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Experimental evidence that oral secretions of northwestern ring-necked snakes (*Diadophis punctatus occidentalis*) are toxic to their prey ☆

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Abstract

Ring-necked snakes (*Diadophis punctatus*) are suspected of being venomous because their Duvernoy's gland secretions have high levels of phospholipase activity, which is characteristic of many viperid and elapid venoms, and because anecdotal reports of feeding behavior are consistent with the use of a venom. We tested the toxicity of northwestern ring-necked snake oral secretions to a natural prey species, northwestern garter snakes (*Thamnophis ordinoides*), by injecting 2–35 µl of oral secretions intraperitoneally. All doses were 100% lethal within 180 min. The dose significantly affected the time to loss of a righting response. Neither injection of saline nor denatured oral secretions resulted in loss of a righting response or any visible detrimental effects. We suggest that northwestern ring-necked snakes may have evolved venom to subdue larger prey items than the snake would otherwise be capable of taking.

Keywords: *Diadophis punctatus*; Northwestern garter snake; Prey; Ring-necked snake; *Thamnophis ordinoides*; Venom

1. Introduction

Until the 1950s, snakes of the family Colubridae were generally considered to be harmless, especially in comparison to the Viperidae, Elapidae, and Atractaspididae. In part, this belief was due to the observation that colubrid dentition and glandular morphology differed from the specialized venom delivery systems of other taxa. However, there have been an increasing number of reports of serious human reactions to bites from colubrid snake species previously assumed to be nonvenomous. At the last count, 36 of the 320 colubrid genera are known to contain at least one venomous species and approximately 1.5% of colubrid species are considered venomous (Ernst and Zug, 1996; Minton, 1996; Pough et al., 2001), although some authors estimate that this number is as high as nearly half of the colubrids (~700 species, Mackessy, 2002).

The topic of colubrid envenomation has been debated both in the medical and in the herpetological literature. In part, this is because venom has

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rarely been precisely defined for use in an ecological or evolutionary context, which has hindered our understanding of the role of venom in the biology of snakes (e.g. Rodríguez-Robles, 1994; Kardong, 1996). In particular, discussion of envenomation from snakes typically revolves around its medical consequences as opposed to its ecological consequences (e.g. Kardong, 1996).

Because of the vast differences in response to venom among prey species (Grogan, 1974; Rodríguez-Robles, 1994) and among populations within a species (e.g. Poran et al., 1987), it is important to determine the toxicity of a snake's oral secretions to sympatric prey species before concluding that the species is venomous in the ecological sense of the word (sensu Kardong, 1996). The standard statistic used to describe the toxicity of venom is the LD_{50}, the dose that is lethal to 50% of laboratory mice (Chippaux, 2006). However, this statistic does not account for potential specificity to a particular prey type. For example, the venom of eastern hog-nosed snakes (*Heterodon platyrhinos = platirhinos*) is lethal to its amphibian prey but has no effect on mice (McAlister, 1963), and the venom of brown tree snakes (*Boiga irregularis*) is much more toxic to lizards and birds than to mice (Mackessy et al., 2006). In light of this interspecific variation in response to oral secretions, we define venom as an oral secretion that facilitates the capture of prey by inducing death or paralysis upon injection in natural prey species (modified from Gans, 1978; Mebs, 1978). While calculations of mouse LD_{50}s are relatively common, few studies have quantified the effects of snake venom dosage on natural prey (Weinstein and Kardong, 1994; Mackessy, 2002).

Ring-necked snakes (*Diadophis punctatus*) do not possess a true venom gland, but do have a Duvernoy's gland, which is derived from the same tissue (Taub, 1967; Mackessy, 2002). Most subspecies of *D. punctatus*, with the apparent exception of the fangless *D. p. edwardsii*, are rear-fanged with the last maxillary teeth larger than the preceding ones and separated from them by a space (Fig. 1; Blanchard, 1942). Just posterior to the eye, the Duvernoy's gland produces secretions that drain to openings above the rear maxillary teeth (Taub, 1966).

