

On the immunity of snakes to their own venom and to the venom of conspecifics across ontogeny

Project Summary:

The assumption that snakes are immune to their own venom is very common; however actual literature on such immunity is minimal and many conflicting conclusions have been made regarding the validity of this assumption. Based on the assumption that snakes are immune to their own venom, much attention has been paid to ontogenetic, or age-related, shifts in venom composition across three venomous snake families, *Viperidae*, *Elapidae* and “*Colubridae*”. Little research has yet been conducted to determine the susceptibility of an individual to the venom of a conspecific – or individual of the same species – of a different ontogenetic stage. My study will attempt to determine if snakes are indeed immune to their own venom, if snakes are immune to the venom of a conspecific at the same ontogenetic stage, and if snakes are immune to the venom of a conspecific at a different ontogenetic stage. The viperids *Bothrops jararaca* and *B. cortiara*, the elapids *Notechis scutatus* and *Oxyuranus microlepidotus*, and the colubrids *Boiga irregularis* and *B. dendrophila* will be obtained and subjected to a series of tests. First, these snakes will be tested for immunity to their own venom. A second series of tests will determine if snakes are immune to venom of conspecifics at various ontogenetic stages. I hypothesize that snakes will be immune to their own venom and that snakes will be susceptible to the venom of conspecifics of any ontogenetic stage.

Introduction and Background:

Three families of snakes, *Elapidae*, *Viperidae* and “*Colubridae*”, use venom for both protection and predation. These venoms consist of hemotoxins and/or neurotoxins, both of which are very effective at immobilizing a victim by either deteriorating tissue or blocking neuronal activity. The assumption that snakes are immune to their own venom is often made; however there is very little evidence of this in peer-reviewed literature. On the contrary, Nichol et al. (1933) concluded that two *Crotalus molossus* (*Viperidae*) (black tailed rattlesnake) individuals died when subjected to each other’s venom. Such observations raise the questions: are snakes really immune to their own venom, and are snakes susceptible to the venom of other individuals of the same species? Recently, there has been a great deal of research on ontogenetic shifts of snake venom composition (Mackessy et al. 2006; Saldarriaga et al. 2003; Furtado et al. 1991). A general pattern of changing lethality and toxicity throughout ontogenetic development has emerged; however the trend of changing venom composition with age is best documented in viperids (Saldarriaga et al. 2003; Meier, 1986). Some elapids display ontogenetic shifts in venom composition and some do not; however the elapids that display venom composition changes show significantly different levels of toxicity and lethality. In venomous colubrids that have been studied, ontogenetic shifts in venom composition is again observed, but more research is needed for both elapids and colubrids to conclusively state that ontogenetic shift in venom composition is a common trend across all venomous snake families. The lacking research on colubrids and elapids will not affect the goals of this study, as the same tests will be performed on each of the six snake

species chosen for this study and full understanding of ontogenetic shifts in venom composition is not necessary information to attain results.

Snakes in the family *Viperidae* are venomous and found worldwide except in Australia, Madagascar and arctic regions. Viperid venom evolved from digestive enzymes by processes of gene divergence and duplication and consists of a complex mixture of proteins and other molecules (Pough et al. 2004). These venoms typically have hemolytic and cytolytic effects on victims. Hemolysins burst red blood cells and release hemoglobin into the surrounding fluid, cytolsins burst cells because of osmotic imbalance, myotoxins destroy skeletal muscle, and hemorrhagins destroy the lining of blood vessels and cause excess bleeding (Pough et al. 2004). All of these compounds are components of Viperid venoms and combine to produce a very potent and destructive predatory and defensive mechanism.

