EVOLUTION OF VENOM VARIATION IN THE FLORIDA COTTONMOUTH,  
Agkistrodon piscivorus conanti

By

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Populations of organisms evolve when they are genetically isolated from each other, have variation within phenotypic characters, and exist in different environments. The evolution of venom in snakes has been examined for many reasons including antivenom research, development of medical research tools, and pharmaceutical drug discovery. Previous studies have disagreed on the manner by which snake venom evolves, however.

In this study, we tried to determine whether snake venom is more influenced by natural selection, gene flow, or genetic drift. To do this, we first developed a safe, standardized, and effective method of venom extraction using Propofol anesthetic and an electric nerve stimulator. We then validated the use of previously-published microsatellite primers for use in the Florida cottonmouth, *Agkistrodon piscivorus conanti*, in order to determine population genetic relationships. Next, we collected 76 venom and 129 deoxyribonucleic acid (DNA) samples from cottonmouths in Florida. The venom samples were collected from live snakes from three different populations, one of which was the focal population of Seahorse Key (SHK). The DNA samples were collected from both live and road-killed specimens from four populations, including SHK.
We analyzed the venom for activities of four enzymes: protease, hyaluronidase, L-amino acid oxidase (LAAO), and phospholipase A₂ (PLA₂).

Because the SHK population primarily feeds on fish dropped or regurgitated by colonially nesting seabirds, and because it is located on an island separated from the mainland by a salt water corridor, we expected the population to be genetically isolated and be evolving through genetic drift. Because the other populations are found on the mainland in diverse habitats, and because the species is known to be generalist in prey preference, we expected that venom enzyme activity would be correlated with local prey, indicating natural selection. We found all four populations to be genetically distinct, but with SHK being very different from the rest and likely inbred. We also found some differences in venom enzyme activity with SHK having higher LAAO and intermediate protease and PLA₂. These results support the idea that the SHK population is under different evolutionary constraints than the mainland populations.
CHAPTER 1
INTRODUCTION

Venoms, Poisons, and Toxins

Toxic secretions of different forms are produced by various animal taxa and may be used for acquisition of prey, digestion of prey, and/or defense against potential. Venoms are potentially toxic secretions produced by one organism to the detriment of another, delivered through some form of specialized morphological adaptation (e.g., fang, stinger, and nematocyst). Poisons, on the other hand, must be ingested (via gastrointestinal tract or skin) to affect the target organism. Both venoms and poisons tend to work in a dose-dependent manner (Mebs, 2002), the main difference between them being the mode of delivery.

Venoms are often confused with toxins, which are chemicals of biological origin that can adversely affect the physiology of another organism. Generally, venoms and poisons both contain toxins, but toxins are pure substances, whereas venoms and poisons are mixtures that may include other biologically active and inert substances.

For a toxic secretion to be considered venom, there must be an assumption of an ecological function (Kardong 1996). While such function is definitely known for the chemical secretions of some animals, it has not been shown for all of them. As an example, the secretion of the cone snail (Conus catus) is used to paralyze fish that are then consumed by the snail (Jakubowski et al., 2005). This indicates, at the very least, that the secretion is used for prey acquisition and can be considered venom. It does not indicate, however, whether the venom is also used for prey digestion or predator deterrence. As a further example, many snakes (such as the cottonmouth, Agkistrodon piscivorus) use oral chemical secretions from specialized glands to immobilize prey—
this indicates that these oral secretions are venoms. These same oral secretions have not been analyzed in many species of snakes (such as the watersnake, *Nerodia fasciata*), so there is debate as to whether they should be considered venoms (Kardong, 2002). Recent publications have championed the opinion that all oral secretions with biologically-active compounds developed in specialized glands should be considered venoms (Fry et al., 2003a), regardless of other knowledge as to specific effects.

**Snake Venoms**

Among venomous animals, snakes are of particular interest for many reasons. Approximately 2700 species of extant snakes currently are recognized, divided into 17 families (Pough et al., 2001), the phylogeny of which has not been conclusively determined (Lawson et al., 2005; Vidal et al., 2007). Four of these families (Atractaspididae, Colubridae, Elapidae, and Viperidae) compose the traditional taxon superfamily Colubroidea (Figure 1), which contains most (approximately 2430) species of snake (Cadle, 1988; Kraus and Brown, 1998; Pough et al., 2001; Slowinski and Lawson, 2002; Vidal, 2002; but see Lawson et al., 2005, Vidal et al., 2007, and Zaher et al., 2009 for recent revisions). All venomous snake species belong to the Colubroidea, and it is generally held that venom arose in the ancestor of this clade followed by alterations in venom composition, glandular morphology, and tooth shape (Vidal, 2002; Jackson, 2003). However, recent research has shown an earlier reptilian origin of venom, with venom being found in multiple lizard families, indicating that only the type of modified venom apparatus (but not venom itself) may be unique to the Colubroidea (Fry et al., 2006). Further confusing the issue, however, is the absence of venom in all of the snakes basal to the Colubroidea, which may indicate multiple loss and gain events throughout snake evolution.
The total number of venomous snake species is not currently known, as many species that have traditionally been considered harmless have not been analyzed for the presence of venom. The discovery of biologically-active oral secretions from “harmless” colubrid snakes has had tragic endings in the cases of Dr. Karl P. Schmidt of the Field Museum of Natural History, who was killed from the bite of a boomslang (<i>Dispholidus typus</i>; Pope, 1958), and Dr. Robert Mehrtens, who was killed by the bite of a twig-mimic snake (<i>Theolttornis capensis</i>; Greene, 1997). Histological studies have indicated the presence of venom glands in species considered to be without venom (Taub, 1967), and studies of gene expression have shown production of toxins from oral glands in snakes considered harmless (Fry et al., 2003b). The increase in popularity of herpetoculture will likely increase the number of snake species known to be venomous.

Variation in venom, both qualitatively and quantitatively, is considerable and occurs at almost every taxonomic level from the individual (i.e. ontogenetic shift: Mackessy, 1988) to the family level (among families of the Colubroidea; Chippaux et al., 1991). In general, snake venoms consist mostly of soluble polypeptides in serous or mucus secretions, but may also include carbohydrates, lipids, metal ions, and other organic compounds, including amines (Mebs, 2001) and purines (Aird, 2002). Up to 90% of the dry weight of most venom is comprised of polypeptides of three size classes: low molecular weight components (< 1.5 kDa), polypeptide toxins (5 to 10 kDa), and enzymes (10 to 150 kDa) (Hider et al., 1991). One individual snake may secrete venom that contains numerous chemical compounds from all three categories.

Venom can affect a wide range of physiological functions in the envenomated organism, and each individual venom component may have a unique function. Most
snake venoms contain phospholipases A$_2$ (PLA$_2$), cell-destroying enzymes that can be edema-inducing, lipolytic, or myolytic, depending on their specific three-dimensional structure (Chijiwa et al., 2003; Lomonte et al., 2003a). They may also be chemically altered to become toxins that can block acetylcholine release from the presynaptic motor end plate (Mebs, 2002). Other compounds that degrade ubiquitous biological structures, such as proteases that degrade peptides or hyaluronidases that destroy hyaluronic acid, are also often found in venoms. Some venoms contain components that affect specific physiological functions, such as enzymes that either potentiate or prevent part of the mammalian blood clotting cascade (Mebs, 2002), while others contain functionally-diverse mixtures of components. Some snake venoms may cause localized swelling in humans, whereas others cause rapid death through loss of neural function.

Components of viperid venom include a wide array of proteins with enzymatic activity and non-enzyme polypeptides. Among the enzymes, the major groups are phosphodiesterases, 5’nucleotidases, alkaline phosphomonoesterases, hyaluronidases, L-amino acid oxidases, metalloproteinases, serine proteases, arginine esterases, and phospholipases A$_2$ (PLA$_2$). Among the non-enzymatic polypeptides are cysteine-rich secretory proteins (CRiSPs), nerve growth factors, PLA$_2$-based neurotoxins, non-PLA$_2$ myotoxins, C-type lectins, disintegrins, bradykinin potentiators, and tripeptide inhibitors (Mackessy, 2009). This is not an exhaustive list of all venom component groupings, as some venom constituents have not as of yet been categorized. Indeed, new venom components are being described often (Pawlak et al., 2006; Nair et al., 2007), and the venom from many species has not been thoroughly studied.
Most venom enzymes work by hydrolyzing biomolecules in the prey, thereby reducing function and altering normal homeostasis of the envenomated organism. These enzymes are usually examined in one of two ways: either they are isolated and characterized for their specific activity (enzyme-based study) or they are examined for their effect on a specific substrate (substrate-based study). This can lead to confusion as one enzyme may affect multiple substrates and may also have more than one moniker, depending on the manner in which it has been studied. Further, there may be overlapping activities from many different enzyme groups. There are, however, generalities in terms of the chemical function and biological activity of many enzyme groups.

Phosphodiesterases, as their name implies, hydrolyze phosphodiester bonds. This catalysis is mostly seen as a degradation of nucleic acids, including DNA and two ribonucleic acids (RNAs), ribosomal RNA and transfer RNA, but the enzymes can affect many other nucleotides and nucleic acids as well (Dhananjaya et al., 2009). Although the overall effect has not been completely determined, it appears that the depletion of such nucleotides results in hypotension and/or shock (Mackessy, 1998). 5’-nucleotidases attack nucleic acid at the 5’ carbon position, degrading the sugar moieties of both DNA and RNA (Dhananjaya et al., 2009; Rael, 1998). The overall effect of 5’-nucleotidases is to release nucleosides from nucleic acids (Mackessy, 2009). A third enzyme group that affects nucleic acids is the alkaline phosphomonoesterases, which hydrolyze phosphomonoesterases at pH above neutral (Dhananjaya et al., 2009; Rael, 1998). The biological effect of the alkaline phosphomonoesterases is uncertain, but
these three groups of enzymes attack nucleotides in different manners, and all three are fair
ly ubiquitous in viperid venoms (Dhananjaya et al., 2009).

Hyaluronidases are also commonly called “spreading factors” because of their capacity to de
graded hyaluronic acid. Hyaluronic acid is a ubiquitous component of the extracellular matrix of tissues, and is at least partially responsible for cementing cells together. Hyaluronidases have been found in all elapid and viperid venom examined thus far (Girish et al., 2002) and although they appear to be at higher activity levels in the elapids, there is a broad range of activity throughout the viperids. When hyaluronic acid is degraded by hyaluronidase, the remaining venom components are able to spread through the tissue, since their movement is delimit
ed. This can lead to localized necrosis, as nearby cells are destroyed by other venom compo
ments, or to systemic effects, as other venom components spread into blood vessels made “leaky” by the hyaluronidase.

L-Amino acid oxidases (LAAOs) are the major exception to venom enzymes in that they catalyze the oxidation of L-amino acids through a two-step deamination process (Chippaux, 2006). The result of the deamination is a general degradation of amino acids that may result in cell damage or apoptosis (Tan and Fung, 2009).

Proteolytic enzymes are those enzymes that lead to the degradation of structural proteins into component peptides or amino acids. They have great digestive capability and can hydrolyze proteins in their native (non-denatured) state through cleavage of peptide and ester bonds (Mebs, 2002). Some of these enzymes have been categorized as metalloproteinases (because of their reliance on metal ion co-factors) and serine proteases (because of their similarity to blood factors). Metalloproteinases cause
hemorrhage and necrosis, but may also be responsible for digestion of prey. Serine proteases disrupt hemostasis and may do so through multiple mechanisms that disrupt the blood clotting cascade (Marsh, 1994; Mackessy, 2009).

Serine proteases are generally placed into three categories, depending on their mode of action. The thrombin-like serine proteases cleave fibrinogen at the same location as thrombin, leading to a rapid depletion of fibrinogen. The resulting fibrin, however, is not coagulable due to a lack of fibrin stabilizing factor leading to an overall anti-coagulation effect and possible circulating clots in the blood stream (Marsh, 1994; Markland, 1998; Swenson and Markland, 2005). Kallikrein-like serine proteases cause release of bradykinin from high molecular weight kininogen and degradation of angiotensin. The result of both of these is a precipitous drop in blood pressure (Nikai and Komori, 1998). Although they have enzymatic activities against peptide and ester substrates, the biological effects of arginine esterases are not fully understood (Mackessy, 2009).

PLA₂s are a very interesting group of enzymes found in almost all venomous species, including viperids, elapids, and some colubrids. In general, PLA₂s are active in destroying the phospholipid layer of cells. However, it is possible for very small changes in the structure of PLA₂s to cause a change in its physiological activity (Chijiwa et al., 2003; Kini and Chan, 1999). As previously noted, PLA₂s can be edema-inducing, lipolytic, myolytic, or acetylcholine release inhibiting (Mebs, 2002). The diversity of function and apparently wide structural variation of PLA₂s makes them interesting for studying molecular evolution (Chijiwa et al., 2003; Creer et al., 2003; Li et al., 2005).
The study of snake venom CRiSPs is fairly new, with most work being done in the last 15 years. CRiSPs have been found to have diverse functions in different species, ranging from disruption of potassium or calcium currents in neurons to induction of hypothermia (Yamazaki and Morita, 2004; Heyborne and Mackessy, 2009). The functions of many similar CRiSPs in other species, however, have not been elucidated.

Nerve growth factors stimulate growth of nerve cells and are found in both viperid and elapid venoms. The actual mechanism of function in prey is not fully understood, nor is the biological function (Kostiza and Meier, 1996), and the number of studies has been limited.

PLA$_2$-based presynaptic neurotoxins function by blocking acetylcholine release from axon terminals, thereby causing a flaccid paralysis. These have very strong neurotoxic functions, and they include the Mojave toxin found in some rattlesnake species (John et al., 1994). Other viperid toxins, the non-PLA$_2$-based myotoxins, disrupt voltage-sensitive sodium channels, leading to prey immobilization and myonecrosis (Mackessy, 2009).

C-type lectins and disintegrins both effectively alter the blood clotting cascade, but in different manners. C-type lectins bind to platelets, and this can either cause initial clotting, or it can cause platelets to be removed from forming clots, depending on the specific form of the lectin (Komori et al., 1999; Du and Clemetson, 2009). Disintegrins act by disrupting platelet aggregation, and they are often found grouped with a metalloproteinase (the combination being called an ADAM—A Disintegrin and Metalloproteinase). Some disintegrins halt initial aggregation, while others work by disrupting formed aggregates (Okuda and Morita, 2001).
Bradykinin potentiating peptides (BPPs) function by effectively being an inhibitor of angiotensin converting enzyme (ACE). ACE is responsible for converting angiotensin I into angiotensin II, and angiotensin II causes vasoconstriction. Besides blocking ACE activity, BPPs may have a direct effect on angiotensin II, but both actions lead to a reduction in vasoconstriction, allowing bradykinin to cause vasodilation and hypotension (Ferreira et al., 1995, 1999).

Besides substances that are effective biologically against prey, some snake venoms also contain factors that appear to help stabilize these other components. These include tripeptide inhibitors (Francis and Kaiser, 1993) and citrate (Freitas et al., 1992). These apparently work by inhibiting the enzyme interactions in the venom, so that the biological functions do not occur until the venom has been injected into potential prey and dispersed.

There are many other venom components that have been characterized from species outside of Viperidae. These include many other toxins, some blood clot disruption factors, and some enzymes. Indeed, colubrid venoms have just recently become the subjects of many studies (Mackessy, 2002), and the total diversity of components has probably only been superficially recognized. As the focus of this introduction is on a viperid snake, non-viperid venom component categories will only be described briefly.

Toxins are a large and diverse group of non-enzymatic peptides that have various functions. They normally affect the nervous system by altering presynaptic or postsynaptic functions at a neuromuscular junction. Toxins that affect these junctions presynaptically have two primary functions: they can interfere with the release of
acetylcholine from the nerve endplate, or they can block specific ion channels, thereby blocking repolarization and increasing the amount of acetylcholine released. In the first case, nerve signals are not fully propagated to the motor unit, so that a flaccid paralysis occurs; in the second case, signals may be propagated unintentionally or with uncontrolled rapidity leading to spasms and possible tetany. Other toxins affect neuromuscular junctions postsynaptically by binding to acetylcholine receptors, thereby blocking acetylcholine uptake and leading to paralysis. While presynaptic toxins are found in both viperids and elapids, they are much more prevalent in elapid venoms and cause a much greater response in prey. Postsynaptic neurotoxins are predominantly of elapid origin.

Another group of toxins, the cardiotoxins, are found primarily in elapids, but may be in smaller concentrations in viperids. These toxins can cause hemolysis of red blood cells, lysis of other cells, depolarization of excitable membranes, and activation of phospholipases A₂ into toxins.

There are other venom components that are mostly limited to elapids. These include acetylcholinesterases and fasciculins. It is logical to assume that acetylcholinesterases function to degrade acetylcholine at the neuromuscular junction (as endogenous forms do). However, in venom these enzymes are so large that they do not seem to actually reach the site where they could do the most harm (i.e. the neuromuscular junction; Mebs, 2002), so their actual effect is not known. Acetylcholinesterases have been found in colubrid venom, as well (Mackessy et al., 2006). The fasciculins have the opposite effect on acetylcholine transmission, as they are potent inhibitors of acetylcholinesterase. By reducing acetylcholinesterase activity,
more acetylcholine travels across the neuromuscular junction and is detected postsynaptically. This can cause spasms and tetany.

The diversity of venom components, both structurally and functionally, is enormous, and is only touched upon here. Often venom components are categorized by function, whereas they are at other times categorized by structure. This leads to much confusion as components of similar structure can cause different effects, and components of different structure can cause similar effects. Also, the number of distinct enzymes and toxins known is increasing, as is the number of known unique toxin families. All of this makes for an interesting and dynamic field of study.

**Venom Delivery Systems**

In snakes, effective use of venom is accomplished by the venom delivery system (VDS). The VDS includes the venom, an associated gland used for production and storage, and specialized teeth used for delivery. The evolution of components of VDSs, including a presumably co-opted salivary gland, allowed for the improved success of snakes because species with these adaptations were more readily able to capture and subdue prey, some of which may have been potentially harmful to the snake. Venomous snakes can either envenomate prey, without any further contact, and consume it after it has died, or they can envenomate the prey while grasping it. In the first case, snakes need not be in contact with prey item except for initial envenomation and final consumption. In the second case, the venom reduces the time the snake is in contact with the prey item (Shine and Schwaner, 1985); this is important because struggling prey can injure or kill snakes. Some species even have the capacity to track envenomated prey through the unique chemical signature of the venom (Furry et al.,
1991; Lavin-Murcio et al., 1993). Reduced prey handling time reduces energy spent struggling with prey and significantly decreases the potential of injury to the snake.

Since the origin of a rudimentary venom gland, the basic pattern has been altered in different snake lineages. Four venom gland morphologies typically are recognized, based on traditional phylogeny: 1) the atractaspidid type; 2) the colubrid type (historically referred to as Duvernoy’s gland); 3) the elapid type; and 4) the viperid type. There is, however, high variability among the morphologies of different species within one family, with the most consistent morphology found in the Viperidae and Elapidae, and the least consistent in the Atractaspidae and Colubridae (Kochva and Gans, 1970).

In general, the atractaspidid venom gland is large, tubular, and elongated posteriorly behind the eye (well beyond the head in some species, such as *Atractaspis engaddensis* (Kochva, 1987)). It comprises mostly unbranched tubules radiating from a central lumen. These glands are not associated with an accessory gland, but mucosal cells line the major portion of the length of the lumen. Also, the terminal duct of the gland empties venom into anterior, tubular fangs (Kochva et al., 1967). In atractaspidids, venom appears to be forcibly ejected through the compression action of an adductor externus medialis muscle that extends from the parietal bone around the posterior end of the gland to the corner of the mouth (Jackson, 2003).

The venom gland of colubrid snakes, if present, is relatively small in most species and also is located posteriorly and ventrally to the eye (Ovadia, 1984, 1985). This gland is somewhat oval in morphology, is laterally compressed, and is considered to be the ancestral state of the Colubroidea (Vidal, 2002; Jackson, 2003). In *Boiga irregularis*,

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one of the few species in which it has been examined, the gland is multi-lobed, with secondary branching occurring within each lobe (Zalisko and Kardong, 1992). The gland is usually encased in a thin connective tissue layer, and consists mostly of serous cells. There is a small central cistern that is analogous to the lumen of other venom glands, but is greatly reduced in comparison (with exceptions such as *Dispholidus typus* and *Thelotornis capensis*—Greene, 1997). One main duct empties the central cistern into the oral epithelium near the corner of the mouth. No accessory glands are associated with the colubrid venom gland, and generally there are no associated compressor muscles. Taub (1967) noted that Duvernoy’s glands contain either purely serous cells (as in *Boiga dendrophila*, *Diadophis punctatus*, *Erythrolamprus bizona*, and others), or a mixture of serous and mucosal cells (e.g., *Nerodia sipedon* and *Thamnophis elegans*), with mucous cells being found in the duct (Kochva, 1987).

Elapid snakes have an oval-shaped serous gland that is associated with a mucous accessory gland. The gland is located ventrally and posteriorly to the eye, with the main part of the gland composed of tubules with simple or complex branching patterns. These tubules empty into a narrow lumen that is surrounded by the accessory gland. After passing through the accessory gland, venom is transported to the anterior fangs. A split adductor externus superficialis muscle that exerts force on the dorsal and ventral aspects of the gland forcibly ejects contents of the gland. It appears that in elapids venom is stored mostly within cells, rather than in the lumen (Jackson, 2003).

The viperid type of venom gland is the most widely studied, and appears to have the most conserved morphology of the families of the Colubroidea (Kochva and Gans, 1970). It is a large, triangular (in profile) gland that is divided into several lobules by
folds in the surrounding tissue (Kochva, 1987). The accessory gland is located just posterior to a secondary duct that empties into large, grooved fangs at the anterior end of the mouth. Between the mucosal accessory gland and the main serous venom gland is a primary duct. This duct is connected to a large lumen fed by multiple, complex branched tubules. The posterior end of the gland expands dorsally to the level of the eye. The large store of venom in the viperid gland lumen is ejected by a complex pattern of musculature that includes the adductor externus profundus muscle (also often termed the compressor glandulae; Jackson, 2003).

Along with these gland types, multiple forms of associated teeth are used to aid in venom delivery by rupture of the skin of the prey and/or conduction of the venom along dental grooves. Tooth types include: 1) aglyphous—small, ungrooved teeth throughout the oral cavity; 2) opisthoglyphous—small (but larger than other teeth in the oral cavity), grooved teeth located in the dorsal, posterior aspect of the mouth in the proximity of the duct that empties the venom gland; 3) proteroglyphous—larger, fixed fangs located anteriorly on the maxilla that have deep grooves (forming partially to fully enclosed tubes in some species); and 4) solenoglyphous—very large, grooved, rotating fangs that are located anteriorly on the maxilla and fold against the roof of the mouth when it is closed. Examples of venom-producing aglyphous snakes include many colubrids (such as *Natrix tessellata* (Ovadia, 1984), *Spalerosophis cliffordi* (Ovadia, 1985; Rosenberg et al., 1985) and *Thamnophis sirtalis* (Kochva, 1965)) as well as some atractaspidids and (under the phylogeny of Lawson et al., 2005) elapids. Opisthoglyphous fangs are seen in many colubrid snakes, including *Boiga irregularis* (Zalisko and Kardong, 1992) and *Telescopus fallax* (Kochva, 1965). The proteroglyphous condition is seen in the snakes
of the Elapidae. All viperids (such as *Causus rhombeatus*—Shayer-Wollberg and Kochva, 1967) are solenoglyphous, as are some atractaspids (such as *Atractaspis engaddensis*—Kochva et al., 1967), although the groups have different fang morphology.

A recent examination of the evolution of different fang types indicates that the ancestral state in snakes is to have no differentiation of teeth along the entire dental lamina (i.e. no fangs). Next, a secondary dental lamina, located in the posterior region of the oral cavity, allowed for elongated and specialized teeth to form in proximity to the venom gland. More recently, in what appears to be two independent occurrences, the anterior dental lamina that originally gave rise to normal teeth was lost in both elapids and viperids. This allowed fangs to be produced in the anterior part of the oral cavity—in neonates of these species the fang originates at the posterior aspect of the oral cavity and then migrates to the anterior position during ontogeny (Vonk et al., 2008).

Venoms historically have been considered either hemotoxic (affecting the blood) or neurotoxic (affecting the nervous system), although those terms do not adequately describe venom complexity. Indeed, a single venom sample may contain compounds with both general functions (e.g., crotaline snakes of the western United States—Glenn et al., 1983; Glenn and Straight, 1985, 1989, 1990; Sánchez et al., 2005). Further, the use of these two terms oversimplifies the action of a mixture that may attack many aspects of physiology other than the circulatory and nervous systems. The physiological functions of snake venoms are diverse and vary by species. Variation within venoms can be found at many taxonomic levels, and is a worthwhile topic for study not simply because of potential human health consequences (antivenom and
pharmaceutical research), but also because it can be used to examine inter- and intra-specific mechanisms of evolution.

**Variables That May Affect Snake Venom Evolution**

Many factors may potentially affect the evolution of snake venom. First among these is the ability to capture prey easier through the use of venom. It is easy to imagine that an ancestral species of snake with a salivary toxin affecting its prey would be more able to subdue prey than snakes lacking this toxin. This increased prey capture ability could translate into increased reproductive success by allowing for rapid growth and enhanced body condition. Rapid growth would allow snakes to avoid some predators because the snakes would reach larger sizes in less time. Enhanced body condition could lead to increased fecundity in females, increased mating success in males of species with male-male combat, and increased male mating success, in general, through differential selection by females. The ability to obtain prey more easily allows organisms to allocate more energy toward reproduction, and less toward foraging.

The ability of the venom to affect different prey species must also be considered. To be successful for prey capture, venom must either contain toxins that are effective toward specific prey species or are general enough to subdue multiple prey species. It is possible that snake species with many different types of toxins (and thus greater variation) would be more able to obtain prey simply by increasing their prey pool. On the other hand, it is also possible that snakes could become more specialized (and thus have less variation) by utilizing an unexploited food source. If this were true, venom variation should be maintained in generalist feeders and reduced in specialists.
The relative danger of potential prey could also influence the evolution of venom composition. If a snake has a prey pool that includes species that can cause the snake physical harm, then more rapidly-acting venom would decrease injury. Conversely, species that consume prey lacking dangerous defenses would likely evolve less toxic venom, assuming that there is a cost of production of strong venom (Li et al., 2005). As the venom would be less toxic, species consuming defenseless prey should have less venom variation than species consuming potentially harmful prey. This lower variation would be due to loss of toxic venom components.

The energetic cost of production and maintenance of venom has not been studied thoroughly, but has been shown to affect metabolic rates in recently-milked snakes (McCue, 2006). If venom has a high energetic cost, then it should be produced only when it confers an advantage to the snake species possessing it. A high energetic cost should also reduce the variation of the venom, as wider variation in chemical production would lead to an unnecessary increase in metabolic cost by co-opting multiple synthesis pathways. On the other hand, if venom is not energetically costly, no such constraint should exist, and individual venoms could easily contain a high diversity of components. Of course, different venom components could have different metabolic costs, confusing the issue even further.

**Studies of Venom Variation—General**

Chippaux et al. (1991) reviewed research on venom variation and the techniques used to analyze it. The review indicated variability in venom composition at multiple taxonomic levels, and gave many examples from each taxonomic level. Interest in venom variation has not diminished. Differences in venom enzymatic activities (Tan and Ponnudurai, 1991, Cavinato et al., 1998, Monteiro et al., 1998, Otero et al., 1998,

**Studies of Venom Variation—Evolutionary Implications**

Previous work has examined the correlation of venom components with other parameters. In an attempt to determine how venom evolves, Daltry et al. (1996b) studied the Malayan pitviper (*Calloselasma rhodostoma*) in the Malay Peninsula (Vietnam, Thailand, and Malaysia) and Java (Indonesia). They visualized whole protein composition of venom from adult snakes using isoelectric focusing and compared it to phylogenetic relatedness, geographic proximity, and local prey type. Relatedness was determined by using restriction-fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA). Prey type was determined by examination of fecal samples from wild snakes combined with gut content analysis of museum specimens from known localities (Daltry et al., 1998).
Daltry et al. (1996b) considered three general hypotheses to explain venom variation over their area of study: 1) geographically close populations have similar venom composition based on gene sharing through local mating or local environmental conditions, indicating that gene flow is a major factor; 2) similarities are based on phylogeny, so that populations with recent common ancestors would have more similar venom composition, indicating that genetic drift is in operation; or 3) variation in local diet explains venom variation because venoms more effective toward locally abundant prey are selected over less locally-effective venom, indicating that natural selection is in action. Their analysis indicated that venom composition was more closely correlated with local prey type than with geographic proximity or phylogeny, and led them to suggest that natural selection was acting through local prey type as the prime factor influencing venom composition over gene flow and genetic drift.

Sasa (1999a, 1999b) expressed skepticism about this result, and postulated a fourth possibility—that venom composition variation has no adaptive value. Under this hypothesis, variation in venom composition does not causally affect fitness and should not show any correlation.

In contrast to Daltry et al. (1996b) is a study of Notechis ater niger by Williams et al. (1988), where venom samples from populations of this Australian elapid snake were subjected to gel filtration chromatography and SDS polyacrylamide gel electrophoresis (SDS-PAGE). Twenty different SDS-PAGE bands were present in venoms from snakes throughout the region, but differed by population. In short, the 12 different populations logically grouped into 7 different elution patterns. The populations of similar banding pattern were not correlated with available prey or with local environment, but rather
geographic distance. The populations studied all were insular, and their venom profile was also correlated with time since separation from nearby landmasses, as determined by water depth. The authors thus concluded that variation in venom composition in this species was more directly linked to vicariance events followed by genetic drift than to any selective pressures.

Creer et al. (2003) examined the viperid *Trimeresurus stejnegeri* in Taiwan to determine correlations among venom composition (as determined by mass-to-charge peak profiles using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)), genetic relatedness (as determined by mtDNA analysis), geographic location, and diet (based on dissections of field-collected snakes (Creer et al., 2002)). They noted that venom composition was not tightly correlated with phylogeny, and postulated that variation in venom composition may be caused by natural selection due to regional diets or by founder effects. The idea that evolution of venom composition may be influenced by different modes of evolution under different environmental conditions has not been directly examined.

More recently, Barlow et al. (2009) found that venom toxicity was correlated with the relative amount of invertebrate prey upon which snakes of the viper genus *Echis* fed. They noted that there was great variation in venom composition among the species examined, and that the same was true for diet variation. However, those species that were known to feed to a greater amount on scorpions had a venom toxicity that was much greater against scorpions than other species (analyzed via lethal dose comparisons). This study indicates natural selection of venom components that are useful against specific prey types.
To fully determine how venom is evolving in snakes, it is necessary to separate out the relative importance of the different modes of evolution. These may be different under various ecological conditions, but they represent a first step towards predicting evolutionary trajectories. Recent work (Reza et al. 2006) has indicated that gene duplication with resulting neo- or sub-functionalization may be responsible for adding to the variation found within venoms. This study, those examining novel venom components, and those analyzing differences in venom activity within and among populations indicate the vast diversity of venom components.

**The Florida Cottonmouth—A Model for Study of Venom Evolution**

The Florida cottonmouth snake (also often referred to as the water moccasin), is a viperid snake common throughout Florida. The species has been well studied (Gloyd and Conant, 1990) and its venom has been the subject of numerous studies (see Chapter 2). It makes a good model species for examinations of venom evolution due to its abundance, well-described ecology, and rich venom literature.

Although this species is considered continuous throughout its range, it is mostly associated with fresh water habitats, so it tends to have local abundance in favorable habitat. Furthermore, there is one population on the island of Seahorse Key that appears geographically isolated and has a very unique ecology. Snakes on this island subsist mostly on fish that are dropped or regurgitated by colonially-nesting seabirds (Wharton, 1969; Lillywhite and McCleary, 2008). This seasonally abundant food source raises questions as to how diet may affect venom composition. As a comparison, snakes on the mainland are known to feed on a wide range of prey and must either actively forage or use sit-and-wait tactics to catch prey. They are also known to be scavengers.
The dichotomy of mainland versus island snakes offers a chance to compare the relative influence of prey on venom composition in the cottonmouth. It also affords the opportunity to characterize natural variation in phenotypic venom characteristics among natural populations. As natural variation is one factor necessary for evolution to occur, simply cataloging any occurring variation is very important. By characterizing inter- and intra-population genetic relatedness, venom characteristics, dietary breadth, and correlations among these, it should be possible to better understand the manner by which these populations, in general, and their venom, in particular, are evolving.
Figure 1-1. Phylogenetic relationships among venomous snake families. This phylogeny, adapted from Pough et al. (2001), shows the traditionally accepted relationships of the advanced snakes, although there is much current dissent in the literature. Many basal families have been excluded as they do not produce venom. Numbers shown indicate the taxa: 1) Colubroidea, 2) Caenophidia, and 3) Macrostomata.
CHAPTER 2
THE VENOM OF THE COTTONMOUTH

Introduction

The toxic secretions of venomous snakes have been studied due to their importance in diagnosis and treatment of snakebite symptoms. Although not as toxic to humans as related rattlesnakes, the cottonmouth is responsible for both lethal and non-lethal bites throughout its range. It is one of the most common snakes in the state of Florida (pers. obs.), and is likely to come in contact with humans in any area where both species utilize resources, which includes most fresh water habitats.

In general, cottonmouth envenomation in humans causes local tissue damage including direct hemolysis, hemorrhaging, and disruptions in the blood clotting cascade. Local tissue damage can be caused by the combined effects of many different enzymes, including phosphodiesterases, 5’-nucleotidases, alkaline phosphomonoesterases, L-amino acid oxidases, metalloproteinases, arginine esterases, and phospholipases. The enzymology of cottonmouth venom has been examined both in terms of the effects of specific venom enzymes (enzyme-based study) and the overall effects of either crude or separated fractions of venom on potential substrates (substrate-based study). While enzyme-based study is difficult because it requires pure enzyme, it results in data specific to one compound, although this may obscure potential synergistic reactions with other venom or target components. Substrate-based studies can indicate overall effect on one substance, but without immediate determination of the responsible enzyme or enzymes. In some cases, multiple enzymes, cofactors, and/or substrates combine to cause an effect.
One enzyme that has been extensively studied in cottonmouth venom is phospholipase A$_2$. This enzyme has been examined for its effects on target tissues, but the specific forms found in cottonmouth venom also have been used as models for studies of interfacial lipid chemistry. Further, cottonmouth PLA$_2$s have been used as a tool in examining dynamics of nerves and cell clusters.

Physiological research using cottonmouth venom has led to examinations of its multiple modes of action in different species. Among these examinations are numerous studies of treatments for various human blood diseases. Besides human testing, other animal species have been utilized as models to examine local and systemic physiological effects of the venom.

Further, cottonmouth venom has been utilized to explore ecological and evolutionary questions. Such explorations include examinations of the effects of venom on chemosensory ability in snakes, comparisons of venom composition among related species, and ability of potential predators and prey to withstand envenomation.

Recent research utilizing aspects of cottonmouth venom have focused on examinations of novel components, such as vascular endothelial growth factor. Still other studies are beginning to examine the shear diversity of venom components expressed utilizing modern sophisticated biochemical and molecular techniques.

The study of cottonmouth venom has mirrored that of the study of snake venoms in general, and has a rich literature reaching back over 150 years (see Ingalls, 1843). While this research has answered a lot of questions and garnered much data, there is still much to be studied. This review is specifically concerned with research on venom of the cottonmouth, *Agkistrodon piscivorus*, and is not an attempt to explain everything
that is known about snake venom. In some cases, extra information from the general
literature is included for clarity. This is an attempt to be as complete as possible, but
the shear volume of work done thus far makes it likely that some ancillary research may
have been missed.

**Human Envenomations by Cottonmouths**

**Demographics of Human Envenomation**

Cottonmouths are occasionally responsible for human envenomations, and
although comprehensive information is lacking, many studies have examined
cottonmouth bites in terms of frequency, location, and other demographic categories.
Interpretation of studies reporting snake bites is difficult because of the potential for
improper identification of species, so most information must be considered carefully.
For many of these, snakebite treatment is not completely standardized, and each case
may have different data collected. Sometimes snake bite data are reported as a group,
regardless of species or demographic information concerning the afflicted individuals.
Often, bite location (anatomical and/or geographic) is not reported or the time frame of
the study is not given (Snyder and Knowles, 1963; Parrish et al., 1965). However, there
are usable data in almost every reported study. One notable exception to this rule was
the case of a human bitten by a “moccasin,” in which the patient recovered after the
anus of two live chickens were used to “draw out” the venom (both chickens died)
and the patient was prescribed ammonia as medication (Ingalls, 1843). Table 2-1
shows a comparison of published studies and indicates (where possible), number and
overall percentage of snake bites attributed to cottonmouths, as well as reports of
mortalities for the specific timeframe and geographic region covered.
Photos of cottonmouth envenomation in humans have been published (Snyder et al., 1968, 1972). Also, detailed case studies of humans bitten by cottonmouths have been described (Parrish, 1967; Henderson and Dujon, 1973; Roberts et al., 1985).

**General Physiological Effects (Human)**

In cases of humans bitten by cottonmouths, the symptoms range from nothing (likely due to lack of venom injection) to death. Case studies of such instances indicate a general sequence of events, but there may be variation depending on the site of the bite, amount of venom injected, and the treatment rendered following the bite. Prior to the development of effective antivenom treatment, symptoms and sequelae were much harsher than in modern bites treated with advanced techniques. Reports of these previous instances give an indication of the general sequence of events in humans with untreated bites.

After envenomation, there is severe pain at the site of the bite. This pain is followed by localized edema and erythema which continues to get worse without treatment (Parrish, 1967) and can lead to compartment syndrome (Roberts et al., 1985). If the venom spreads throughout the circulatory system, hemolysis of blood and fibrinolysis of clots lead to a drop in blood pressure, which can render the victim very weak and eventually helpless (McCollough and Gennaro, 1966; Parrish, 1967). If the envenomation is not treated, the loss of blood pressure can lead to organ failure and eventually death; although death from cottonmouth bites is quite rare (Table 2-1).

The sequence of symptoms leading up to death has been described for dogs (Brown, 1941; Vick et al., 1967) and cats (Russell and Bohr, 1962) and is similar to that in humans.
Specific Physiological Effects (Comparative)

Epithelial tissue

*Agkistrodon piscivorus* venom has been used in comparative physiological research to examine direct effects on skin. Direct application induces purpura in rat skin (Spaet, 1952), and petechial hemorrhaging in the hamster cheek (Arendt et al., 1953; Fulton et al., 1956) and dog lung surface (Bonta et al., 1969, 1970a, 1970c).

Subdermal tissue

In studies where venom is injected intradermally, there are local effects ranging from edema to necrosis. Likely due to the presence of hyaluronidase, venom often spreads from the injection site to other areas, making it difficult to separate strictly local effects from potentially systemic effects. The induction of edema in the rat paw has been used to study potential anti-inflammatory substances (Rocha e Silva et al., 1969) and venom inhibitors (Marshall et al., 1989), and such untreated edema eventually leads to hemorrhage and myonecrosis (Mebs et al., 1983).

Blood—hemolysis

Very early in the study of cottonmouth venom, the ability to lyse red blood cells (RBCs) was either conjectured or shown. Mitchell and Reichert (1886) postulated that hemolysis may be occurring in the pigeons upon which they did their studies. Flexner and Noguchi (1902) noted that a very dilute concentration of cottonmouth venom had great ability to hemolyse dog and rabbit blood in vitro, even when they heated venom to 96°C prior to use. They noted that RBCs separated from serum were not lysed by venom, indicating a factor in serum was necessary for hemolysis to occur. Further work during this time noted that separated horse RBCs hemolysed within 40 min of exposure.
to venom (Noc, 1904), and confirmed the low doses needed (Noguchi, 1909) and general temperature insensitivity (Madsen and Noguchi, 1904).

Decades later, it was stated that the hemolytic activity of cottonmouth venom was very sensitive to heat, was most stable at pH 5.0 to 7.0, quickly lost activity in solution, and was not affected by exposure to chloroform (Peck and Marx, 1937). These researchers also indicated that the hemolytic activity was caused by two different substances, called hemolysins A and B, that could be separated electrophoretically (Marx and Peck, 1938). The hemolytic factor of the venom works rapidly to destroy lymphocytes, but polymorphonuclear leukocytes are more resistant to lysis (Schrek, 1943). In general, a dose-dependent effect of venom on hemolysis using separated human RBCs was noticed, and the addition of serum increased this action (Philpot, 1949).

The hemolytic ability against RBCs was further examined in rabbits (Minton, 1956) and guinea pigs (Bhargava et al., 1970; Vincent et al., 1972), and one study showed hemolysis of RBCs from humans, capuchin monkeys, dogs, pigeons, rabbits, snakes (Bothrops asper) and toads (Bufo marinus) (Gomez-Leiva, 1976). A further examination showed 35 to 95% hemolysis for RBCs from ten different mammal species (Kelen et al., 1960-62). Addition of heparin (Vincent et al., 1972), dextrane sulfate (Vincent et al., 1972), and albumin (Gul et al., 1974) increased the hemolytic activity; but ethylenediaminetetraacetic acid (EDTA) reduced hemolysis (Deutsch and Diniz, 1955). Interestingly, A. piscivorus RBCs appear to be resistant to hemolysis by venom (Minton, 1976).
Using Sephadex G-50 to separate the venom, no hemolysis activity was seen in any of the three resulting fractions (Bonta et al., 1970b). However, activity was seen in fractions collected after ion exchange chromatography of whole venom (Clark and Higginbotham, 1971).

There is some disagreement as to the relative level of hemolysis brought about by cottonmouth venom. Deutsch and Diniz (1955) state that *A. piscivorus* venom has the highest hemolytic rate of the snake venoms they examined, but Rosenfeld et al., (1960-62) state that it has only weak hemolytic activity. Utilizing blood agar plates and fractionated venom, Gennaro and Ramsey (1959a) found only minor hemolytic activity.

**Blood—coagulation**

While the overall effect of *A. piscivorus* venom on blood is anti-coagulant, some aspects of the venom enhance coagulation, leading to clot formation. Boiling venom prior to injection into an animal model causes at least initial coagulation of blood (Mitchell and Reichert, 1886), and fairly small concentrations are necessary for initiation of coagulation (Noguchi, 1909). When venom is combined with horse fibrinogen or prothrombin (precursors to blood clots) at low concentration, there is a coagulant effect that disappears with time or higher concentrations (Eagle, 1937). Likewise, rabbit RBCs show coagulation at low venom concentrations (Schrek, 1943; Minton, 1956), and human RBCs show great initial agglutination in short time periods with concentrated venoms (Philpot, 1949). These quickly decline with time, and the same is true for dogs injected with venom (Houssay and Sordelli, 1919). Oshima et al. (1969) indicate that fibrin clots appear within 2 hours of venom mixing with RBCs. *Agkistrodon piscivorus* venom does contain lectins, which are known to aid in agglutination and may account for initial clotting activity (Ogilvie and Gartner, 1984).
Blood—anti-coagulation

Inhibition of blood coagulation has been noted for some time (Mitchell and Reichert, 1886; Noc, 1904) as has the increase in clotting time of blood mixed with cottonmouth venom (Houssay and Sordelli, 1919). Taylor and Mallick (1936) suggested that the prolongation of clotting time indicated that *A. piscivorus* venom was anti-thrombic, as did Fleckenstein and Fettig (1952), and Eagle (1937) indicated that the anti-coagulant effect was due to some form of proteolysis of blood clotting proteins. Others later suggested that the venom was either fibrinogenolytic (Didisheim and Lewis, 1956) or fibrinolytic (Rosenfeld et al., 1959). This has led to disagreements in the literature, with some indicating low (Mebs, 1970) or high (Bhargava et al., 1972) kinin-releasing activity, no (Bajwa et al., 1982) or mild (Copley et al., 1973) thrombin activity, and no (Kornalík, 1966; Bajwa et al., 1982) or very low (Kornalík and Stýblová, 1967) plasminogen activation.

Although there have been many examinations of the way venom may disrupt different parts of the blood clotting cascade, research in this area is ongoing. What is known is that cottonmouth venom contains a protein C activator (Exner et al., 1985), which can act as an anti-coagulant. This substance in venom is 39 to 42 kDa in molecular weight and is stable after heating to 70°C at pH 3.0 (Stocker et al., 1986). Two fibrinogenolytic enzymes, piscivorase I and II, (Hahn et al., 1995; Markland, 1998) have been purified from cottonmouth venoms, as have two disintegrins—applaggin and piscivostatin. All of these substances have resulting anti-coagulant activities, and they all may work in combination to exacerbate overall disruption of the blood clotting cascade.
Muscle

Because of their proximity, it is sometimes difficult to separate the effects of venom on skin and underlying tissue such as muscle or small blood vessels. However, examination of muscle effects generally show swelling at the site of venom injection with underlying hemorrhaging and eventual myonecrosis (Homma and Tu, 1971). Mebs et al. (1983) found venom injected intramuscularly into rats caused creatine kinase production (an indicator of muscle degradation) at four hours that was reduced at 16 and 48 hours. Also, direct injection into pigeon breast muscle showed major hemorrhaging of surrounding tissue (Mitchell and Reichert, 1886).

Envenomation in dogs makes skeletal muscle unresponsive to direct or nervous stimulation, but this is likely a secondary effect (Hadidian, 1956). Venom added directly to guinea pig muscle showed decreased invoked muscle contractions followed by complete block (Russell and Long, 1961). Yamazaki et al. (2003) indicate that the presence of cysteine-rich secretory proteins in venom leads to reduced muscle contraction in rat tails, and the venom fractions responsible for myonecrosis have been separated using Sephadex G-75 and CM cellulose chromatography (Mebs and Samejima, 1986).

The Lys49 (AppK49) form of A. piscivorus venom phospholipase A₂ has been determined to cause direct myotoxicity by binding long-chain fatty acids (Pedersen et al., 1995). By creating a synthetic peptide containing the C-terminal sequence of AppK49, Núñez et al. (2001) demonstrated its ability to lyse skeletal muscle, and the amino acid residues 115 through 129 have been shown to be the most important structural aspects for this function (Lomonte et al., 2003b; Yamazaki et al., 2005a, 2005b). AppK49 is actually a homodimer, and dissociation of its tertiary structure
greatly reduces myotoxic activity (Angulo et al., 2005). Lomonte et al. (2003a) have reviewed the myotoxic action of AppK49.

**Nervous tissue**

Rosenberg and colleagues have conducted a series of experiments using nerves, especially the giant squid axon, to examine venom effects. They found that, in general, small concentrations of venom make the nerve susceptible to other exogenous compounds, by increasing nerve membrane permeability (Rosenberg and Podleski, 1962; Rosenberg and Ng, 1963; Rosenberg and Podleski, 1963; Hoskin and Rosenberg, 1965; Rosenberg, 1965; Rosenberg and Hoskin, 1965; Rosenberg and Dettbarn, 1967; Condrea et al., 1967). A similar phenomenon was shown using frog nerves (Hadidian, 1956), and the factor responsible for this action was hypothesized to be PLA$_2$ (Rosenberg and Podleski, 1962; Rosenberg and Hoskin, 1963; Rosenberg and Ng, 1963; Condrea, 1967).

Russell and Long (1961) showed that venom added to guinea pig phrenic nerve preparations initially caused muscle contractions that decreased in amplitude until neuromuscular block at 21 min post-application. Pre-treatment with tri-O-cresylphosphate made hen sciatic nerve preparations more susceptible to $A.\ pisivorus$ venom effects (Morazain and Rosenberg, 1970). Guinea pig brain preparations were used to examine the ability of $A.\ pisivorus$ and three other venoms to inhibit brain enzymes that oxidize pyruvate, and boiled cottonmouth venom was found to have the lowest inhibitory effect of the venoms studied (Braganca and Quastel, 1953).

Cottonmouth venom contains a nerve growth factor (Levi-Montalcini and Cohen, 1956; Cohen, 1959), although its mode of action in envenomated prey has not been
examined. Although neurotoxic effects have been hypothesized (Micheel and Jung, 1936; Brown, 1941; Braganca, 1955; Jimenez-Porras, 1968), it is unlikely that cottonmouth venom contains a strict neurotoxin, and that any symptoms resembling neurotoxic effects are secondarily caused by other venom components.

**Respiratory system**

Alterations in respiration have been noted in animals injected with cottonmouth venom. Rabbits have shown immediate increases in rate, followed by decrease and, in some cases, cessation (Mitchell and Reichert, 1886). Dogs injected directly into the femoral vein with higher venom concentrations died from respiratory arrest (Brown, 1941), but it was also noted that most dogs do not die from (or do not have) respiratory complications (Hadidian, 1956). In short, respiratory effects appear to be secondary symptoms of envenomation.

**Circulatory system**

In general, the venom of the cottonmouth affects the circulatory system by causing hemorrhaging of blood vessels, hemolysis of RBCs, and disruption of the blood clotting cascade. This triple effect results in a precipitous drop in blood pressure that incapacitates or kills prey.

Cottonmouth venom has been found to cause hemorrhagic activity in pigeons (Mitchell and Reichert, 1886; Taylor and Mallick, 1936), rabbits (Mitchell and Reichert, 1886; Peck and Marx, 1937; Flowers, 1963), chicken embryos (Witebsky et al., 1935), mice (Ohsaka et al., 1966; Ownby et al., 1994), and dog lung surface (Bonta et al., 1970a, 1970b, 1970c; Bhargava et al., 1970), but not on rat lung (Almeida et al., 1977). The mode of action is characterized as diapedesis (leaking through basement
membranes) or loss of structural integrity of small blood vessels (Malucelli and Mariano, 1980).

The metal chelators EDTA (Goucher and Flowers, 1964; Bonta et al., 1970a) and α-mercapto-β-(2-furyl)acrylic acid (MFA; Giroux and Lachemann, 1981) both reduce the hemorrhagic effect of cottonmouth venom, indicating the presence of metal ions in the hemorrhagic factor. This fact led to the determination of metalloproteinases as the major factors responsible for blood vessel damage (Spiekerman et al., 1973). Hemorrhagic activity was also reduced or destroyed by X-ray (Flowers, 1963) or ultraviolet (Tejasen and Ottolenghi, 1970) irradiation. Treating the venom with heparin causes a precipitate to form but does not appear to affect hemorrhagic activity (Bhargava et al., 1970; Bonta et al., 1970b, 1970c), nor does treatment with chloroform (Peck and Marx, 1937).

Hemorrhagic activity is greatest when pH is between 6.0 and 8.0, but the factor appears to be denatured outside this range (Peck and Marx, 1937). Boiling of the venom does not seem to destroy the hemorrhagic effect (Mitchell and Reichert, 1886). Hemorrhagic activities have been reported for all three subspecies of *A. piscivorus* (Tan and Ponnudurai, 1990).

The hemorrhagic factor was initially described as a basic protein of approximately 10 kDa molecular weight (Bonta et al., 1970b, 1970c), but has more recently been characterized as an acidic protein of 69 kDa molecular weight (Dinh et al., 1985). This latter value is more in line with known metalloproteinases (Mackessy, 2009). The hemorrhagic factor can be separated from hemolytic factors via electrophoresis (Marx and Peck, 1938), and also can be separated from other venom components via
Sephadex G-50 (Bhargava et al., 1970; Bonta et al., 1970b), Sephadex G-100 (Dinh et al., 1985) and ion exchange (Clark and Higginbotham, 1971; Dinh et al., 1985) chromatography.

Cottonmouth venom appears to inhibit angiotensin converting enzyme (ACE) through the action of a bradykinin potentiating peptide, AppF (Ferreira et al., 1995, 1999). The overall effect of such a substance is to reduce or stop vasoconstriction, thereby adding to an overall drop in blood pressure. Bhargava et al. (1970) also described a hypotensive substance called slow reacting substance C.

Weak dilutions of venom affect the heart, although no cardiotoxin has been isolated. Magenta (1922) found that very weak dilutions of venom placed directly on the heart of *L. ocellatus* (genus not reported) caused cessation of beating within 44 min, while isolated frog hearts showed dose-dependent declines in both heart rate and stroke volume (Brown, 1940). Further, isolated rabbit hearts quickly ceased activity after venom was placed on them (Essex, 1932). By using radio-labeled *A. p. piscivorus* venom injected into mice, it was found that large amounts of radioactivity localized to the heart and lungs (Gennaro and Ramsey, 1959b). Other circulatory effects are based on direct effects on blood (hemolysis) and disruptions in the blood clotting cascade (see Blood section above).

**Immune system**

Guinea pig serum mixed with *A. piscivorus* venom showed reduced activity of complement proteins C2, C3c, and C4 in one study (Zarco et al., 1967) and of C2 through C9 in another (Birdsey et al., 1971). Very little activity against C1 was seen (Birdsey et al., 1971).
Mast cell research has been conducted utilizing cottonmouth venom, as mast cells release histamines at the site of envenomation (Zahl and Nowak, 1951). Injection of venom intradermally into guinea pigs causes mast cell degranulation (Raab and Kaiser, 1965). Clark and Higginbotham (1971) described *A. piscivoros* venom as having a mastocytolytic cationic protein that induces local and systemic allergic reactions.

**Lymphatic system**

Cottonmouth venom injected into tissues causes edema formation in rats (Rocha e Silva et al., 1969; Bhargava et al., 1972; Mebs, 1983) and mice (Marshall et al., 1989). Sephadex G-50 separation was used to isolate the substance responsible for edema formation, and it was found to have a molecular weight of about 8 kDa (Bhargava et al., 1972). Marshall et al. (1989) suggested that edema formation may be caused by phospholipase activity via generation of eicosanoids and platelet-activating factor.

**Excretory system**

Very little research has been conducted on the effects of venom on the excretory system of envenomated prey. Huang et al. (1972) used tritiated venom injected into rats to examine excretory patterns. They found an increase in both urinary and fecal excretion of tritium up to 20 hours after envenomation followed by return to normal levels over the subsequent 60 hours. Fecal excretion was the major route of clearance of venom, and liver and intestinal tissue showed the greatest radioactivity distribution.

Direct effects of venom on kidneys have also been examined. When venom is injected into rats, alkaline phosphatase and leucine aminopeptidase activities increase in the urine, indicating nephrotoxicity (Raab and Kaiser, 1966a, 1966b).
Treatment of Cottonmouth Bite

Medical treatment

Treatment for cottonmouth bite follows guidelines for typical pit viper bites in North America (Smith and Bush, 2009). General recommendations include keeping the envenomated patient calm and immediately seeking professional medical attention. The use of traditional snake bite treatments such as suction, cutting, pressure bandages, and tourniquets are not helpful and may actually cause secondary problems. Spread of tissue damage in rats injected with cottonmouth venom was not affected by local application of either cold or heat (Cohen et al., 1992).

Medical treatment generally includes observation of symptoms followed by antivenom use, if warranted (Smith and Bush, 2009). Although previous studies have found fasciotomy to be useful for compartment syndrome (Roberts et al., 1985), it is currently not advised in favor of vigorous antivenom use to reduce compartment pressure (Smith and Bush, 2009).

Antivenom research

Agkistrodon piscivorus venom has been used for antivenom development, as cottonmouths are responsible for human envenomation annually. Early on, it was found that antivenom had specificity for individual venoms, although some cross-reactivity was also noticed (Noguchi, 1906a, 1906b). These initial studies showed that antimoccasin antivenom was more efficacious in reducing toxicity and hemolysis effects, and that this efficacy was both time- and dose-dependent. In one early clinical report, the instance of death in humans bitten by cottonmouths was lower when antivenom was used (4/167 or 2.4%) than when it was not (4/27 or 14.8%), as is expected (Githens, 1935). Current antivenom is more efficacious, and mortality is highly unlikely in treated individuals.
Although the normal mode of antivenom production is to inject small amounts of venom into a laboratory animal and then collect the resulting antibodies, some research has gone into finding alternatives to this method. In one series of tests, researchers used venom initially exposed to the metal chelator EDTA to reduce negative effects of the venom on antibody-producing animals (Goucher and Flowers, 1964; Flowers and Goucher, 1965). They found that local tissue damage and necrosis was reduced in animals receiving venom mixed with EDTA, but that other venom effects were not significantly reduced (Flowers and Goucher, 1965). X-ray irradiated venom was found to lose lethality but maintain antigenicity in laboratory rabbits (Flowers, 1966), and thiabendazole treatment of venom inhibited hemorrhagic activity in guinea pigs (Stone et al., 1966). In this last study, there was also a reduction in venom effects when thiabendazole was injected into animals following envenomation. Further, photooxidized venom injected into mice and rabbits over 3 to 4 weeks afforded some protection against native venom (Kocholaty and Ashley, 1966). Mice given serum from inoculated rabbits were also afforded protection from native venom, and the intravenous route of antivenom was found to be more efficacious than the intraperitoneal (IP) route (Kocholaty and Ashley, 1966; Kocholaty et al., 1968).

In North America, polyvalent antivenoms are created by combining antibodies against the venoms of locally common crotalid snakes (usually genera *Crotalus* and *Agkistrodon*). Examination of polyvalent antivenoms have shown the ability to neutralize 40.8 murine median lethal doses (LD$_{50}$s) per mg antivenom (Gingrich and Hohenadel, 1956), and the ability of polyvalent antivenom to protect dogs against *A. piscivorus* venom also has been shown (Snyder et al., 1968). More recently, polyvalent
antivenom was effective against 2 (but not 5) LD$_{50}$s of cottonmouth venom in mice, in terms of hemorrhagic, myotoxic, and defibrinating activities (Arce et al., 2003). Sánchez et al. (2003) examined three commercially-produced antivenoms and found that fibrinolytic and hide powder azure activities were not inhibited by any of these, but hemorrhagic and gelatinase activity inhibition was case-specific.

**Venom-Associated Anatomy**

The venom apparatus of *A. piscivorus* is typical of that for viperid snakes, and is paired, with both left and right apparatuses. It contains a main gland with a lumen in which venom is stored, a primary duct through which venom leaves the main gland, an accessory gland into which the primary duct empties, a secondary duct through which venom travels, and a tubular fang through which venom leaves (Gennaro et al., 1960, 1963, 1968). The histology of the glandular tissue indicates that the main gland is serous in nature and the accessory gland is mucous-secreting (Gennaro et al., 1963; Rhoades et al., 1967). Venom is produced in the cells of the main gland, and stored in vesicles prior to being secreted into the lumen (Gennaro et al., 1968). Within the lumen, the venom is serous in nature and does not appear to have a mucosal aspect to it until it passes through the accessory gland (Gennaro et al., 1963). The microstructure of both the venom and accessory glands has been examined with electron microscopy (Odor and Gennaro, 1960; Odor, 1965; Andrews et al., 1968; Gennaro et al., 1968), and basic histological staining techniques for the glands have been described (Gennaro et al., 1960).

It has been suggested that the accessory gland may be responsible for increasing the toxicity of the venom (Gennaro et al., 1963; Rhoades et al., 1967). Rhoades et al. (1967) noted that the histology of the accessory gland was typical of that seen in
mucous-secreting glands and that it contained much more uronic acid than the main gland. More recently, a factor that causes lung collapse in frogs was isolated from the accessory gland, but was not found in the main venom gland (Gennaro et al., 2007).

As part of a cDNA library construction, the gene for parvalbumin was isolated from venom gland tissue and cloned (Jia and Perez, 2009). It was localized to the gland, but not to the venom, indicating that it is not secreted by the gland into the lumen.

The musculature surrounding the glandular region, including those muscles involved in venom ejection, have been described in detail by Kardong (1973). The effects of occlusion of the primary duct have been examined in *A. p. piscivorus* and *A. p. leucostoma* (Glenn et al., 1973), and no adverse health effects were seen in the snakes, as it appears that the venom gland initially fills and then stops producing venom.

**Venom Characteristics**

**Basic Description**

**Volume**

Although the amount of venom stored within the lumen of the main venom gland is likely correlated with animal size, an average of 550 µl per extraction was found for 315 animals (sizes not given) over a two year period (Wolff and Githens, 1939a), 320 µl for “young” snakes, 420 µl for adults, and 530 µl for “old” specimens (Do Amaral, 1928). These researchers found that the volume ejected by individuals did not decrease with multiple extractions over the two year duration of their study. Maximum amounts of venom have been reported as 3.5 and 4.0 ml of venom at two different times from one individual (Wolff and Githens, 1939b), and an average of 1.05 ml for “exceptional” specimens (Do Amaral, 1928).
In a typical predatory strike, snakes do not empty their entire venom store. To this effect, it has been stated that *Agkistrodon* (species not stated) eject only 25 to 75% of their venom store in any one bite through a rubber membrane (Do Amaral, 1928). Using radioiodine as a marker, it was also noted that cottonmouths deliver less venom to smaller prey and less overall than *Crotalus atrox* (western diamondback rattlesnake; Gennaro et al., 1961).

**Specific gravity**

Mitchell and Reichert (1886) report *A. piscivorus* venom as having a specific gravity of 1.032 g/ml. This is the only known study to specifically state specific gravity of this venom.

**Protein content**

While some studies examine protein content to report activities on a per mg protein basis, there is one report of protein comprising 68% of *A. piscivorus* venom (Kocholaty et al., 1971). This indicates that enzymes and peptides comprise the majority of the venom.

**Glycoprotein content**

Of carbohydrate associated with *A. piscivorus* venom (51.7 µg/mg venom protein), neutral sugars account for 15.0 µg/mg, amino sugars account for 28.2 µg/mg, and sialic acid accounts for 8.5 µg/mg. The ratio of neutral sugars (D-galactose: D-mannose: L-fucose) was determined to be 1.0:0.81:0.72 (Oshima and Iwanaga, 1969).

**Dry mass**

As venom is often dried for storage and then reconstituted in solution prior to use, many authors have given numbers for the dry mass of venom per individual. Unfortunately, these numbers are not accompanied by the mass of the animals. It has
been reported that the dry mass of venom was 27.42% initial wet mass (Mitchell and Reichert, 1886); 28 to 28.6% initial wet mass, depending on animal size (Do Amaral, 1928); and 28 g/100 ml venom (28%; Elliott, 1978 quoting Wolff and Githens, 1939a, 1939b). The Mitchell and Reichert (1886) value is reported in their paper as the percent loss on drying, which would indicate a dry mass of 71.58% initial mass, but this was interpreted as 27.42% by Do Amaral (1928) and is much closer to other reported values. Dry mass per extraction has been reported in terms of average as 98 mg (for 264 specimens; Githens, 1935), 100 to 150 mg (Minton, 1974), and 158 mg (Wolff and Githens, 1939a). One study that grouped animals into size classes gave dry mass per extraction as 90 mg for adults, 120 mg for older animals, and 300 mg for exceptional individuals (Do Amaral, 1928), and a maximum (of 264 specimens) for another study was reported as 190 mg (Githens, 1935). Average dry mass, without information on animal size, has been reported as 150 mg (Vick, 1973), 90 to 148 mg (Kochva, 1978) and 90 to 170 mg (Russell, 1980).

**Spectrophotometry**

Singer and Kearney (1950a) published the absorbance spectrum (250 to 560 nm) for *A. piscivorus* venom showing the whole enzyme. They also provide a spectrogram of the prosthetic (flavin adenine dinucleotide) group.

**Stability**

Although storage methods for venom vary, reports specifically for *A. piscivorus* venom indicate that different methods of preservation yield different results, indicating that preservation method is important (Schöttler, 1951b). This study noted that opening vials of venom multiple times reduced activity. Jones (1976) reported that lyophilized
venom gave the same activity one year after drying, and Gennaro and Hood (1961) reported that phospholipase was still active 8 and 10 years after drying.

**Variation**

The many components and activities found within cottonmouth venom have been compared against those of many other related and unrelated species. However, very little work has been done on natural variation of venom components within the species, among subspecies, among populations, or within populations. Potential variation is very important because it can influence the efficacy of antivenom. It is also interesting as a phenotypic character that could be used in studying evolutionary relationships among and within snake species. Early on, variation was noticed in its ability to abate the Shwartzman phenomenon (Peck and Rosenthal, 1935) and stop nosebleeds (Goldman, 1936) in the same patient. The high level of venom variation found within populations of snakes has indicated that venom is not a good phenotypic character for determining taxonomic relationships among snake species and populations (Jones, 1976; Gibson, 1977). Cottonmouths appear to detect their individual venom in prey and use it as a search cue, so venom variation may be important for prey tracking (Chiszar et al., 1992; Greenbaum et al., 2003). Perhaps the best example of venom variation within the cottonmouth is the wide values found using commercially-supplied venom from three sources to examine multiple physiological activities (Tan and Ponnudurai, 1990).

**Toxicity**

The median lethal dose (LD₅₀) of cottonmouth venom has been determined in many different studies, usually utilizing mice, and by different routes of administration. These are shown in Table 2-2, as are other lethality values for animals either using a different toxicity statistic or animals with special physiological conditions.
Many different measures of toxicity have been reported, and it is often difficult to compare them because of the sheer diversity of techniques or lack of important information. One of the earliest examinations of *A. piscivorus* venom effects on pigeons showed that heating venom to 78ºC did not affect the toxicity of the venom, but boiling caused a reduction (Mitchell and Reichert, 1886). In the same study the following items were noted: filtering venom through “animal charcoal” rendered venom non-toxic, co-injection of an iron chloride solution reduced venom effects, and addition of iron to the venom solution made no difference. Further, it was noted that IP injection of boiled, filtered venom was lethal to pigeons, but without the hemorrhaging normally seen. In another early study a rabbit injected with venom was dead within 11 min and did not show signs of intravascular blood coagulation (Houssay and Sordelli, 1919).

Gloyd (1933) reported a cottonmouth envenomating and killing one *Crotalus atrox*, and Conant (1934) reported one captive cottonmouth that envenomated and killed two rattlesnakes (*Crotalus confluentus oreganus*) and another cottonmouth. A study examining the effects of venom on other snake species indicated that *A. contortrix mokeson* may have some immunity toward cottonmouth venom, as do conspecifics, but both can die from the evenomation (Swanson, 1946).

Minimum and other (average, maximum) lethal doses for cottonmouths have also been reported in a number of studies (Table 2-3). It was calculated, in one study, that the cottonmouth had enough toxic venom to kill 1755 pigeons (Wolff and Githens, 1939), and a later study calculated average time to death in mice (98 min) and dogs (240 min; Vick et al., 1967). Another study showed less than 8 hour survival time in dogs bitten by cottonmouths in a laboratory environment (Vick, 1973).
Separation of venom by Sephadex G-75 column chromatography showed primary toxicity in the first peak (Clark and Higginbotham, 1965a), and separation by P-10 bio-gel column chromatography showed highest lethality in fraction Ic (Moran and Geren, 1979). Further, using coarboxymethyl cellulose ion-exchange chromatography, lethality was noted in all four fractions, but with fractions 1 and 2 having greater potency than fractions 3 and 4 (Powlick and Geren, 1981). Recently, Gennaro et al. (2007) noted that a dose of 0.5 mg/kg was lethal to rabbits but not cats, whereas a dose of 5.0 mg/kg was lethal to both.

UV irradiation of venom reduced its toxicity (Tejasen and Ottolenghi, 1970) in mice, as did heating to 80°C before use (Noc, 1904). Wolff and Githens (1939) found that multiple venom extraction events did not reduce toxicity toward pigeons. Heatwole et al. (1999) found that the LD_{50} of venom against adult bullfrogs was higher than the LD_{50} against tadpoles of the same species, indicating an ontogenetic shift whereby animals more likely to be envenomated are afforded more protection. Cottonmouth venom also has been found to be toxic against tumor cells, but was not found to preferentially kill tumor cells over normal ones (Tu and Giltner, 1974). The concentration of venom that killed 50% of stomach cancer cells was found to be <3.0 µg/ml (Ahn et al., 1997).

**Separation techniques**

Separation techniques are commonly used to isolate specific components of venoms, and *A. piscivorpus* venom has been separated using many different techniques. In the earliest report, it was noted that solids precipitate from liquid venom simply through the effects of gravity (Mitchell and Reichert, 1886). Through dialysis with water, these researchers separated "globulins" from the remainder, which contained
“peptones.” “Globulins” were defined as proteins insoluble in distilled water and coagulable by boiling. “Peptones” were considered to be proteins soluble in water and not coagulable by boiling. Further, “globulins” were broken into the categories of water-venom-, copper-venom-, and dialysis-venom-globuins based on the separation technique that yielded them.

Because separation procedures such as precipitation, extraction, and absorption often led to destruction of venom components, and because much of the venom was thought to be protein, electrophoresis was attempted with _A. piscivorus_ venom (Marx and Peck, 1938). It was found that two substances they called hemolysins A and B migrated to different ends of the electrophoresis chamber, and that the hemorrhagic factor could be separated from the hemolysins. It was also determined that the hemorrhagic factor’s isoelectric point was between pH 4 and 5. Later, the isoelectric point of L-amino acid oxidase was found to be between pH 5.5 and 5.6 (Singer and Kearney, 1950a).

Among the forms of electrophoresis reported for separating _A. piscivorus_ venom, paper electrophoresis isolated 8 bands (Habermann and Neumann, 1954), starch electrophoresis showed 13 bands (Bertke et al., 1966), disc electrophoresis yielded 4 to 8 major and 3 to 9 minor bands depending on pH (Basu et al., 1969, 1970; Mebs and Samejima, 1986), and gel electrophoresis found ten bands (Powlick and Geren, 1981). Moving boundary electrophoresis (Wagner et al., 1968) and cellulose acetate strips have also been used, the latter examining all three subspecies (an Illinois population indicated as _A. p. conanti_ is well outside of its range, and could only be _A. p. piscivorus_ based on locality; Jones, 1976).
Chromatography methods used include paper (Franklin et al., 1951), Sephadex G-50 (four fractions, Bhargava et al., 1970), Sephadex G-75 (three peaks, Clark and Higginbotham, 1965a; Mebs and Samejima, 1986), Sephadex G-100 (three peaks Bajwa et al., 1982), P-cellulose (12 fractions, Clark and Higginbotham, 1965b), CM cellulose (Mebs and Samejima, 1986), anion exchange (Wagner et al., 1968), gel filtration (Wagner et al., 1968), ion exchange (16+ fractions, Clark and Higginbotham, 1971; Spiekerman et al., 1973; Prescott and Wagner, 1976; six peaks, Dinh et al., 1985; Ramírez et al., 1999), P-10 Bio-gel column (Moran and Geren, 1979; Powlick and Geren, 1981), carboxymethyl cellulose ion exchange (four peaks, Powlick and Geren, 1981; four peaks, Dinh et al., 1985), high-performance liquid (Ramírez et al., 1999), and concanavalin A Sepharose (specifically for A. p. leucostoma, Soper and Aird, 2007).

Combinations of separation techniques have been used for secondary separation of primary fractions (e.g. Clark and Higginbotham, 1971; Powlick and Geren, 1981; Dinh et al., 1985). Sedimentation also has been used for separation of venom components (Wagner et al., 1968).

Separation techniques have been utilized in an attempt to isolate fractions containing specific activities or components of the venom. These have been done to examine allergenicity (Clark and Higginbotham, 1965b), L-amino acid oxidase (Powlick and Geren, 1981), aminopeptidase (Wagner et al., 1968; Prescott and Wagner, 1976), bradykinin-releasing activity (Powlick and Geren, 1981), esterase (Prescott and Wagner, 1976; Powlick and Geren, 1981), fibrinolytic activity (Retzios and Markland, 1990; Hahn et al., 1995; Ramírez et al., 1999), hemolytic activity (Powlick and Geren, 1981), hemorrhagic activity (Powlick and Geren, 1981; Dinh et al., 1985), hyaluronidase

**Specific Components**

**Enzymes**

Studies of venom enzyme activity are many and varied. Although they are a method for distinguishing specific activities (either enzyme-based or substrate-based), their real value comes in comparisons based among individuals, groups, species, or higher levels. It is especially difficult to compare reported values against each other, as there are many different enzymatic techniques that utilize various assay conditions and report results using varied and (sometimes) confusing units. Luckily, the majority of results are reported on a per mg venom or per mg protein basis, which allows some conformity.

**Phosphodiesterase.** Cottonmouth venom has been shown to have phosphodiesterase activity that has been characterized as low to moderate (Mebs, 1970), moderate (Gulland and Jackson, 1938a; Tan and Ponnudurai, 1990), and moderately high (Kocholaty et al., 1971). Reported values of phosphodiesterase activity include 7800 enzyme units/mg dry venom (Richards et al., 1965), 47 units/mg protein (Kocholaty et al., 1971), 0.046 ± 0.011 international units/mg (Moran and Geren, 1979), and 10 to 27 nmol/mg/min (Tan and Ponnudurai, 1990). Carboxymethyl cellulose
ion-exchange chromatography shows phosphodiesterase elution in the second fraction (Powlick and Geren, 1981), and UV irradiation of venom appears to destroy phosphodiesterase activity in a time-dependent manner (Tejasen and Ottolenghi, 1970). Tan and Ponnudurai (1990) examined pooled venom samples of all three A. piscivorus subspecies from commercial sources and found that A. p. conanti had low variation, while A. p. piscivorus and A. p. leucostoma both had high variation.

5'-nucleotidase. After initial indications that it does have 5'-nucleotidase activity (Gulland and Jackson, 1938b; Zeller, 1951), moderate (Mebs, 1970) and moderate to high (Tan and Ponnudurai, 1990), and high (Braganca, 1955) activity has been noted for A. piscivorus venom. Gulland and Jackson (1938b) indicated that venom could dephosphorylate both adenosine- and inosine-5-phosphate. The two specific values reported for this activity are 495000 enzyme units/mg dry venom (Richards et al., 1965) and 3.2 to 7.3 nmol phosphate produced/min/mg venom (Tan and Ponnudurai, 1990).

Alkaline phosphomonoesterase. Multiple reports of phosphomonoesterase in A. piscivorus venom indicate that there is little activity. Specifically, no activity (Gulland and Jackson, 1938a; Zeller, 1951; Braganca, 1955), 550 enzyme units/mg dry venom (Richards et al., 1965) and 2 to 11 nmol/mg/min (Tan and Ponnudurai 1990) have been reported. This enzyme also was called monophosphatidase by Richards et al. (1965).

Hyaluronidase. Presence of hyaluronidase was initially seen by mixing venom with India ink and noting a dose-dependent increase in diameter of lesions produced in rabbit skin (Duran-Reynals, 1938, 1939). At the time, it was considered a “spreading factor,” but Zeller (1948) equates this term with the enzyme hyaluronidase.
A period of confusion in the literature is seen when Haas (1946a) indicated cottonmouth venom hyaluronidase should be called “invasin,” to which mammals had an inhibitor termed “antinvasin” in their serum. In the same series of papers, “proinvasin I” was described as a venom substance that destroyed “antinvasin I” (Haas, 1946b), and it was hypothesized that this allowed hyaluronidase to be active (Zeller, 1948). Further confusing was the description of “antinvasin II” which destroys “proinvasin I” as well as the hypothesized existence of “proinvasin II” and “antinvasin III” (Haas, 1946c). Later work determined that “proinvasin I” was simply the proteolytic capability of the venom, which degrades the serum that normally inactivates hyaluronidase. By doing so, hyaluronidase is effectively activated (Hadidian, 1953, 1956).

Boquet et al. (1958) determined the activity of \textit{A. piscivorus} venom hyaluronidase to be 16.1 total reducing units/s/mg venom, which was the lowest of the species they studied. Contrary to this, a more recent examination of the enzyme indicated high activity and variability at 66 to 200 National Formulary units/mg venom (Tan and Ponnudurai, 1990). Powlick and Geren (1981) used carboxymethyl cellulose ion-exchange chromatography to isolate hyaluronidase.

\textbf{L-amino acid oxidase.} L-amino acid oxidase (LAAO) degrades L-amino acids via a two step process whereby the amino group is replaced by an oxygen molecule that is double covalently bonded to the central carbon, the overall mechanism being oxidation (Chippaux, 2006). Although the general activity of the enzyme has been examined in \textit{A. piscivorus} venom, the majority of details concerning LAAO have been discovered in other species, so what work that has been done specifically on \textit{A. piscivorus} LAAO is
fairly sparse. Besides its direct activity, LAAO also has been implicated in activating proteases in the same venom (Zeller, 1951)

The enzyme itself has been described as about 59 (Powlick and Geren, 1986) to 60 kDa (Singer and Kearney, 1950b), with a flavin adenine dinucleotide (FAD) prosthetic group (Singer and Kearney, 1950a). The enzyme’s cellulose electrophoresis mobility is reported as -1.78 to -1.81 X 10^-5 cm^2/s/V (Wellner and Meister, 1960), and its isoelectric point is reported as pH 5.5 to 5.6 (Singer and Kearney, 1950a).

In general, the concentration of substrate limits the reaction, with high concentrations reducing activity. However, in the presence of pure oxygen, higher substrate concentrations will not reduce activity (Meister and Wellner, 1963). Under anaerobic conditions, activity is reduced but still occurs with L-leucine as substrate (Braganca and Quastel, 1952). The enzyme reaches optimal activity around pH of 7.4 (Wellner and Meister, 1960), with a steady decline with lower pH and a sharp decline at higher pH (Meister and Wellner, 1963). Phosphate, in the form of phosphate buffer, has a reversible (Kearney and Singer, 1951b; Meister and Wellner, 1963) inhibitory effect (Kearney and Singer, 1949, 1951c), as does pure water, but presence of chloride ions maintains activity (Kearney and Singer, 1951a). When the pH is lowered, the enzyme appears to dissociate into smaller fragments, indicating the presence of one or more labile covalent bonds (Powlick and Geren, 1986). The optimal reaction temperature has been reported as 38°C (Singer and Kearney, 1950b)

Singer and Kearney (1950c) give information on isolating LAAO from crude venom, but later attempts to crystallize the pure form were unsuccessful (Wellner and Meister, 1960). The carboxymethyl cellulose ion-exchange chromatography profile for
A. piscivorus venom has been published, and it indicates where LAAO separates (Powlick and Geren, 1981). This same study further separated the LAAO-containing fraction on a bio-gel column.

As with other venom enzymes, reported activity values suffer due to lack of standardization of assay conditions, assay methods, and units reported. Relative descriptions of A. piscivorus LAAO activity have included 1) higher than most elapids, but medium to low for viperids (Zeller, 1948); 2) very high (Mebs, 1970); 3) in the midrange of those tested (Kocholaty et al., 1971), and 4) moderate activity with marked variation (Tan and Ponnudurai, 1990). Activity of A. piscivorus venom LAAO has been reported as 380 µl O₂ consumed/h/mg venom (Zeller, 1948), 3100 molecules substrate oxidized/enzyme molecule/min (Singer and Kearney, 1950b), 277 units/mg protein (based on absorbance change X1000; Kocholaty et al., 1971), 0.40 ± 0.03 international units/mg (Moran and Geren, 1979), 105 to 301 National Formulary units/mg venom (Tan and Ponnudurai, 1990), and 7.28 U/mg venom (Ahn et al., 1997).

**Metalloproteinase.** Although the hemorrhagic capacity of A. piscivorus venom has been well studied, very few examinations of the specific form of metalloproteinase responsible for this action have been conducted. Leucostoma peptidase A was described based on its size (MW = 22.5 kDa) and its ability to hydrolyze many L-amino acids (Wagner et al., 1968). This same paper reported its relative amino acid composition, determined its isoelectric point at pH 6.5, and considered it an endopeptidase protease (also called arylamylase). It was later considered a metalloproteinase due to its Ca²⁺ to Zn²⁺ ratio (2:1), and its failure to act when mixed with the metal chelator EDTA (Spiekerman et al., 1973). This study also confirmed the
molecular weight of 22.5 kDa. It appears to be an endopeptidase because it only hydrolyzes peptides with basic or aromatic C-terminal residues. It is active over the entire pH range studies (7.0 to 10.0), but is optimal at pH 8.5. Leucostoma peptidase A can be purified via a published protocol (Wagner et al., 1968; Prescott and Wagner, 1976).

More recently, Jia et al. (2009) utilized a cDNA library to characterize two different forms of metalloproteinase from A. p. leucostoma venom gland. Both forms showed activity against fibrinogen.

**Serine protease I (thrombin-like).** Although much work has been done examining the effects of cottonmouth venom on the blood clotting cascade, very little of that work has focused on the presence of thrombin-like activity. Copley et al. (1973) indicated that the venom shows mild thrombin-like activity; whereas Bajwa et al. (1982) state that it has no thrombin-like activity. Utilizing venom from all three subspecies, Tan and Ponnudurai (1990) found no thrombin-like activity.

Hadidian (1956) states that A. piscivorus venom destroys prothrombin, indicating it has activity reducing any thrombin-like effects. Other studies have also indicated antithrombin activity (Kornalík, 1966, 1971), including activity against antithrombin III, the major thromboprotective plasma protein (Kress and Catanese, 1980). These studies indicate a potentially complex interaction of venom with multiple factors leading to (or reducing) thrombin activity of converting fibrinogen to fibrin.

**Serine protease II (kallikrein-like).** Cottonmouth venom appears to have kallikrein-like activity (Bonta et al., 1970a), which may be important for alterations in the blood clotting cascade. Venom mixed with human plasma releases bradykinin in a
dose-dependent manner (Philpot et al., 1978), indicating kallikrein activity of cleaving
kininogen (Webster and Pierce, 1963). Compared to other venomous snakes,
cottonmouth venom is reported to have moderate kallikrein-like activity (Deutsch and
Diniz, 1955; Oshima et al., 1969). Using carboxymethyl cellulose ion-exchange
chromatography, activity was isolated in the first and second fractions (Powlick and
Geren, 1979). A kallikrein-like enzyme with a molecular weight of 29 kDa has been
isolated and characterized as cleaving kininogen and the Bβ chain of fibrinogen (Nikai
et al., 1988a), and kinin-releasing activity in A. p. piscivorus venom has been reported
as 5.1 ± 0.6 µg kinin released/min/mg venom (Bailey et al., 1991).

**Serine protease III (arginine esterase).** Arginine esterase (also called arginine
ester hydrolase) activity in A. piscivorus venom has been reported as 4.6 µmol N-α-tosyl
L-arginine methyl ester hydrolyzed/min/mg protein (Oshima et al., 1969) and 6.8 to 10.8
µmol α-benzoyl-L-arginine ethyl ester consumed/min/mg venom (Tan and Ponnudurai,
1990). Moderate activity toward p-tosyl-L-arginine methylester also has been reported
(Kocholaty et al., 1971).

**Phospholipase I (phospholipase A₂).** Of the research conducted on
cottonmouth venom, by far the most studied component has been the enzyme
phospholipase A₂ (PLA₂). A. piscivorus venom has very high PLA₂ activity (Mebs,
1970) and has been reported as having higher activity than many other venoms
(Kocholaty et al., 1971), including those of *Crotalus adamanteus, Daboia russelli, Naja
naja*, and *Ophiophagus hannah* (Rosenberg and Ng, 1963). The A₂ forms of
phospholipases work generally to hydrolyze phospholipids bound to membranes (e.g.
those found in the plasma membrane of cells) at the sn-2 acyl bond, resulting in
arachidonic acid and lysophospholipid products (Augustyn and Elliott, 1970; see Ownby et al., 1999, for a review). Types of cells affected include RBCs, which results in hemolysis (Gul et al., 1974). Phospholipase also has been referred to in the older literature as phosphatidase (Chargaff and Cohen, 1939), and the forms found in A. piscivorus venom have been used for studies of venom activity, nerve conduction, and interfacial chemistry.

Initially, PLA$_2$ was examined for activity either from whole venom or from separated fractions. In these cases, A. piscivorus venom phospholipases have been found to hydrolyze many different lipid substrates, including egg yolks (Chargaff and Cohen, 1939; Tan and Ponnudurai, 1990), lecithin (Chargaff and Cohen, 1939; Fairbairn, 1945), cephalins (Fairbairn, 1945), ovolecithin (Long and Penny, 1957) phosphatidylcholine (Rosenberg, 1976), phosphotidylethanolamine (Rosenberg, 1976), phosphatidylserine (Rosenberg, 1976), 1-acyl-2-acyl-glycero-3-phosphorylcholine (Waku and Nakazawa, 1972), 3-(acyloxy)-4 nitrobenzoic acids (Cho et al., 1988b), 1,2-dioctanoyl-3-$sn$-glycerophosphorylcholine (Van den Bergh et al., 1988), and 2-arachidonoyl-1-stearoyl-L-3-phosphatidylcholine (Yamaguchi et al., 1997). Other substrates that are not hydrolyzed include cerebrosides (Fairbairn, 1945), sphingomyelins (Fairbairn, 1945), acetal phospholipids (Fairbairn, 1945), lysophospholipids (Fairbairn, 1945), cephalin (Chargaff and Cohen, 1939, contrary to above), sphingomyelin (Rosenberg, 1976), 1-O-alkenyl-2-acyl-glycero-3-phosphorylcholine (Waku and Nakazawa, 1972), and 1-O-alkyl-2-acyl-glycero-3-phosphorylcholine (Waku and Nakazawa, 1972).
Conditions for assaying PLA₂ activity have been quite diverse, with many different additives and assay conditions (e.g. temperature, pH) being tested. Substances found to enhance activity include Ca²⁺ (Marinetti, 1965), Mg²⁺ (small—Marinetti, 1965), albumin (Gul et al., 1974), lysolecithin (Bell and Biltonen, 1989a), fatty acids (Bell and Biltonen, 1989a), and glycerides (Bell et al., 1995). Indeed, it has been determined that Ca²⁺ presence is nearly essential for proper PLA₂ function (Bell and Biltonen, 1989a; Van den Bergh, 1989; Lathrop and Biltonen, 1992; Scott et al., 1992) and that the overall (Long and Penny, 1957; Bell and Biltonen, 1989a) and initial (Bell and Biltonen, 1989a) rates of reaction are Ca²⁺ concentration-dependent. It also has been shown that Ca²⁺ reduces dimerization of PLA₂. Cho et al. (1988b) found that activity was fairly similar when either Ba²⁺ or Sr²⁺ was substituted for Ca²⁺ (when using benzoic acid substrates). No observable effect was found with addition of Mn²⁺ (Marinetti, 1965), Fe³⁺ (Marinetti, 1965), sodium iodoacetate (Long and Penny, 1957), sodium p-chloromercuribenzoate (Long and Penny, 1957), or sodium mercaptoacetate (Long and Penny, 1957). Inhibition was noted after the addition of Al³⁺ (Marinetti, 1965), Cu²⁺ (Long and Penny, 1957; Marinetti, 1965), Mg²⁺ (Cho et al., 1988b—for benzoic acid substrates), Zn²⁺ (Marinetti, 1965; Cho et al., 1988b—for benzoic acid substrates), EDTA (Long and Penny, 1957), heparin (Dua and Cho, 1994), and fucoidin (Angulo and Lomonte, 2003—specifically for the Lys49 PLA₂).

Activity patterns have been noted in multiple studies, with higher temperatures generally yielding higher activities, up to a point. Marinetti (1965) found higher activity at 41°C than at 25°C (Marinetti, 1965), and optimal temperature between 25 and 75°C was found to occur at 65°C (Nair et al., 1976). However, another study showed the
The maximal temperature (between 38 and 48°C) was 41 to 44°C, depending on assay conditions (Bell et al., 1995). Increased dimerization has been seen with increased pH (over the range 4.2 to 7.0; Myatt et al., 1991), and optimal pH for lecithin hydrolysis is 6.5 to 7.0 (Magee and Thompson, 1960). General studies of traditional enzyme kinetics have also been conducted (Fairbairn, 1945; Magee and Thompson, 1960; Marinetti, 1965).

Phospholipase activity was reduced when venom was exposed to X-ray (Flowers, 1963) or UV radiation (Tejasen and Ottolenghi, 1970). In terms of stability, Gennaro and Hood (1961) found phospholipase to be active in dried *A. piscivorus* venom extracted 8 and 10 years earlier.

Many techniques for separation of the PLA2-containing fraction of cottonmouth venom have been described, including those utilizing sephadex G-50 (Bhargava et al., 1970; Bonta et al., 1970b), gel filtration (Augustyn and Elliott, 1970), carboxymethyl cellulose ion-exchange chromatography (Powlick and Geren, 1981), diethylaminoethyl (DEAE) cellulose chromatography (Powlick and Geren, 1981), sephadex G-75 (Marinetti, 1965), and sedimentation (Augustyn and Elliott, 1970). Initial estimates of the molecular mass of purified PLA2 were 13.5 ± 0.25 kDa by gel filtration and 13.3 to 15.4 kDa by sedimentation.

Reported values for activity of PLA2 suffer in that they are not standardized by substrate type, assay conditions, or reported activity units. Using clearing of an egg yolk emulsion as indication of activity, Marinetti (1965) found three values of 25, 200, and 270 absorbance units/min. Other values include 54 µequivalents of fatty acids titrated/mg protein (Kocholaty et al., 1971), 18.9 ± 1.4 international units/mg (Moran and
Geren, 1979), 10.80 turbidimetric units/µg PLA$_2$ (fractionated; Bhargava et al., 1970),
and 306 to 825 µmol product/min/mg (603 to 825, conanti; 306 to 625, leucostoma; 430
to 771, piscivorus; Tan and Ponnudurai, 1990).

Studies of A. piscivorus PLA$_2$ published prior to 1984 have suffered in that what is
reported is activity based on substrate used or product produced. This is a non-specific
assay, in which results are clouded by the presence of multiple forms of PLA$_2$ and
possible effects from other venom components. While these types of studies are useful,
they do not give indications of the separate mechanisms of action of the two known
PLA$_2$ forms. The two forms differ in some small ways, but are named based on the
amino acid residue located in the 49$^{th}$ position. The first group of described PLA$_2$s is
termed Asp-49 (or AppD49, for A. p. piscivorus aspartate 49) due to the presence of an
aspartate, and this was thought to be the case for all PLA$_2$s. However, a form in which
the aspartate is replaced by a lysine residue (group name Lys-49; AppK49) was
subsequently described (Maraganore et al., 1984). AppD49 is found in dimeric form
with a specific conformation that allows Ca$^{2+}$ ions to activate it, but AppK49 does not
have this binding region (Maraganore and Heinrikson, 1986).

The mechanism of action of AppD49 against phospholipids has been extensively
studied, and the general scheme follows. A monomer of AppD49 normally binds a Ca$^{2+}$
ion, thereby giving a conformational change that allows the PLA$_2$ to bind a lipid
substrate (Bell and Biltonen, 1989a). Although AppD49 may also bind without Ca$^{2+}$, it
has no enzymatic function without it (Scott et al., 1986). Following binding of Ca$^{2+}$, each
PLA$_2$ undergoes autocatalytic acylation and dimerizes to become an active enzyme
(Cho et al., 1988a). It appears that the monomer form of AppD49 has very little
enzymatic activity, and that the time required for it to dimerize accounts for the lag time seen in enzymatic reactions (Bell and Biltonen, 1989b, 1992; Bell et al., 1995). Lathrop et al. (2001) further examined lipid binding of AppD49 and conjectured that there is either a conformational change when the enzyme is activated, or there is a ternary complex formed by the combination of lipid, enzyme, and Ca$^{2+}$. Further exhaustive studies of the kinetics of A. piscivorus PLA$_2$ have examined the molecular dynamics, including electrostatic interactions (Stahelin and Cho, 2001; Tatulian, 2001; Leidy et al., 2004; Diraviyam and Murray, 2006).

AppK49 was, for a time, considered an inactive form of PLA$_2$ because of the inability to dimerize and lack of lipid-catalyzing enzymatic activity (Scott et al., 1992; Baker et al., 1994). However, it was later determined that AppK49 has physiological functions that do not mirror those of AppD49, and is just as active in other respects (Dhillon et al., 1987). Although it was initially thought to have lower lipid catalytic ability, AppD49 does not catalyze lipid substrates at all (Van den Bergh et al., 1988). It does, however, affect tissues in mouse heart and nerve diaphragm preparations (Dhillon et al., 1987; Condrea, 1989) and acts as a myotoxin (Pedersen et al., 1995) by binding to long-chain fatty acids. By utilizing the terminal amino acid sequence of AppK49 and making a synthetic peptide, it has been determined that the myotoxic effects are a direct result of the sequence of amino acids in the 115 to 129 residue range (Núñez et al., 2001; Lomonte et al., 2003b). AppK49 works as a homodimer and also has been determined to be a vascular endothelial growth factor (Yamazaki et al., 2005a, 2005b). The synthetic C-terminal end sequence has been utilized to examine effects of AppK49 on toad bladder cells, where it did not increase permeability (Leite et al., 2004), and has
been found to not discriminate between normal and tumor muscle cells (Araya and Lomonte, 2007). Further, the synthetic peptide (which is only in monomer form) has reduced activities with lower pH (Angulo et al., 2005).

The structures of both forms have been elucidated as to their amino acid chain, and crystal and NMR structures. They have also been examined for active sites via site-directed mutagenesis. The primary amino acid structures of both forms have been reported (Maraganore and Heinrikson, 1986, 1993; Maraganore et al., 1987; Van den Bergh et al., 1989; Welches et al., 1993), and indicate many conserved regions, but with important differences. The failure to bind Ca$^{2+}$ has been conjectured as the reason for lack of lipid catalytic activity in AppK49 (Van den Bergh et al., 1989), as the conformation of this form apparently causes the space where Ca$^{2+}$ would normally bind to be filled, thereby blocking Ca$^{2+}$ (Scott et al., 1992). The 7 amino acid extension found on the AppD49 form is not considered the reason for their ability to dimerize (Welches et al., 1993). Angulo et al. (2005) examined AppK49 and indicated that although dimerization increases myotoxic and cytotoxic activities, it is not essential for some effect.

AppD49 has been cloned into bacterial cells to produce the molecule for studies of its structure (Lathrop et al., 1992). The crystal structure has been examined for both AppD49 (Han et al., 1997) and AppK49 (Scott et al., 1986, 1992; Holland et al., 1990), and the secondary structure of AppD49 has been determined via NMR spectroscopy (Jerala et al., 1996).

Detailed examinations of the dynamics of PLA$_2$ have been conducted for elucidating the catalytic network (conserved, functional residues—Demaret and Brunie,
1992) and examining effects of rippling bilayers (Leidy et al., 2004) on activity. Other studies have modulated assay conditions to examine interfacial kinetics between PLA₂ and lipid substrates (Bell and Biltonen, 1989a; Bell et al., 1992; Dua and Cho, 1994; Baker et al., 1994; Bell et al., 1995; Sheffield et al., 1995), and it has been proposed that the two PLA₂ isoforms may act synergistically to affect a larger array of lipid structures than either form could individually (Shen and Cho, 1995b).

There has been some slight disagreement in the classification of PLA₂, with some considering it an acidic protein (Powlick and Geren, 1981; Lynch, 2007) or a basic one (Augustyn and Elliott, 1970). Possibly clarifying the issue, Maraganore et al. (1987) stated that there is an acidic dimer (the dimerized form of AppD49) and two basic monomers (the two components of the dimerized AppK49).

In a series of experiments, *A. piscivorus* venom applied to squid nerve axons allowed better transport of neurotransmitters and other chemicals across the cell membrane (Rosenberg and Ng, 1963; Rosenberg and Podleski, 1963; Hoskin and Rosenberg, 1965; Rosenberg, 1965; Rosenberg and Hoskin, 1965; Condrea et al., 1967; Rosenberg and Dettbarn, 1967), indicating PLA₂ activity. This occurred both with whole venom and an isolated fraction that contained PLA₂ (Condrea et al., 1967). Treatment also reduced electrical activity normally elicited by acetylcholine (Rosenberg and Podleski, 1963). This effect was seen more with crudely-dissected axons, indicating the venom may be responsible for disrupting Schwann cells, but not the axolemma (Rosenberg and Hoskin, 1965). Further evidence of this was the Schwann cell structural changes (vacuolation of cytoplasm) seen in some preparations (Martin
and Rosenberg, 1968). Venom from Agkistrodon contortrix was not as effective at making squid cells competent to curare (Rosenberg and Podleski, 1963).

Cottonmouth venom also has been examined for its effects on the electroplax of the electric eel, Electrophorus electricus. It causes irreversible depolarization at higher concentrations and antagonizes depolarization caused by carbamylcholine at lower concentrations (Bartels and Rosenberg, 1972), both of which indicate activity of PLA₂. At the concentrations used in this series of studies, PLA₂ was found to disrupt mitochondrial function without destroying them (Rosenberg, 1976). Previous studies have also noticed the ability of App PLA₂ to cause increased mitochondrial respiration with small concentrations and severe inhibition of electron transport at higher concentrations (Petrushka et al., 1959; Augustyn et al., 1970). Another study utilized A. piscivorus PLA₂ to examine changes in the bacteria Escherichia coli after exposure to bactericidal/permeability-increasing proteins from neutrophils (Forst et al., 1987).

Further animal studies with A. piscivorus venom have indicated that PLA₂ is responsible for edema formation in the mouse paw model (Marshall et al., 1989). It is reported that response is dose-dependent, and the mode of action may be via a histamine/serotonin release from basophils or mast cells (Calhoun et al., 1989). When AppK49 was used alone, edema occurred, but skeletal muscle was also lysed (Núñez et al., 2001). Cottonmouth PLA₂ has been investigated for potential treatment of hypercholesterolemia in humans (Shen and Cho, 1995a), and also has been studied as a potential method of molecular packaging, due to its conformation (Shen et al., 1994).

**Phospholipase II (phospholipase B).** Two studies have indicated the presence of phospholipase B (PLB) activity in cottonmouth venom. This activity was found only at
high pH, and increased from pH 8.5 to 10.5, with no activity being seen from pH 4.5 to 8.0 (Doery and Pearson, 1964). Using lysophosphatidylcholine as substrate, the activity of PLB was recorded as 0.51 ± 0.10 µequivalents of free fatty acids at pH 9.4 (Fletcher et al., 1979).

**Other enzymatic activities**

**Aminopeptidase.** *A. piscivorus* venom has been found to have leucine aminopeptidase activity because it strongly (Michl and Molzer, 1965) or moderately (Tu and Toom, 1967) degrades the substrate L-leucyl-β-naphthylamide. This activity has been seen in other studies, as well, by separating a fraction that contains the activity (Wagner et al., 1968; Prescott and Wagner, 1976). Although they later indicate aminopeptidase activity, Wagner and Prescott (1966a, 1966b) initially indicated that hydrolysis of L-leucyl-β-naphthylamide and L-alanyl-β-naphthylamide were not done by a true aminopeptidase.

**ATPase.** Activity toward ATP has been seen at both a low (Zeller, 1950, 1951) and moderate (Mebs, 1970) level. Addition of Mg$^{+2}$ did not affect activity (Zeller, 1950).

**Cholinesterase.** Despite multiple attempts, no cholinesterase activity has been found in *A. piscivorus* venom (Zeller, 1947, 1948, 1949; Hadidian, 1956; Mebs, 1970). Because multiple studies yielded the same results, this seems to indicate either complete absence or below-detection-level activities of cholinesterase in *A. piscivorus* venom.

**Collagenase.** Direct digestive effects of *A. piscivorus* venom on collagen have been examined, with basically no or low activity reported. Although no collagen digestion was seen, mice injected with venom showed an increase in urine hydroxyproline, which is an indicator of collagen degradation (Kaiser and Rabb, 1971).
Rat collagen shows a reduction in viscosity when exposed to *A. p. leucostoma* venom (Simpson et al., 1971). Weak collagenase activity has been seen (Moran and Geren, 1979), but has been attributed to the action of proteases (Szabo and Gennaro, 1978).

**Elastase.** One study has indicated elastinolytic activity in *A. piscivorus* venom, and indicated that it was among the highest of those species examined (Bernick and Simpson, 1976). However, no other examinations of this enzyme in cottonmouth venom have been conducted.

**Fibrinogenase.** Although thrombin-like enzymes cause the conversion of fibrinogen into fibrin, thereby enhancing initial blood clotting, fibrinogenases are enzymes responsible for cleaving fibrinogen in a manner that does not lead to clotting (Markland, 1991, 1998). The factor responsible for fibrinogenase activity in cottonmouth venom has been characterized and is called β-fibrinogenase (Nikai et al., 1988b). It is a 33.5 kDa molecular weight molecule with an isoelectric point at pH 4.5 and a venom concentration of 2.5 mg/g crude venom. It is stable at pH 2 to 10 and when mildly heated, and is inactivated by benzamidine, 2-mercaptoethanol, N-bromosuccinimide, and diisopropylfluorophosphate, which indicates its serine structure is important for activity. It has both esterase and kinin-releasing activity.

Hahn et al. (1995) have described two fibrinolytic enzymes (piscivorase I and II) that also have fibrinogenolytic activity. Piscivorase I has a molecular weight of 23.4 kDa and cleaves both the Aα and Bβ chains of fibrinogen, while piscivorase II is 29.0 kDa in size and primarily cleaves the Aα chain.
**Nucleosidase (NADase).** Two studies have examined cottonmouth venom nicotinamide adenine dinucleosidase (NADase). In these studies, NADase had either weak (Moran and Geren, 1979) or moderate (Tatsuki et al., 1975) activity.

**Non-specific esterase.** Other than studies of specific activities of phosphodiesterase, alkaline phosphomonoesterase, and arginine esterase, many researchers have examined the ability of snake venom to hydrolyze ester compounds in terms of overall venom activity. Cottonmouth venom is reported to have low activity toward t-BOC-L-alanine p-nitrophenyl esterase (Bernick and Simpson, 1976) and no activity toward β-naphthyl acetate.

Using Sephadex G-50 column separation, general esterase activity was found to be greatest in the first fraction (Bonta et al., 1970b; Bhargava et al., 1970, 1972). Venom esterase was not inhibited by snake serum from an unreported species (Philpot et al., 1978).

**Non-specific protease.** Multiple proteolytic enzymes occur in cottonmouth venom, and many studies have examined general protease activity toward a substrate rather than isolating specific enzymes. The terms protease and proteinase are interchangeable and can be found in both forms throughout the literature. Many studies reporting proteolytic activity have utilized ester-containing substrates for hydrolysis studies. These are not included here (see Non-specific esterase section above).

Proteolytic activity (hydrolysis) has been found against prothrombin (Hadidian, 1956), fibrinogen (Hadidian, 1956), urea-denatured hemoglobin (Wagner and Prescott, 1966), casein (Kocholaty and Ashley, 1966; Wagner and Prescott, 1966; Kaiser and Rabb, 1967; Oshima et al., 1969; Mebs, 1970; Kocholaty et al., 1971; Moran and

Proteolytic activity against hemoglobin was not affected by presence of Mg\(^{2+}\), Ca\(^{2+}\) or Zn\(^{2+}\) (Wagner and Prescott, 1966). General protease activity was inhibited by the use of cyanide (Hadidian, 1956); Co\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\) or Cd\(^{2+}\) ions (Wagner and Prescott, 1966); EDTA (Philpot, 1959; Wagner and Prescott, 1966); p-chloromecuribenzoate (Wagner and Prescott, 1966), cysteine (Wagner and Prescott, 1966), penicillamine (Philpot, 1959), and MFA (Giroux and Lachemann, 1981). Protease activity was also found to be inhibited by the presence of serum (Philpot et al., 1978), presence of human plasma α2 macroglobulin (Kress and Catanese, 1981), and exposure to ultraviolet light (Tejasen and Ottolenghi, 1970). In this last study, they noted that the loss of activity was not strictly a time-dependent function, indicating there may be more than one proteolytic factor. Bonnett and Guttman (1971) found that protease activity was inhibited by the serum of the kingsnake.

The factor responsible for proteolytic activity in cottonmouth venom has been described as having a molecular weight of 22.5 kDa, with an isoelectric point at pH 6.5 (Wagner et al., 1968). In another study, it was determined to have a molecular weight of 26 kDa, contain 1 mole of Zn and 2 of Ca\(^{2+}\) per mole of enzyme, and function
optimally at pH 8.5 (Wagner and Prescott, 1966). Kornalík (1971) indicated that its function and structure are not identical to either plasmin or trypsin and others have stated that is not the same as kallikrein or trypsin (Bhargava et al., 1972).

Separation studies have utilized ion exchange (Clark and Higginbotham, 1970) and Sephadex A-50 column chromatography (Prescott et al., 1976). This last study noted five different proteolytic components from their second fraction, indicating multiple factors with proteolytic capacity.

**Non-specific Peptidase.** Outside of studies of aminopeptidase, the only substrate-based examination of the ability of cottonmouth venom to degrade small peptides was conducted by Tu and Toom (1968). They found most di-, tri-, and tetra-peptides examined were degraded by venom.

**Nonenzymatic proteins/peptides**

**Cysteine-rich secretory proteins.** One instance of cysteine-rich secretory proteins (CRISPs) has been reported in *A. piscivoros* venom (Yamazaki et al., 2003). The authors isolated the protein, reported its amino acid sequence, and used it to show reduced muscle contractility of injected rat tails.

**Nerve growth factors.** Although its function in envenomated prey has not been examined, a nerve growth factor (NGF) has been isolated from cottonmouth venom (Cohen and Levi-Montalcini, 1956a). This factor was isolated (by DEAE cellulose electrophoresis) and used to increase growth in both sensory and sympathetic nerve ganglia (Cohen and Levi-Montalcini, 1956b). Both crude venom and the isolated fraction were found to increase nerve growth in a linear fashion, and venom had 1000x the potency of mouse tumor growth factor (Levi-Montalcini and Cohen, 1956).
factor is approximately 20 kDa in molecular weight (Cohen, 1959) and is similar, immunologically, to other snake, but not mouse, NGFs (Bailey et al., 1976).

**C-type lectins.** *A. piscivoros* venom contains lectins, which aid in agglutination. Gartner and Ogilvie (1984) reported lectin concentrations of 9.8 mg/g venom and indicated that the venom had agglutination capability of $4.7 \times 10^8$ units/mg lectin (Gartner and Ogilvie, 1984). They described the isolated lectin as dependent on Ca$^{2+}$ and inhibited by chelators such as EDTA and dithiothreitol. Their lectin had a molecular mass of 28 kDa (two 14 kDa units connected by disulfide bridges) and an isoelectric point at pH 7.2 to 7.3. A later study described APL, the specific *A. piscivoros* lectin, as being composed of homodimers, each having a molecular weight of 16.2 kDa, linked by a disulfide bridge (Komori et al., 1999).

**Disintegrins.** Two forms of disintegrins, which are inhibitors of platelet aggregation in the blood, have been described from *A. piscivoros* venom: applaggin and piscivostatin. Applaggin was initially described as a homodimer because of its 17.7 kDa size when unreduced by thiol and 9.8 kDa when reduced (Chao et al., 1989), but others have indicated it does not dimerize (Wencel-Drake et al., 1993). Regardless of dimerization capability, the monomeric form is 71 amino acids in length and binds to the Arg-Gly-Asp region of the integrin GPIIb/IIIa, which is necessary for normal blood clotting activity (Savage et al., 1990). By binding to this region, applaggin disrupts the clotting cascade. Attempts to determine the structure of applaggin by X-ray crystallography have not been successful, due to low diffraction (Arni et al., 1999).

The second *A. piscivoros* venom disintegrin, piscivostatin, was more recently described as having a heterodimeric structure with $\alpha$ and $\beta$ chains of 65 and 68 amino
acids, respectively (Okuda and Morita, 2001). It appears to work by inhibiting both platelet aggregation and (if aggregation does occur) platelet disaggregation (Okuda and Morita, 2001). The crystal structure of piscivostatin has been examined (Fujii et al., 2002). Studies on both disintegrins have been reviewed in comparison with other known disintegrins from snake venom (McLane et al., 2007).

**Bradykinin potentiating protein.** *A. piscivoruss* venom appears to have activity similar to the bradykinin potentiating peptide (BPP) found in *Bothrops jararaca*. BPP is an inhibitor of both angiotensin converting enzyme (ACE) and angiotensin I, so it works to reduce vasoconstriction, leading to a drop in blood pressure. Cottonmouth venom has been found to be a mild inhibitor of ACE in rabbits (Sander et al., 1972). Ferreira et al. (1995) found one separated fraction, which they termed AppF, to have BPP activity on isolated guinea pig ileum and a molecular weight of 1224.2 Da, determined by mass spectrometry. They later examined the isolated AppF using NMR spectroscopy and found it to have two conformations (Ferreira et al., 1999).

**Small organic compounds**

**Citrate.** Citrate has been found to be the major organic acid in *A. piscivoruss* venom (Freitas et al., 1992). It is likely that presence of citrate stabilizes venom activity by inhibiting enzymes from catalysis within the venom gland.

**Neurotransmitters.** Some work has been done in an attempt to find neurotransmitters in *A. piscivoruss* venom, with somewhat equivocal results. Norepinephrine was present in venom gland extract, but neither it nor serotonin was detected in cottonmouth venom (Anton and Gennaro, 1965). Indole-reacting compounds have been detected in cottonmouth venom using three different assay methods, although the author of this study cautioned that the non-specificity of the
assay makes it impossible to definitively state that serotonin is present (Welsh, 1966). In a similar study, an acetylcholine-like substance was detected (Welsh, 1967).

**Toxins**

*Neurotoxins.* Although it has been suggested that cottonmouth venom has a neurotoxic effect (Brown, 1941), a proven neurotoxin has never been isolated. Indeed, venom effects on nerves seem to be limited to phospholipase A$_2$ activity causing degradation of cell membranes. Hadidian (1956) indicated some evidence for neurotoxicity, but concluded that loss of nerve impulses were secondary effects. One study (Braganca, 1955) has reported a neurotoxin of molecular weight 4 kDa, but another (Micheel and Jung, 1936) reported an inability to purify a neurotoxin. If a neurotoxin does exist in cottonmouth venom, it does not seem to have a major effect.

*Mojave toxin.* *A. piscivor*us venom has not been found to contain Mojave toxin, unlike some species of crotaline rattlesnakes (Weinstein et al., 1985). While it is likely that this particular component is not found in cottonmouths, it is possible that it is simply present below detection limits.

**Vascular endothelial growth factor**

The Lys49 form of *A. piscivor*us phospholipase A$_2$ has been characterized as a vascular endothelial growth factor because of its ability to stimulate cell proliferation in *vitro* (Yamazaki et al., 2005a, 2005b). The snake-derived form is called KDR binding protein (KDR-bp), and it also induces hypotension in rats and binds with high affinity to kinase insert domain-containing receptor (KDR, also known as vascular endothelial growth factor receptor 2). KDR-bp is a weak vascular permeability enhancer (Yamazaki et al., 2007), and its functional binding site appears to be the C-terminal loop (Fujisawa et al., 2008).
Potential Components

Recently, Jia et al. (2008) constructed and analyzed a complementary DNA (cDNA) library for cottonmouth (A. p. leucostoma) venom gland tissue. This information can now be used to examine homology among similar components in different species and to find and characterize novel toxins. From this work the first venom gland-specific, non-venom component—parvalbumin—has been characterized and expressed in E. coli (Jia and Perez, 2009). The cDNA library also has been used to characterize two different forms of metalloproteinase from the venom, both of which have activity against fibrinogen (Jia et al., 2009).

Venom Studies

Human Disease Clinical Research

Cottonmouth venom has been examined as potential treatment for a number of human diseases, although it was noted that intradermal injection of venom in humans causes reddening of the skin (Essex, 1932). Most of these studies occurred in the 1930’s and were concerned with various hematological conditions. In general, intradermal injection of weak concentrations of venom causes a wheal with surrounding redness and ecchymoses, and these conditions usually disappear after multiple injections (Peck, 1933a; Peck and Rosenthal, 1935). However, in some cases (75% of patients considered “allergic” and 50% of patients considered “non-allergic), hypersensitivity occurs and is expressed as a hot, red, tender swollen area (Peck, 1933a). In such cases, desensitization by reducing venom concentration was usually successful (Peck, 1933a; Peck and Rosenthal, 1935). In a number of cases, patients had bad reactions and needed to be removed from venom therapy (Peck and
Goldberger, 1933), and there appears to be a balance between the efficacy of treatment and hypersensitivity (Peck and Rosenthal, 1935).

Venom was used as a treatment, with at least some success, for thrombocytopenic purpura (Peck, 1932; Greenwald, 1935), Schonlein-Henoch’s purpura (Peck, 1932), hemophilia (Peck, 1932), uterine bleeding (Peck and Goldberger, 1933; Goldberger and Peck, 1937), postpartum bleeding (Davin et al., 1937), epistaxis (Goldman, 1936; Dack, 1935), urticaria (Cohen, 1951). Venom therapy does not seem to have an effect on congenital hemophilia (Peck and Rosenthal, 1935), scarlet fever (Schneierson et al., 1936), or uterine bleeding caused by fibromyomas (Goldberger and Peck, 1937). For the study of the use of venom in reducing post-partum bleeding, it was found that reduced blood loss during birth occurred when venom was given prior to birth. The offspring of such births were apparently not affected by the pre-partum venom, as indicated by a lack of antivenom in the cord blood (Davin et al., 1937). In a study of the use of venom in treating thrombocytopenic purpura, active bleeding was stopped, general bleeding was much reduced, and platelet counts increased, indicating that venom was working to reduce blood loss from capillaries (Greenwald, 1935).

The venom also has been used as a supplementary test for diagnosis of various blood diseases via examination of capillaries. A positive test (when capillaries rupture due to the venom) can indicate or rule out certain diseases, and a change in this test result can indicate recovery (Peck et al., 1936, 1937a). Diseases in which this test has shown to be diagnostic or non-effective have been tabulated (Peck, 1937b). Brabec and Kornalík (1977) examined the effect of A. piscivorus venom on blood from normal and diseased patients. They found that, compared to RBCs from normal patients,
RBCs from patients with autoimmune hemolytic anemia were not differently lysed, those from patients with hereditary spherocytosis showed slightly greater lysis, and those from patients with paroxysmal nocturnal haemoglobinuria showed much greater lysis. These effects only occurred in the presence of plasma, indicating complement is activated. In an additional study of the effects on A. piscivorus venom on blood from patients with 16 different hematological diseases, high hemolysis was only seen in blood from patients with paroxysmal nocturnal hematoglobinuria, and only if complement was also present (Žák et al., 1984; Brabec et al., 1985).

Immunology

Cottonmouth venom has been used for immunological studies because of its ability to reduce the Shwartzman phenomenon normally seen with multiple exposures to allergens. This reduction was seen both in humans (Peck and Rosenthal, 1935) and rabbits (Peck and Sobotka, 1931; Peck, 1933b), and this ability was reduced in the presence of either A. piscivorus antivenom or horse serum (Peck, 1934).

Agkistrodon piscivorus venom has been examined for immunological reactivity toward venoms or antivenoms produced using the venom of other species. It was found that neither Daboia nor Echis antivenom protected pigeons against cottonmouth venom, although Daboia antivenom did specifically neutralize the hemorrhagic effects (Taylor and Mallick, 1965). In agar plate immunodiffusion studies, there was evidence of antigens that were similar to or shared with Agkistrodon contortrix and Crotalus atrox venom (Minton, 1957). Tu and Adams (1968) used immunodiffusion to show A. piscivorus venom to be more similar to A. contortrix than to A. (=Calloselasma) rhodostoma venom. Using other venoms, it was found that cottonmouth venom did not immunoreact with Bungarus fasciatus or Naja naja venom or antivenom, although it did
show precipitation with polyvalent crotalid antiserum (Munjal and Elliott, 1972).

Polyvalent antivenom, conspecific antivenom, and heparin all visibly complexed with cottonmouth venom in vitro (Clark and Higginbotham, 1965a).

Although both treatments cause loss of toxic activities, venom exposed to X-ray (Flowers, 1963, 1966) or ultraviolet radiation (Tejasen and Ottolenghi, 1970) maintains its antigenicity. Both of these studies were attempts to make inoculation of antivenom-producing animals less toxic while still creating useful antivenom.

The ability of serum from other snake species to neutralize cottonmouth venom has been examined for both venomous and non-venomous species. Some neutralizing capacity was seen in serum from the venomous species *Crotalus adamanteus* (Philpot and Deutsch, 1956; Philpot et al., 1978), *Agkistrodon contortrix* (Philpot et al., 1978; Weinstein et al., 1991), and conspecifics (Philpot et al., 1978). Non-venomous genera showing neutralizing capacity include *Coluber, Natrix, Elaphe*, and *Lampropeltis* (Philpot et al., 1978). The genus *Lampropeltis* (king and milk snakes) has been examined in depth due to their habits of consuming venomous snakes as part of their diets. *Lampropeltis* species known to show venom neutralizing effects in their sera include the species *L. getula* (Ditmars, 1928; Bonnett and Guttman, 1971) and the subspecies *L. g. floridana* (Philpot and Smith, 1950a, 1950b; Philpot and Deutsch, 1956; Weinstein et al., 1992). Besides *L. g. floridana*, Weinstein et al. (1992) also found neutralizing capacity in *L. alterna*, *L. calligaster*, *L. g. getula*, *L. g. californiae*, *L. g. holbrooki*, *L. mexicana greeri*, *L. triangulum hondurensis*, *L. t. triangulum*, and *L. ruthveni* (but not for *L. g. splendidida*). Resistance to cottonmouth venom also has been seen in mammals including the opossum (*Didelphis viriginiana*—Kilmon, 1976; Werner and Vick, 1977;
Ramírez et al., 1999), the woodrat (*Neotoma micropus*—Perez et al., 1978; Ramírez et al., 1999) the Mexican ground squirrel (*Spermophilus mexicanus*—Ramírez et al., 1999), and the cotton rat (*Sigmodon hispidus*—Ramírez et al., 1999).

Monoclonal antibodies developed using *Crotalus atrox* venom had strong reactions against *A. piscivorus* venom (Perez et al., 1984), as did anti-protein C activator developed using *A. contortrix* venom (Stocker et al., 1987). A further cross-reaction was found between antifibrolase antibody from *A. contortrix contortrix* and fibrinolytic enzyme from *A. p. conanti* (Chen et al., 1991).

**Conclusion**

Studies of the venom of *A. piscivorus* are diverse and many. While they have examined overall effects of venom on humans and different animal models, they have also specifically scrutinized individual venom components to the molecular level. This research has indicated that cottonmouth venom has many different components with various physiological effects, some of which have been characterized, and some of which remain to be studied. Most of the work has focused on physiological effects or efficaciousness of antivenom, and much has also been done using venom as a research tool. However, much work remains to be done in the areas of ecology and evolution of the venom in order to truly understand it.
Table 2-1. Published records of cottonmouth bites and bite-caused fatalities. Where possible, number of mortalities directly credited to cottonmouths and geographic locations are given. Each study was done slightly differently and had different methods of analyzing data. Unreported values are indicated by “--.” Overall, the data show that cottonmouths are responsible for a high percentage of snake bites in the United States, but are not responsible for many deaths.

<table>
<thead>
<tr>
<th>Bites by cottonmouths</th>
<th>Bites by all snakes</th>
<th>Cottonmouth bites (%)</th>
<th>Mortalities</th>
<th>Time span</th>
<th>Geographic location</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>458</td>
<td>9.4</td>
<td>1</td>
<td>1928</td>
<td>United States</td>
<td>Hutchison, 1929</td>
</tr>
<tr>
<td>194</td>
<td>1973</td>
<td>9.8</td>
<td>8</td>
<td>--</td>
<td>United States</td>
<td>Githens, 1935</td>
</tr>
<tr>
<td>7</td>
<td>51</td>
<td>7.3</td>
<td>0</td>
<td>1948</td>
<td>Florida</td>
<td>Andrews and Pollard, 1953</td>
</tr>
<tr>
<td>46</td>
<td>179</td>
<td>25.7</td>
<td>--</td>
<td>1962</td>
<td>Florida</td>
<td>Sowder and Gehres, 1963</td>
</tr>
<tr>
<td>33</td>
<td>461</td>
<td>7.2</td>
<td>--</td>
<td>1958-1959</td>
<td>Texas</td>
<td>Parrish, 1964</td>
</tr>
<tr>
<td>133</td>
<td>1538</td>
<td>8.6</td>
<td>--</td>
<td>1958-1959</td>
<td>10 US states a</td>
<td>Parrish et al., 1965, 1966</td>
</tr>
<tr>
<td>9</td>
<td>64</td>
<td>14.1</td>
<td>0</td>
<td>1927-1965</td>
<td>Florida and Georgia b</td>
<td>Watt and Gennaro, 1965</td>
</tr>
<tr>
<td>208</td>
<td>2836</td>
<td>7.3</td>
<td>--</td>
<td>1958-1959</td>
<td>United States c</td>
<td>Parrish, 1966</td>
</tr>
<tr>
<td>755</td>
<td>6680</td>
<td>11.3</td>
<td>--</td>
<td>1959</td>
<td>United States d</td>
<td>Parrish, 1967; Parrish and Donnell, 1967</td>
</tr>
<tr>
<td>69</td>
<td>382</td>
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<td>0</td>
<td>1963-1966</td>
<td>Florida</td>
<td>Andrews et al., 1968</td>
</tr>
<tr>
<td>6</td>
<td>204</td>
<td>2.9</td>
<td>--</td>
<td>--</td>
<td>Western United States e</td>
<td>Russell, 1969</td>
</tr>
<tr>
<td>46</td>
<td>177</td>
<td>26.0</td>
<td>--</td>
<td>1962</td>
<td>Florida</td>
<td>Sowder and Gehres, 1968</td>
</tr>
<tr>
<td>38</td>
<td>168</td>
<td>22.6</td>
<td>--</td>
<td>1963</td>
<td>Florida</td>
<td>Sowder and Gehres, 1968</td>
</tr>
<tr>
<td>38</td>
<td>185</td>
<td>20.5</td>
<td>--</td>
<td>1964</td>
<td>Florida</td>
<td>Sowder and Gehres, 1968</td>
</tr>
<tr>
<td>38</td>
<td>198</td>
<td>19.2</td>
<td>--</td>
<td>1965</td>
<td>Florida</td>
<td>Sowder and Gehres, 1968</td>
</tr>
<tr>
<td>49</td>
<td>190</td>
<td>25.8</td>
<td>--</td>
<td>1966</td>
<td>Florida</td>
<td>Sowder and Gehres, 1968</td>
</tr>
<tr>
<td>32</td>
<td>158</td>
<td>20.3</td>
<td>--</td>
<td>1967</td>
<td>Florida</td>
<td>Sowder and Gehres, 1968</td>
</tr>
<tr>
<td>7</td>
<td>45</td>
<td>15.6</td>
<td>--</td>
<td>1969-1976</td>
<td>South Carolina f</td>
<td>Sabback et al., 1977</td>
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<tr>
<td>26</td>
<td>107</td>
<td>24.3</td>
<td>0</td>
<td>1927-1977</td>
<td>Florida and Georgia f</td>
<td>Watt, 1978</td>
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<tr>
<td>1</td>
<td>32</td>
<td>3.1</td>
<td>0</td>
<td>1970-1977</td>
<td>United Kingdom g</td>
<td>Reid, 1978</td>
</tr>
</tbody>
</table>

a The states in this study were Arkansas, Arizona, Georgia, Louisiana, Mississippi, North Carolina, Oklahoma, South Carolina, Texas, and West Virginia. b Two counties were analyzed from each state. c Based on data reported by hospitals. d Same data as Parrish (1966), except combined with physicians reports and estimated for non-participating hospitals. e Includes non-native, captive snakes. f Both studies included data from one hospital. g Instances of bites from non-native specimens.
Table 2-2. Published toxicity values for cottonmouth venom. Median lethal dose (LD$_{50}$) is the concentration of venom required to kill 50% of study animals. Units are mg lyophilized venom/kg body weight, unless otherwise noted. The routes of administration are: IP—intraperitoneal, IV—intravenous, SC—subcutaneous. For comparison, variations on these studies are listed at the bottom of the table. These studies included determinations of LD$_{50}$ in physiologically altered animals and determinations of alternate toxicity values (LD$_{99}$ and LD$_{100}$).

<table>
<thead>
<tr>
<th>LD$_{50}$</th>
<th>Route</th>
<th>Animal</th>
<th>Source</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.11</td>
<td>IP</td>
<td></td>
<td>Russell, 1967$^b$</td>
<td></td>
</tr>
<tr>
<td>4.844</td>
<td>IP</td>
<td>mouse</td>
<td>Kocholaty et al., 1971</td>
<td></td>
</tr>
<tr>
<td>5.10</td>
<td>IP</td>
<td>mouse</td>
<td>Russell, 1980</td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td>IP</td>
<td>mouse</td>
<td>Weinstein et al., 1991</td>
<td></td>
</tr>
<tr>
<td>5.33</td>
<td>IP</td>
<td>mouse</td>
<td>Weinstein et al., 1992</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>IP</td>
<td>mouse</td>
<td>Powlick and Geren, 1981</td>
<td></td>
</tr>
<tr>
<td>6.85</td>
<td>IP</td>
<td>mouse</td>
<td>Minton, 1956</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>IP</td>
<td>mouse</td>
<td>Huang et al., 1972</td>
<td></td>
</tr>
<tr>
<td>4.00</td>
<td>IV</td>
<td></td>
<td>Russell, 1967$^b$</td>
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<tr>
<td>60.0$^a$</td>
<td>IV</td>
<td>mouse</td>
<td>Ohsaka et al., 1966</td>
<td></td>
</tr>
<tr>
<td>64$^a$</td>
<td>IV</td>
<td>mouse</td>
<td>Clark and Higginbotham, 1965a</td>
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<tr>
<td>75 ± 5.5$^a$</td>
<td>IV</td>
<td>mouse</td>
<td>Clark and Higginbotham, 1971</td>
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<td>2.044</td>
<td>IV</td>
<td>mouse</td>
<td>Kocholaty et al., 1971</td>
<td></td>
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<td>3.35</td>
<td>IV</td>
<td>mouse</td>
<td>Flowers, 1963</td>
<td></td>
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<td>Gingrich and Hohenadel, 1956</td>
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<td>4.17</td>
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<td>mouse</td>
<td>Russell, 1980</td>
<td></td>
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<tr>
<td>11.3 to 13.6</td>
<td>SC</td>
<td>mouse</td>
<td>Moran and Geren, 1979</td>
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<td>25.10</td>
<td>SC</td>
<td>mouse</td>
<td>Russell, 1980</td>
<td></td>
</tr>
<tr>
<td>25.85</td>
<td>SC</td>
<td>mouse</td>
<td>Minton, 1956</td>
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Table 2-2. Continued

<table>
<thead>
<tr>
<th>LD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Route</th>
<th>Animal model</th>
<th>Source</th>
<th>Notes</th>
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<tr>
<td>Other toxicity studies:</td>
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<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IV</td>
<td>mouse</td>
<td>Clark and Higginbotham, 1965a</td>
<td>Immunized, adrenalectomized</td>
</tr>
<tr>
<td>39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IV</td>
<td>mouse</td>
<td>Clark and Higginbotham, 1965a</td>
<td>Immunized</td>
</tr>
<tr>
<td>44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IV</td>
<td>mouse</td>
<td>Clark and Higginbotham, 1965a</td>
<td>Non-immunized, adrenalectomized</td>
</tr>
<tr>
<td>3 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IV</td>
<td>mouse</td>
<td>Clark and Higginbotham, 1971</td>
<td>Adrenalectomized, sensitized</td>
</tr>
<tr>
<td>20</td>
<td>IV</td>
<td>mouse</td>
<td>Flowers, 1963</td>
<td>X-ray irradiation</td>
</tr>
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<td>5.26</td>
<td>IV</td>
<td>mouse</td>
<td>Vick et al., 1967</td>
<td>LD&lt;sub&gt;99&lt;/sub&gt;</td>
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<tr>
<td>0.75</td>
<td>IV</td>
<td>dog</td>
<td>Vick et al., 1967</td>
<td>LD&lt;sub&gt;99&lt;/sub&gt;</td>
</tr>
<tr>
<td>40</td>
<td>IP</td>
<td>frog</td>
<td>Hall and Gennaro, 1961</td>
<td>LD&lt;sub&gt;100&lt;/sub&gt;</td>
</tr>
<tr>
<td>6.2</td>
<td>IP</td>
<td>mouse</td>
<td>Hall and Gennaro, 1961</td>
<td>LD&lt;sub&gt;100&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values listed are in µg/animal.  <sup>b</sup>Animal model not reported.
Table 2-3. Reported minimum and mean lethal doses for cottonmouth venom.

Minimum lethal doses are defined as the lowest dose used that killed the species studied. Mean lethal dose is the average of doses that killed animals in the study. The routes of administration are as follows: IP—intraperitoneal, IV—intravenous, SC—subcutaneous.

<table>
<thead>
<tr>
<th>Value</th>
<th>Units</th>
<th>Animal model</th>
<th>Route</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>4 mg/kg</td>
<td>mouse</td>
<td>IP</td>
<td></td>
<td>Gennaro and Ramsey, 1959a</td>
</tr>
<tr>
<td>7 mg/kg</td>
<td>mouse</td>
<td>IP</td>
<td></td>
<td>Gennaro and Ramsey, 1959a</td>
</tr>
<tr>
<td>2.5 mg/kg</td>
<td>rabbit</td>
<td>IP</td>
<td></td>
<td>Noguchi, 1909</td>
</tr>
<tr>
<td>0.06 mg/animal</td>
<td>pigeon</td>
<td>IV</td>
<td></td>
<td>Githens, 1935</td>
</tr>
<tr>
<td>0.1 mg/animal</td>
<td>pigeon</td>
<td>IV</td>
<td></td>
<td>Taylor and Mallick, 1936</td>
</tr>
<tr>
<td>0.15 mg/kg</td>
<td>mouse</td>
<td>IV</td>
<td></td>
<td>Izard and Boquet, 1953</td>
</tr>
<tr>
<td>2 mg/kg</td>
<td>rabbit</td>
<td>IV</td>
<td></td>
<td>Izard and Boquet, 1953</td>
</tr>
<tr>
<td>2.46 mg/kg</td>
<td>dog</td>
<td>IV</td>
<td></td>
<td>Vick, 1973</td>
</tr>
<tr>
<td>0.015 mg/animal</td>
<td>mouse</td>
<td>SC</td>
<td></td>
<td>Schöttler, 1951a</td>
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<tr>
<td>1 mg/animal</td>
<td>mouse</td>
<td>SC</td>
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<td>Noc, 1904</td>
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<td>30 to 50 mg/animal</td>
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<td>SC</td>
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<td>Noc, 1904</td>
</tr>
<tr>
<td>0.10 mg/kg</td>
<td>mouse</td>
<td>SC</td>
<td></td>
<td>Izard and Boquet, 1953</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>guinea pig</td>
<td>SC</td>
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<td>Izard and Boquet, 1953</td>
</tr>
</tbody>
</table>

Mean lethal dose:

<table>
<thead>
<tr>
<th>Value</th>
<th>Units</th>
<th>Animal model</th>
<th>Route</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.11 mg/animal</td>
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<td></td>
<td>Githens, 1935</td>
</tr>
<tr>
<td>32.4 mg/kg</td>
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<td>SC</td>
<td></td>
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</tr>
</tbody>
</table>

*Venom heated to 80°C prior to use.*
CHAPTER 3
VENOM EXTRACTION FROM ANESTHETIZED FLORIDA COTTONMOUTHS

Introduction

Researchers who work with venomous organisms must ensure the well-being of both their animals and themselves through good safety practices. These precautionary practices are complex, especially when studies require venom extraction from live snakes. Collection techniques should facilitate the study’s goals and experimental design, proper treatment of the animals, and safety of those coming into contact with the animals. Safety precautions will, therefore, vary depending on the study goals. In commercial antivenin production the goal is to maximize venom yield. In long-term studies of recaptured wild snakes, however, consistency, repeatability, and portability in the field are equally important. These goals require different extraction techniques.

Many studies utilizing venom do not detail collection methods, but previously described venom collection techniques for snakes include spontaneous ejection (where the snake bites through a membrane into a collecting cup—di Tada et al., 1976; Tare et al., 1986), forced ejection by glandular massage (di Tada et al., 1976; Mackessy, 1988) and evoked ejection using electrical stimulation (Johnson, 1938; Gans and Elliott, 1968; Glenn et al., 1972; Marsh and Glatston, 1974; Johnson et al., 1987; and Vieira et al., 1988). For any method the animal can be conscious or anesthetized. The use of halothane for inhalant anesthesia was first described by Hackenbrock and Finster (1963), and other inhalants (i.e. methoxyflurane, isoflurane, and sevoflurane) have also been utilized. Some studies have used injectable anesthetics (i.e. ketamine, propofol),
and others have combined anesthesia with saliva-inducing chemicals such as pilocarpine to increase venom yield (Hill and Mackessy, 1997).

Grasping snakes behind the head and allowing them to bite through a membrane over a collecting vessel (spontaneous ejection) yields large amounts of venom. It can be, however, unsafe and difficult to standardize. The snakes are made to bite multiple times, with decreased venom yields in consecutive bites, indicating incomplete emptying of stored venom. Further, there is contradicting evidence concerning the ability of snakes to meter venom ejection (Hayes, 1993, 1995; Hayes et al., 1995; Young et al., 2002, Young and Zahn, 2001), a further factor potentially adding to the difficulty of quantifying total venom volume for snakes that are conscious. Any physical restraint during extraction is likely stressful to the animals, possibly resulting in altered behavior and physiology. Most importantly, manual restraint of venomous snakes increases the chance of human envenomation.

Anesthesia has previously been used to increase safety and potentially reduce stress in snakes during venom collection by reducing the contact time in which the animals are conscious. In most studies the venom glands were massaged to eject the venom into a collecting vessel or capillary tube (Mackessy, 1988). Although this technique works well with large viperids, it does not ensure collection of the entire venom store due to variation in squeeze force and massage technique. Further, massage may alter venom composition, which in at least one case has been observed as differences in turbidity and color (Mackessy, 1988). Also, in the neotropical rattlesnake (Crotalus durissus), massage has been associated with higher incidence of glandular infection (di Tada et al., 1976).
Electrical stimulation is common for venom collection from arthropods (Hemiptera—Sahayaraj et al., 2006; Hymenoptera—Davies et al., 2004; Brochetto-Braga et al., 2006; Araneae—Escoubas et al., 2002; and Scorpiones—Candido and Lucas, 2002) and other invertebrates (Anthozoa—Malpezzi et al., 1993). While it can cause death of small invertebrates, it has been used in conscious snakes without apparent adverse effect (Johnson, 1938; Gans and Elliott, 1968; Glenn et al., 1972; Marsh and Glatston, 1974; Johnson et al., 1987; and Vieira et al., 1988). A few studies have compared collection techniques with ambiguous conclusions. The authors do, however, agree that manual manipulation and electrical stimulation yield more venom than does spontaneous ejection in the same snakes (di Tada et al., 1976; Tare et al., 1986).

Here we describe venom collection from anesthetized Florida cottonmouths (*Agkistrodon piscivorus conanti*) using electrical stimulation to enhance quantification, and to decrease handling time and envenomation hazard to researchers. Further, our technique uses a relatively inexpensive, portable human medical nerve stimulator that can be used in either the laboratory or the field.

**Materials and Methods**

**Animals**

As part of a larger study, 70 wild cottonmouth snakes (*Agkistrodon piscivorus conanti*) were collected from three locations in Florida. While in captivity, snakes were maintained individually in commercial fiberglass or plastic caging with newspaper substrate and *ad libitum* access to water, but were not fed until after venom collection. Venom was collected mostly from adult snakes, but some juveniles were also used for
comparison. Further, three clutches of neonate snakes (n = 17) born in captivity from wild-caught gravid females, were compared.

Anesthesia

Isoflurane (5% induction, 2.5% maintenance) in oxygen (2 L/min) administered with a precision vaporizer and flow-meter was used initially for anesthesia (n = 2). This technique produced consistent, reversible anesthesia but was rejected because of prolonged induction and recovery times, and difficulty of use in remote field situations. A combination of medetomidine (100 µg/kg) and ketamine (10 mg/kg) administered intramuscularly (IM) in the paravertebral musculature produced short-term immobility in a third (n = 1) snake (395 g), but a higher combination dosage (200 µg/kg and 20 mg/kg IM, respectively) failed to produce reliable, safe immobility in a fourth (n = 1) snake (434 g). Glandular massage was attempted in these four and two other snakes (n = 2) anesthetized with propofol (10 mg/kg). We were unable to eject venom from any of these snakes using this technique, so alternate methods of extraction were explored. These six individuals are not included in statistical analysis.

Anesthesia in the rest of the snakes (total n = 81; 64 wild caught, 17 captive reared) was accomplished by an intravascular injection of propofol (Rapinovet, Schering-Plough Animal Health Corporation; 10 mg/ml, 8 to 10 mg/kg body mass) into the ventral coccygeal vessels posterior to the cloaca (Heard, 2001). For physical restraint each snake was induced to enter a hard plastic, transparent snake tube (Lock, 2008) which allowed safe access to the vessels for injection and visualization of the snake during induction. The snake was assessed to be sufficiently relaxed when it could not right itself, could not raise its head when in dorsal recumbency, and did not respond
to a strong pinch in the cloacal region. If induction did not occur within 10 to 15 min, a supplemental propofol injection (n = 33), isoflurane (5%) in oxygen and nitrous oxide (n = 4) insufflation into the snake tube, or a sequential combination (n = 2) was administered. These last two groups of animals are not included in subsequent data analysis so that only animals anesthetized solely with propofol (n = 75) are compared. Supplemental propofol injections were administered via caudal vein (n = 24) or by intracardiac injection (n = 9; Isaza et al., 2004).

**Venom Extraction**

Once the snake was determined to be sufficiently relaxed, its head was extended beyond the end of the snake tube (Figure 3-1). If the animal should move in this position, it could rapidly be pulled back into the tube by grasping its tail and caudal body. Venom was extracted by electrical stimulation using a portable constant current peripheral nerve stimulator (Fisher & Paykel Healthcare). Depending on size, the amperage was set at 10, 20, or 30 mA, with 20 mA being effective for most snakes. Initial attempts using a single twitch or a “train of four” failed to elicit venom expulsion. All successful venom collections used a tetanic pulse of 5 s duration.

The alligator clips of the stimulator probes were attached across the venom gland, pinching the loose skin near the articulation of the jaw and directly behind or under the eye (Figure 3-2). To provide good contact for current transmission, the clips were moistened with alcohol. The right and left venom glands were stimulated in succession, and venom from both glands was pooled into a clean beaker for quantification. Stimulation of one gland sometimes yielded venom from both, indicating the pulse was sufficient to elicit muscle contraction over both glands. Crude venom from each
individual was centrifuged at 5000 rpm for 2 min, and the supernatant was aliquoted in volumes of 25 to 100 µl for quantification to the nearest 25 µl.

**Demographic Data**

The sex of each animal was determined using commercial stainless steel sexing probes, and the following measurements were made: mass, snout-to-vent length (SVL), and tail length. Relationships between propofol dose or induction time and animal mass were analyzed using linear regression analysis.

**Results**

Induction times after administration of propofol into the ventral coccygeal vessels were 12.2 ± 8.5 min (mean ± 1 standard deviation), whether with (19.0 ± 7.8 min; n = 33) or without (6.8 ± 4.1 min; n = 42) supplemental doses of propofol only (no isoflurane). We initially used 10 mg/kg body mass propofol to induce unconsciousness, and although lower concentrations (9.8 ± 1.7 mg/kg) were sufficient in some cases, at other times supplemental doses (total 18.8 ± 5.6 mg/kg) were needed (P << 0.001; Figure 3-3). Time to induction was mass-specific, with larger animals taking longer to be affected by anesthesia (P < 0.001; Figure 3-4). For the animals for which we have specific data (n = 15), initial recovery (time when animals started moving on their own) occurred in 74.1 +/- 38.2 min.

Venom volume was directly correlated with snout-vent length (P << 0.001; Figure 3-5), and mass (P << 0.001; data not shown) with larger animals having greater volume. A few animals produced less venom than would be expected based on their mass, including one animal that was later found to have an abscessed liver and one that was
likely hit by a car prior to collection. When repeatedly stimulated, however, no more venom was ejected, suggesting complete emptying of the glands.

**Discussion**

A commercially-available medical nerve stimulator produced reliable, safe collection of venom from anesthetized cottonmouth snakes. The short-acting anesthetic propofol induced anesthetic immobility in most snakes at dosages of 8 to 10 mg/kg IV. Induction times were variable when the drug was administered into the vessels of the ventral tail, and several snakes required supplemental injections. Induction after intracardiac administration was very rapid, although overall induction times were calculated after initial ventral tail injection.

Most of the snakes continued breathing throughout the procedure. Further, the snakes recovered fairly rapidly, allowing them to quickly resume normal behavior. A major problem with the use of propofol in these and other animals is that it must be injected IV to be effective. This requires skill in venepuncture in these and smaller snakes, and even then, many of the animals require supplemental injections. The prolonged induction times in snakes administered propofol in the ventral coccygeal vessels may be due either to failure to inject the full dose into the caudal vein or prolonged circulation from the caudal portion of the body. The rapid and consistent responses to intracardiac injection of the lower propofol dosages (5 mg/kg) support this. Although intracardiac injection is relatively easier to achieve, care must be taken to prevent injury to the heart. The other concern is administering a cardiodepressant drug in high concentration to the myocardial tissue. Although the majority of this study was conducted using propofol injected IV, we have subsequently found IC injection to reduce
handling time further (data not shown). Such injections should only be conducted, however, by those familiar with the technique to prevent damage to the heart. A consistent and efficacious injection is important for this technique to work in a timely manner, and practice is needed under proper guidance.

A major advantage of propofol for field work in reptiles is that it can be readily transported and requires no more equipment than a syringe and needle to administer. A hand-held ventilator, such as an Ambu bag, and endotracheal tube are all that are necessary to ventilate an anesthetized animal, and these are not typically needed. The constant current peripheral nerve stimulator simplifies venom extraction both in a field situation and in the laboratory. The model described is portable and uses AA batteries, making it very convenient. The combination of anesthesia and electrical stimulation leads to greater safety, better repeatability, easier quantification, and lowered handling time.

Since the animals are unconscious during the procedure, they are unlikely to bite, thereby enhancing safety. They are also unable to resist muscle flexion around the gland, increasing repeatability and quantification, and are unaware of handling and sample collection, likely reducing stress. Care must still be taken to avoid accidental contact, and we do this by maintaining the animal’s heads within clear plastic restraining tubes, except during venom extraction.

It is possible some venom remains in the gland after electrical stimulation, creating errors in volume measurement. With repeated stimulation, however, we saw no greater venom yield. In comparison, spontaneous ejection results in more venom with greater number of bites. A further problem with quantification of yield from wild snakes is that
conditions prior to capture can not be determined. It is always possible the animal recently fed or used venom in defense, thereby reducing available volume. Captive neonates fed two weeks after venom collection were able to subdue prey with venom, but many held prey after envenomation, suggesting a possible reduction in available venom (data not shown). It is also possible that removing all venom from the glands increases the amount of cellular material ejected with the venom. As most studies centrifuge venom and remove cellular debris after collection but prior to lyophilization, this may not present a problem.

We recaptured three snakes within twelve months of initial capture and release. All of these animals were in good health, similar body condition, and with equal or greater venom yield during the second collection. While this is a small sample size, it does indicate that our methods do not irreversibly harm the animals. Future research into the effects of this method on snakes should include multiple captures or long term maintenance in the laboratory as well as hormonal assays for typical stress indicators, such as cortisol. Likewise, a thorough comparison of venom yield among this and other extraction techniques would be useful.

In summary, the combination of injectable propofol anesthetic and electrical stimulation for venom extraction yield measurable, repeatable results. This technique allows for reduced handling time for the animals and increased safety for the researchers, and is portable to most field situations. It should be considered as an alternative or addition to other extraction techniques.
Figure 3-1. Anesthetized Florida cottonmouth (*Agkistrodon piscivorus conanti*) in tube with only head protruding. The animal is lying in dorsal recumbency and easy to bring back into the tube by pulling the anterior portion of the body, should it prematurely revive.
Figure 3-2. Anesthetized Florida cottonmouth (*Agkistrodon piscivorus conanti*) showing placement of electrodes on the skin surface across the venom gland.

Figure 3-3. Anesthesia induction in Florida cottonmouths, *Agkistrodon piscivorus conanti*. Snakes (*n* = 75) were initially injected with 10 mg/kg body mass or less, and some (*n* = 33) had supplemental injections (up to 10 mg/kg body mass). Induction occurred in 12.2 ± 8.5 min of initial injection. There was a significant mass-associated positive trend between amount of propofol needed for induction and animal mass (regression analysis).

\[ y = 0.0144x - 0.9484 \]

\[ R^2 = 0.8078 \]

\[ P << 0.001 \]
Figure 3-4. Mass-specific effects of propofol on induction time in Florida cottonmouths, *Agkistrodon piscivorus conanti*. Snakes were initially injected with propofol (10 mg/kg body mass), and monitored for loss of righting reflex and failure to respond to a mild cloacal pinch. Induction time was positively correlated with snake mass (regression analysis).

![Figure 3-4](image)

\[ y = 0.004x + 9.5399 \]
\[ R^2 = 0.1046 \]
\[ P < 0.005 \]

Figure 3-5. Mass-specific venom yield from Florida cottonmouths, *Agkistrodon piscivorus conanti*, by electrical stimulation. All animals were anesthetized with propofol (10 mg/kg body mass) prior to venom collection, and venom yield was significantly correlated with animal snout-vent length (regression analysis).

![Figure 3-5](image)

\[ y = 1.0197x - 271.91 \]
\[ R^2 = 0.6523 \]
\[ P << 0.001 \]
In the United States, there are three genera of viperid snakes, and all are members of the subfamily Crotalinae (rattlesnakes and their allies). These genera are: *Crotalus* (rattlesnakes), *Sistrurus* (pigmy rattlesnakes), and *Agkistrodon* (copperheads and cottonmouths). All are venomous and range in abundance, depending on habitat specificity.

The Florida cottonmouth (*A. piscivorus conanti*) is associated with fresh water habitats throughout most of the state of Florida, where it feeds on a wide range of mostly vertebrate prey. The cottonmouth in general has been the focus of many wide-ranging studies of ecology and evolution (Gloyd and Conant, 1990), and previous phylogenies have utilized mtDNA to examine phylogeography and relationship with the copperhead, *A. contortrix* (Guiher and Burbrik, 2008). However, previously no microsatellite loci have been specifically developed for use in this species.

Many previous studies of snakes have developed microsatellites to analyze genetic relatedness among or within populations of a certain species (hereafter termed target species; Villarreal et al., 1996; Gibbs et al., 1998; Burns and Houlden, 1999; McCracken et al., 1999; Prosser et al., 1999; Jordan et al., 2001; Matson et al., 2001; Garner et al., 2002; Holycross et al., 2002; Carlsson et al., 2003; Bond et al., 2005; Manier and Arnold, 2005; Stapley et al., 2005; Lane et al., 2008; Munguia-Vega et al., 2009). Some of these studies have examined the utility of previously-developed loci in species other than the target species (hereafter termed non-target species) and have yielded different
results including: 1) no PCR amplification (Gibbs et al., 1998; Prosser et al., 1999; Holycross et al., 2002; Blouin-Demers and Gibbs, 2003; Carlsson et al., 2003; Stapley et al., 2005; Meister et al., 2009), 2) amplification but without reporting allelic diversity (Gibbs et al., 1998; Prosser et al., 1999; Blouin-Demers and Gibbs, 2003; Bond et al., 2005; Clark et al., 2008), and 3) amplification with multiple alleles reported (Jordan et al., 2001; Matson et al., 2001; Hille et al., 2002; Holycross et al., 2002; Carlsson et al., 2003; Manier and Arnold, 2005; Stapley et al., 2005; Anderson, 2006; Lane et al., 2008; Meister et al., 2009). This last category of studies indicates that some snake microsatellite loci may be broadly useful for genetic studies of non-target snake species. Indeed, a few studies have utilized loci developed in one species to answer population genetics questions in another (Scott et al., 2001; Manier and Arnold, 2005; Clark et al., 2008).

**Materials and Methods**

As part of a larger study, Florida cottonmouth (N = 89) DNA samples were collected from two locations in Florida: Paynes Prairie Preserve State Park (PP) and Lower Suwannee National Wildlife Refuge (LS), both located in northern Florida, separated by approximately 90 km. To preserve DNA, tissue samples were either mixed with lysis buffer (for blood from live animals; 0.1 M Tris-HCl with 100 mM EDTA and 10 mM NaCl, pH 8.0) or tissue buffer (for body wall from road-killed animals; saturated NaCl solution with 250 mM EDTA and 20% DMSO, pH 7.5). DNA extraction was done using the Qiagen DNEasy kit, and resulting DNA was examined quantitatively and qualitatively using a NanoDrop 8000, with initial concentrations ranging from 8.6 to 90.0 ng/µl. Primers from previously published (Villarreal et al., 1996; Gibbs et al., 1998; Holycross et
al., 2002; Carlsson et al., 2003; Goldberg et al., 2003; Oyler-McCance et al., 2005; