus*, Panobs = *P. obsoletus*

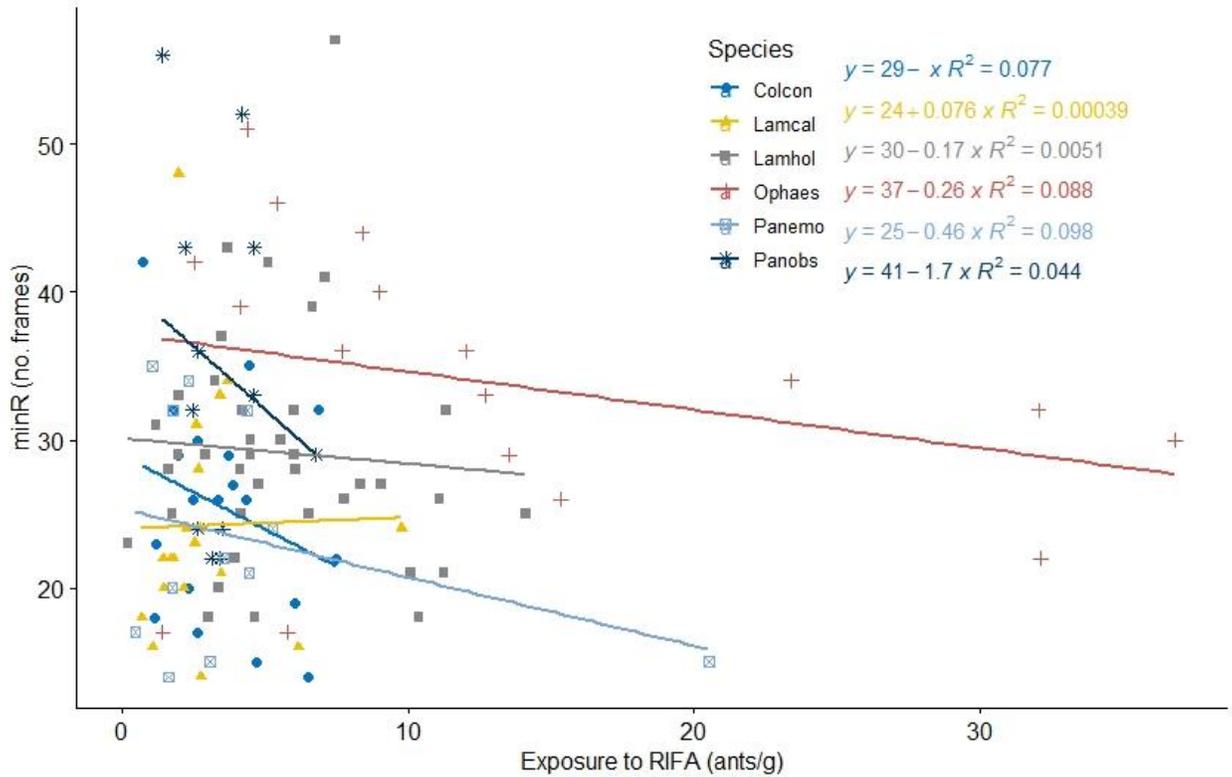


Figure 2: Righting response (minR) by exposure to fire ants (ants/g) across species. Equations are listed for linear relationships between righting ability and ants/g by species. Species abbreviations: Colcon = *Coluber constrictor*, Lamhol = *Lampropeltis holbrooki*, Panemo = *Pantherophis emoryi*, Lamcal = *L. calligaster*, Ophaes = *Opheodrys aestivus*, Panobs = *P. obsoletus*.

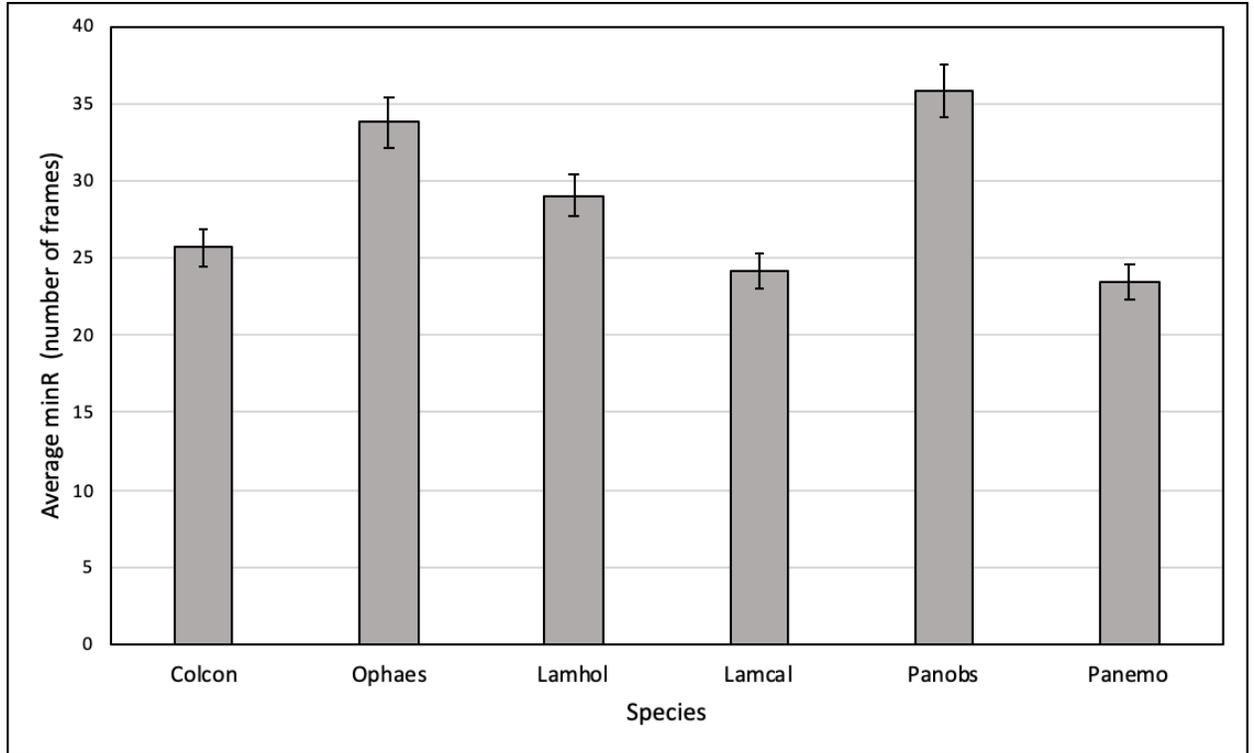


Figure 3: Average minimum righting response time (minR) by species. Species abbreviations:

Colcon = *Coluber constrictor*, Lamhol = *Lampropeltis holbrooki*, Panemo = *Pantherophis emoryi*, Lamcal = *L. calligaster*, Ophaes = *Opheodrys aestivus*, Panobs = *P. obsoletus*.