In addition to the morphology of *D. punctatus*, several anecdotal reports of feeding and defensive behaviors provide support for the hypothesis that at least some subspecies are venomous. Early speculation as to the toxic nature of the oral secretions followed reports of bites from *D. punctatus* producing a burning sensation in humans (Myers, 1965). Observations of *D. punctatus's* feeding behavior also indicated envenomation of the prey. Gehlbach (1974) observed that regal ring-necked snakes (*Diadophis punctatus regalis*) and prairie ring-necked snakes (*Diadophis punctatus arnyi*) would chew or hold onto their prey until it became immobile. Anton (1994) reported a bite by *D. p. regalis* that resulted in the death of an ornate tree lizard (*Urosaurus ornatus*) in 71 min. Hill and Mackessy (2000) reported that bites by *D. p. regalis* proved fatal to specimens of neonatal cornsnake (*Elaphe guttata*), wandering garter snake (*Thamnophis elegans vagrans*), and western patch-nosed snake (*Salvadora hexalepis*) within 6 min, but that no local reactions were observed. In addition, we have observed northwestern ring-necked snakes (*Diadophis punctatus occidentalis*) chewing on long-toed salamanders (*Ambystoma macrodactylum*) with their rear teeth imbedded until the salamanders became immobile (pers. obs.).

Reports of defensive bites are less common. In addition to Myer's report of a human bite, which may have been defensive, Rossi and Rossi (1994) reported an apparent defensive bite by the southern ring-necked snake (*Diadophis punctatus punctatus*), which resulted in the death of a long-nosed snake (*Rhinocheilus lecontei*) that had ingested most of the posterior portion of the ring-necked snake's body. The ring-necked snake eventually crawled out of the dead long-nosed snake.

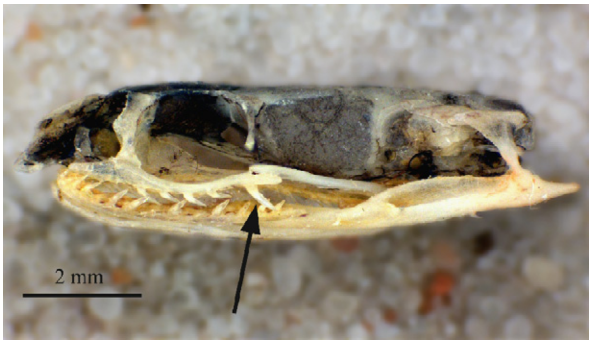

Fig. 1. Lateral view of the skull of a northwestern ring-necked snake, *Diadophis punctatus occidentalis*. Anterior is to the left. An enlarged rear fang is indicated by the arrow. The specimen, MVZ 172389, was loaned courtesy of the Museum of Vertebrate Zoology, University of California, Berkeley. It was photographed with the assistance of B. Webster and J. Pitts.

Further evidence that some *D. punctatus* may be venomous is provided by chemical analyses of the oral secretions of *D. p. regalis*. Hill and Mackessy (2000) found that oral secretions of this subspecies contained low phosphodiesterase and moderate or high levels of phospholipase A_2 activity. The specific role of phosphodiesterases in venom is unclear, but they generally degrade cyclic AMP and may involve disruption of events mediated by cyclic AMP and ADP (Hill and Mackessy, 2000; Mackessy, 2002). Phospholipase A_2 is a common component of elapid and viperid venom. Its primary function in venom is to catalyze reactions that harm musculature and nerves, leading to paralysis and death, but it has a wide range of other functions as well (Kini, 2003).

Anecdotal accounts of feeding and defensive behavior combined with chemical analyses of Duvernoy's gland secretions provide strong support for the hypothesis that *D. punctatus* is venomous. However, these data do not eliminate the possibility that the prey species died from the wounds inflicted by the ring-necked snake's chewing. These data also do not eliminate the possibility that the oral secretions do not act as venom in natural prey due to species specificity, evolved immunity, or resistance. Accordingly, some still consider evidence for venom in this species to be meager (McCallum et al., 2006).