The genus *Bothrops* (*Viperidae*) found in Central and South America contains thirty-two recognized species that are commonly referred to as lanceheads. *Bothrops* venom causes edema, myonecrosis, blistering, hemorrhaging, nephrotoxicity and defribination of tissues (Saldarriaga et al. 2003). Ontogenetic shifts in *Bothrops* venom composition are common and especially well documented. However, there is variation within the genus as to the ontogenetic stage that employs more potent venom. A study by Furtado et al. (1990) showed that *Bothrops cortiara* displayed increased protein content, decreased fibrinolytic activity, and decreased MCD-F (minimum coagulant dose in fibrinogen) activity with increasing age. The results of the study by Furtado et al. (1990) suggest an overall decrease in toxicity and lethality of venom with age. Conversely, the same study by Furtado et al. (1990) showed that *Bothrops jararaca* has decreased protein

content, decreased procoagulant activity, and increased frequency of blood incoagulability, but similar MCD-F activity with increasing age, resulting in an overall increase in toxicity and lethality with age.

Snakes in the family *Elapidae* are also venomous and found worldwide in subtropical and tropical regions, including the Indian and Pacific oceans (Pough et al. 2004). Unlike Viperid venom, which is often hemotoxic, Elapid venom typically consists of neurotoxins that act either presynaptically, preventing the release of neurotransmitters into the neuromuscular junction, or postsynaptically, preventing neurotransmitters from binding to postsynaptic receptors (Hill et al. 2004), or both. Little literature on the auto-susceptibility and auto-immunity of snakes that employ neurotoxic venoms has been published. Additionally, research has not revealed as significant an ontogenetic shift in venom composition in Elapid snakes as in other venomous snake families.

The common tiger snake, *Notechis scutatus* (*Elapidae*), is found in coastal regions and wetlands in southern Australia and Tasmania. *N. scutatus* is highly venomous and employs prothrombin activators and pre-and postsynaptic neurotoxins that cause blood coagulation by inhibiting anti-clotting factors, cause skeletal muscle deterioration, and cause paralysis in victims (Tan et al. 1993a). Tan et al. (1993a) compared juvenile and adult venoms of *N. scutatus* to determine if an ontogenetic shift in venom composition occurs. The results of the aforementioned study showed that, although there is no significant change in enzymatic activity between juveniles and adults, there is a change in protein composition and an increase in acetylcholinesterase, 5'-nucleotidase, and c-amino acid oxidase that result in increased toxicity with age. Tan et al. (1993b) conducted a similar study comparing the venom composition of juvenile and adult *Oxyuranus*

microlepidotus, the inland taipan, to determine if venom composition is age dependent. As with *Notechis scutatus*, *Oxyuranus microlepidotus* employs presynaptic neurotoxins and strong prothrombin activators, however no change in biological activities, protein composition, or lethality was observed between juveniles and adults in *O. microlepidotus*. Further studies of Elapid snake venom are necessary to determine if an overall trend of ontogenetic shifts in the venom composition of Elapid snakes occurs.

“*Colubridae*” is a paraphyletic group that contains two-thirds of all snake species. Some, but not all, colubrids are venomous. The Duvernoy’s gland, which is the venom secretory gland in colubrids, is significantly different than the venom gland found in viperids and elapids and therefore produces different kinds of venoms. The brown treesnake, *Boiga irregularis* (“*Colubridae*”), is native to northeastern Australia, New Guinea, and the Solomon Islands but can also be found in Guam, where it is invasive (Mackessy et al. 2006). Mackessy et al. (2006) found that *Boiga irregularis* employs a neurotoxin that has been shown to cause inhibition of postsynaptic neuromuscular activity in the chick biventer cervix muscle. Another colubrid species, *Boiga dendrophila*, was found to employ alpha-neurotoxins, which competitively bind to nicotinic acetylcholine receptors (Fry et al. 2003). The study by Mackessy et al. (2006) compared juvenile and adult *B. irregularis* venom to determine if an ontogenetic shift in venom composition occurs. The results of the study by Mackessy et al. (2006) showed that protein content varied significantly across ontogeny, with juvenile venom consisting of forty-eight percent protein and adult venom consisting of eighty percent protein. Furthermore, a decrease from thirteen peptides in juvenile venom to nine peptides in adult venoms was observed. These data suggest that venom of juveniles is nearly two

times more toxic than venom of adults. Further research is needed on *Boiga dendrophila* both to determine if there is an ontogenetic shift in venom composition and if these snakes are immune to their own venom.

For this study, I propose to use the discussed literature as a starting point to understand the immunity and susceptibility of snakes to their own venom and to the venom of conspecifics at the same ontogenetic stage and at a different ontogenetic stage. I will conduct experiments that reveal if these six species of snakes are indeed immune to their own venom as is so widely assumed. Also, susceptibility to venoms from individuals of the same species at different ontogenetic stages will be tested in an attempt to either prove or disprove common assumptions made regarding the intraspecific interactions of venomous snakes in the families *Viperidae*, *Elapidae*, and “*Colubridae*”.

Hypotheses:

H: Snakes are immune to their own venom and snakes are susceptible to the venom of conspecifics.

H₀: There is no difference between susceptibility or immunity of snakes to their own venom and to the venom of conspecifics at any ontogenetic stage.

H_a: Snakes will show immunity to both their own venom and to venom of conspecifics at any ontogenetic stage.

Proposed study:

Multiple individuals of each of the six snake species listed above (*Bothrops jararaca* and *B. cortiara* [*Viperidae*], *Boiga irregularis* and *B. dendrophila* [“*Colubridae*”], *Notechis scutatus* and *Oxyuranus microlepidotus* [*Elapidae*]) will be obtained for this experiment. The snakes will be raised on similar diets and under similar,

monitored conditions in order to eliminate confounding factors having to do with prey type, food availability, and other unforeseen environmental differences wild snakes encounter that could potentially alter venom composition. Snakes of the same species will originate from the same area to eliminate potential differences in venom toxicity due to geographic variation. Once the snakes have been obtained, they will be allowed a period of time to acclimate to the laboratory environment. Once snakes have acclimated, each snake will have its venom extracted following the method outlined by Klauber (1972). First, the snake will be inserted into a plastic tube to reduce the risk of injury both to the snake and to the researcher. The mouth of the snake will be opened and the jaw tilted back using a metal hook. The venom cup, a small container with a thin parchment layer as a cover, will be slipped under the fangs while the jaw is steadied with the hook. The hook will then be removed and the fangs inserted into the parchment layer of the container. Pressure will be put on the venom glands to ensure maximum venom yield. The venom will be stored appropriately to ensure that the venom does not lose potency or denature. Care will be taken to not harm the snake during the venom extraction process and each snake will have venom extracted only as often as is necessary and healthy for the animal.

Once venoms have been obtained from each snake, the tests will commence. The first test will determine if snakes are immune to their own venom. Multiple snakes of each species will be manually injected with their own venom that was previously extracted. The second test will determine if snakes are immune to the venom of conspecifics of the same ontogenetic stage. Multiple snakes of each species will be injected with the venom from conspecific individual at the same ontogenetic stage. The

first two tests will be performed on both juveniles and adults to determine if there is changing susceptibility with age. The third test will determine the susceptibility of snakes to venom of conspecifics at a different ontogenetic stage. For this test, multiple juveniles will be injected with venom from conspecific adults and multiple adults will be injected with venom from conspecific juveniles. For each test, enough individuals will be tested to obtain sufficient data to display trends. Additionally, the same per gram volume of venom will be manually injected to reduce differences in toxicity due to volume in each test. The venom will be injected into the same area of each snake to reduce confounding factors having to do with circulation and penetration of venom. Susceptibility and immunity will be measured by assessing physical affects of venom like necrosis, excessive bleeding, lethargy, restlessness, and possibly death. Table 1 summarizes each test that will be carried out for purposes of clarification.

Description of Tests
Adult injected with own venom
Juvenile injected with own venom
Adult injected with venom of conspecific of same ontogenetic stage
Juvenile injected with venom of conspecific of same ontogenetic stage
Adult injected with venom of juvenile conspecific
Juvenile injected with venom of adult conspecific

Table 1: Descriptions of each of six tests that will be performed on each of six snake species chosen for this experiment.

If snakes show immunity to their own venom but not to the venom of conspecific individuals at any ontogenetic stage, the hypothesis made by my study would be supported. Further verification of the common assumption that snakes are immune to their own venom would be achieved and more information regarding ontogenetic shifts in venom composition would be understood. However, if snakes are not immune to their own venom and/or if snakes show no difference in immunity to the venom of conspecifics, the hypothesis would not be supported and more research would have to be done both to determine if the assumption that snakes are immune to their own venom is a fallacy and what the significance of ontogenetic shifts in venom really is. Whether or not the hypothesis put forth in this study is supported, many future studies on snake venom composition and the immunity of snakes to venom will stem from this study.

References:

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Research Budget and Justification:

Duration of study:

Time must be allowed to attain the snakes before any kind of acclimation or experimentation can take place. I will allow two months for capture and shipment of snakes from Australia and South America. After I have received the snakes, the snakes must be allowed to acclimate to laboratory conditions for three months before tests can commence to make sure that conditions are controlled. Once the snakes have acclimated, the study will proceed fairly quickly. Time is a constraint because once venom is extracted from snakes, the venom must be used for the experiments quickly to reduce the risk of the venom losing toxicity or denaturing. All time factors considered, this study will require eighteen months for completion.

Salary

-One undergraduate assistant will be paid \$10/hour with 10% benefits.

$$\$10 * 20\text{hrs} * 45\text{wks} = \$9,000$$

$$\$9,000 * 0.10 = \$900$$

$$\$9,000 + \$900 = \mathbf{\$9,900/year/assistant}$$

One undergraduate will be sufficient to help with lab and animal maintenance and feeding because certain qualifications are required to handle such venomous snake species as will be used for this study. Because qualified personnel are expensive, a closely monitored assistant and I will be conducting all experiments.

Equipment

-Use of lab

I am assuming that I will have access to a lab with much of the necessary equipment for snake care and handling, such as snake sticks, tongs, lights, venom extraction equipment, etc., at the University of Arizona.

-Animals

I will obtain snakes from Australia and South America through universities with access to proper permits and qualified personnel to deal with the snakes. The costs of the snakes used for this study are highly variable, as they are not typically found on pet markets. Since this is a scientific research project, I hope to obtain snakes at a reasonable price. Estimates of such a price are \$75 per snake. $\$75 * 180 = \mathbf{\$13,500 \text{ for snakes}}$

Each snake will only be used for one test. Therefore, in order to carry out one series of tests, I will need six individuals of each of the six species, equaling 36 snakes. Because my goal is to get conclusive results from my study, I will have to test at least five individuals per species per test. I will therefore need 180 snakes. Costs of shipping vary, but I will assume \$75 shipping fee per snake. $\$75 * 180 = \mathbf{\$13,500 \text{ for shipping}}$

-Food

-Each snake requires 1 mouse per week. Each mouse costs \$0.25. If I am taking care of 180 snakes in my lab, I will need 180 mice per week.

180 mice * 72 weeks = 12,960 mice for the entirety of the study
12,960 * \$0.25 = **\$3,240 for mice**

-Enclosures

Snakes will be kept in Rubbermaid™ containers that cost \$3 each.
180 snakes * \$3.00 = **\$540 for enclosures**

Travel

-Anticipated travel costs include: airfare to South America and/or Australia to work out details of acquiring animals, airfare/lodging for conferences, etc.

I estimate **\$5,000** for travel costs for myself for the entirety of the project.

Publication costs

-I estimate **\$3,000** for publication costs from preliminary writing stages through publication in a scientific journal.

Total amount requested: \$48,680

Duration of Study: 18 months