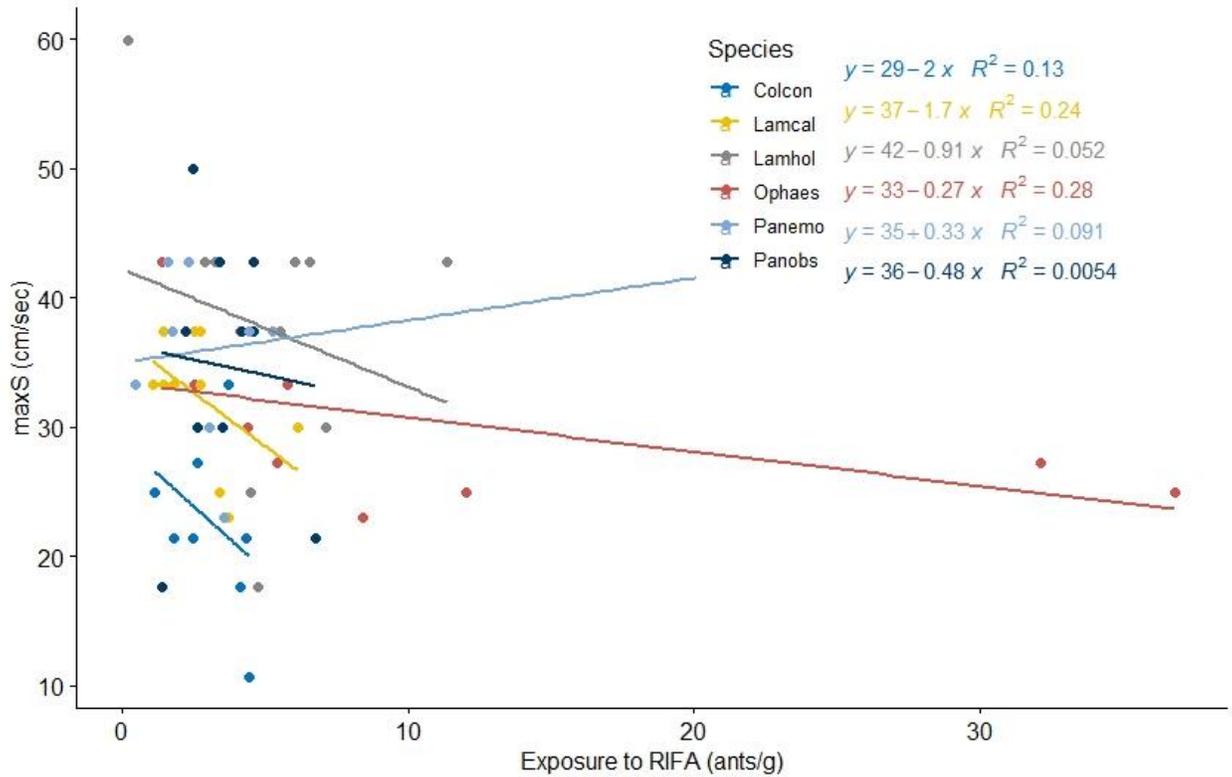


Figure 4: Maximum swim speed (maxS; maximum cm/sec over 10 cm interval) relative to exposure to fire ants (ants/g) across species. Equations are listed for linear relationships between swim speed and ants/g by species. Species abbreviations: Colcon = *Coluber constrictor*, Lamhol = *Lampropeltis holbrooki*, Panemo = *Pantherophis emoryi*, Lamcal = *L. calligaster*, Ophaes = *Opheodrys aestivus*, Panobs = *P. obsoletus*.

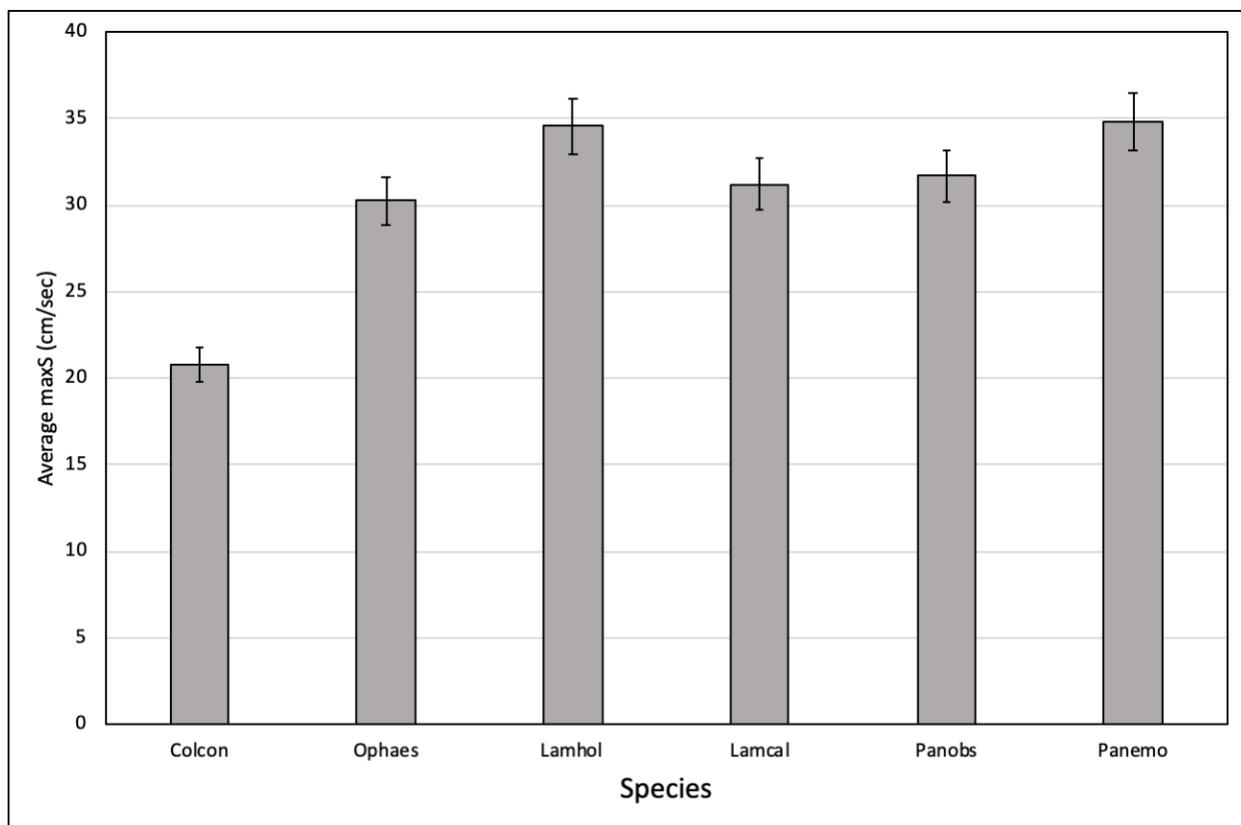


Figure 5: Average maximum swimming speed (maxS; maximum cm/sec over 10cm interval) of hatchlings of six oviparous terrestrial snake species. Species abbreviations: Colcon = *Coluber constrictor*, Lamhol = *Lampropeltis holbrooki*, Panemo = *Pantherophis emoryi*, Lamcal = *L. calligaster*, Ophaes = *Opheodrys aestivus*, Panobs = *P. obsoletus*.

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