We examined the stomach contents of a small series of *D. p. occidentalis* to provide initial data on their prey at our study site. We then tested whether the oral secretions of northwestern ring-necked snakes are toxic to their prey, and thus whether these secretions constitute venom by aiding in the capture and subduing of prey. We compared the effects of injecting oral secretions to denatured secretions and saline solution in a sympatric prey species to experimentally evaluate the hypothesis that the secretions are effective in subduing natural prey and that mortality is not due to the physical damage inflicted by ring-necked snakes' chewing.

2. Material and methods

2.1. Collection and maintenance of snakes

Thirty-three northwestern ring-necked snakes (snout-to-vent length [SVL] 17.2–52.5 cm, mean 36.1 cm; mass 2.5–34.3 g, mean 14.3 g) were collected from the E.E. Wilson Wildlife Area, 16 km north of Corvallis, Benton County, OR, USA (N44°42′, W123°12′). All specimens were collected between 2 June and 28 July 2001 and kept in glass tanks with screen lids in an environmentally controlled room (humidity—day 65%, night 55%; temperature—day 24 °C, night 18 °C, 12:12 light:-dark cycle). Food was offered in the form of live long-toed salamanders (*A. macrodactylum*) collected from the same locality. Water was provided ad libitum.

2.2. Examination of stomach contents

Upon capture, individuals were examined to determine the presence of stomach contents by visual inspection and then by gently running a finger along the snake's ventral surface. When prey items were detected, we induced regurgitation by pressing a thumb against the posterior end of the prey item and moving this digit anterior while maintaining a constant pressure. Regurgitation was only induced when prey items were externally detected, and thus some of the smallest and softest-bodied prey may have escaped detection.

2.3. Collection of oral secretions

Some subspecies of ring-necked snake, including *D. p. occidentalis*, produce droplets of clear viscous liquid at the corners of the mouth when handled (Blanchard, 1942; Stebbins, 1954; but see Myers, 1965). We collected oral secretions from these droplets using a method modified from Vest (1981). We held a snake behind its head and positioned the tip of a sterile 1 ml insulin syringe near the edge of the upper labial scale adjacent to the rear maxillary teeth. The oral secretion was then collected by slowly drawing back the syringe plunger. This process was repeated on both sides of the snake's mouth and the collected liquid was placed into a sterile 1 ml Eppendorf tube. Oral secretions were collected from each snake only once. Variation among individuals in the volume produced was apparent but not quantified. The secretions collected from all 33 snakes were pooled and then stored on ice until used in our toxicity experiments within 24 h. Pooling samples provided the advantage of standardizing the toxicity of our treatments, with the drawback of obscuring potential ontogenetic or other variation in toxicity of oral secretions.

2.4. Determination of toxicity

We tested the effects of ring-necked snake oral secretions on neonatal northwestern garter snakes (*Thamnophis ordinoides*). Subject snakes were captive-born offspring of wild-caught females that had been collected from the same locality as the ring-necked snakes. *T. ordinoides* ranged from 14 to 21 days old during testing (SVL 13.7–16.4 cm, mean 14.9 cm; mass 1.0–2.7 g, mean 1.6 g).

Doses of the oral secretion (2, 8, 12, 16, 20, 25, or 35 μl) were brought to one of two standard volumes by diluting as necessary with reptilian Ringer's solution ("saline"; Guillette, 1982). Bringing oral secretion doses to standard volumes was intended to minimize any potential effects of the volume of fluid injected. Oral secretions of 2–16 μl were brought to 20 μl, and the 25 μl dose was brought to 35 μl. Doses of 20 and 35 μl were not diluted, resulting in 20 μl injections containing 2–20 μl of oral secretions, and 35 μl doses containing 25–35 μl of oral secretions. Each dose was injected intraperitoneally into 5–11 *T. ordinoides* (total $N = 41$) under a ventral scale at approximately one-third of the body length anterior to the cloaca. We qualitatively recorded behavioral responses to the injection, and recorded the time passed until the snake no longer righted itself in response to being turned onto its dorsum. The loss of a righting response is a biologically significant point of incapacitation where the ability to escape or injure a predator has been lost.

Two separate control trials were conducted. One control group of 13 neonate *T. ordinoides* were each injected with 20 μl of the saline only, with no ring-necked snake oral secretions. A second control group of seven *T. ordinoides* were injected with denatured oral secretions as follows. We boiled one 140 μl sample of venom for 10 min to denature its enzymes. After cooling the denatured oral secretions to room temperature for 1 h, 20 μl was injected into each *T. ordinoides*. Both types of control injections were performed identically to the treatment injections.

2.5. Statistical analyses

Regression analysis was used to investigate the relationship between the time to loss of the righting response and the dose of oral secretion injected. To account for the variation in the masses of the subject snakes, we calculated dose as the microliters of oral secretions that were injected per gram of body mass of the subject. Times to loss of the righting response were transformed to a logarithmic scale to meet assumptions of equal variance, and were normally distributed after transformation.

3. Results

3.1. Examination of stomach contents

We detected stomach contents in three of the 33 ring-necked snakes we collected. These three individuals contained the remains of one garter snake each, including two red-spotted garter snakes (*Thamnophis sirtalis concinnus*) and one northwestern garter snake. Each of the three prey items had been ingested head first and was partially digested such that the anterior end of the prey was no longer present, prohibiting measurement of original prey sizes.

3.2. Determination of toxicity

Individual *T. ordinoides* injected with small dosages (2 μl oral secretions diluted to 20 μl total volume) exhibited no initial response, but after 30 min the snakes exhibited signs of paralysis starting from the location of the injection, accompanied by muscle spasms and followed by regurgitation. Shortly before the loss of the righting response the snakes became lethargic and could be stimulated to move only by contact. The onset of these symptoms seemed to occur faster with increasing dosage (data not recorded), and snakes injected with $\geqslant 25$ μl of oral secretions showed symptoms immediately.

Effects of a given dose varied with the mass of the subject snake (Fig. 2). For this reason, we conducted subsequent analyses using a mass-adjusted dose (microliters of oral secretions per gram of subject body mass) as the independent variable. The time to loss of the righting response was significantly positively related to the mass-adjusted dose of oral secretions administered (Fig. 3; $r^2 = 0.72$, $P < 0.001$). Data were not normally distributed, but conversion of times to a natural logarithmic scale before analysis normalized the data. The best-fit line for this regression is described by the equation ln(time to loss of the righting response) = $-0.073 \times \text{dose} + 4.76$, with time in minutes and dose in microliters of oral secretions per gram of subject mass. While the loss of a righting response may indicate paralysis and not necessarily death, none of the snakes that lost their righting response recovered.

None of the 13 control injections of 20 μl saline solution resulted in fatalities or symptoms of envenomation within 24 h, the maximum period of observation. Also, none of the seven injections of 20 μl denatured oral secretion controls elicited mortality or symptoms of envenomation within 24 h.

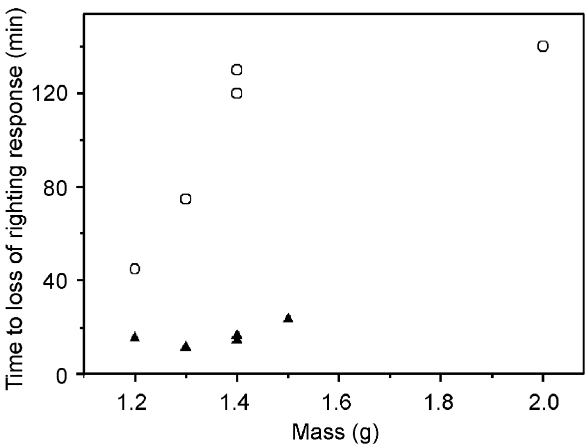

Fig. 2. Time to loss of the righting response for neonate northwestern garter snakes (*Thamnophis ordinoides*) as a function of body mass for two representative doses of northwestern ring-necked snake (*Diadophis punctatus occidentalis*) oral secretions. Open circles represent doses of 2 μl oral secretions and filled triangles represent doses of 35 μl.

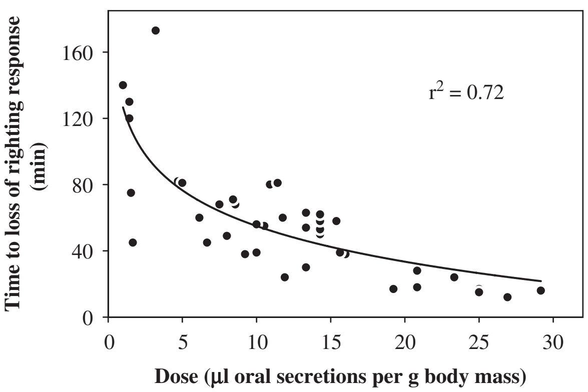

Fig. 3. Time to loss of the righting response for neonate northwestern garter snakes (*Thamnophis ordinoides*), as a function of mass-corrected dose of northwestern ring-necked snake (*Diadophis punctatus occidentalis*) oral secretions. Time was natural log transformed for statistical analysis, which normalized the data, but is shown here on a linear scale.

4. Discussion

Results from this study show that the oral secretions of *D. p. occidentalis* are venomous to a prey species, *T. ordinoides*. Regression analysis of the results shows that the time taken to lose the righting response is dependent upon the dose of oral secretions injected.

The doses we administered were biologically relevant. While our method of collecting venom in 1 ml syringes (where precise measurements of collected volume were not possible because of volumes left in the needle of the syringe) prohibited exact quantification of yield from each donor, we can calculate a minimum mean yield by totaling the doses that were administered from our total. We administered a total of 850 μl, giving a minimum mean yield of 25 μl of oral secretions per snake. Because each dose of $\geqslant$2 μl was 100% lethal, each *D. p. occidentalis* yielded, on average, enough venom to subdue at least 12 neonatal *T. ordinoides*.

In this study, we were primarily concerned with the toxic effect of *D. p. occidentalis* oral secretions on their natural prey and therefore we did not attempt to separate the effects of saliva and Duvernoy's secretions. Because our denatured oral secretion controls did not elicit the same response as raw oral secretions, we conclude that the toxic nature of the oral secretion is due to its protein content. Based on the distribution and types of proteins contained in each secretion (Hill and Mackessy, 1997, 2000), we believe the responses reported here are due to the secretions of the Duvernoy's gland rather than the saliva. However, isolation of these two glandular secretions would be required to confirm this.

It is interesting to note that, although all doses were lethal and the relationship between dose and time to loss of the righting response was significant, only 72% of variation in the time to the loss of the righting response was accounted for by variation in the dose administered. Because venom strength did not vary, a portion of the remaining variation must be due to variation among individuals in susceptibility to the venom. Inter-individual variation in susceptibility to toxins has been shown previously and is important because it provides the raw material for the evolution of resistance to the toxin if heritable (Poran et al., 1987; Daltry et al., 1996; Brodie et al., 2002). Our study site occurred in an area of overlap between the ranges of northwestern ring-necked snakes and northwestern garter snakes, constituting less than half of the range of the latter. Gene flow from northwestern garter snake populations outside of the selective pressure of ring-necked snake venom may limit the ability of northwestern garter snakes to adapt to this pressure.

Considering the kinds of prey ingested and the toxicity of oral secretions to natural prey, we suggest that *D. p. occidentalis* venom, probably produced by the Duvernoy's glands, contributes to the subduing of prey before or during ingestion. This assertion would be strengthened by data showing the quantity and effectiveness of venom that is actually delivered during prey handling by *D. p. occidentalis*.

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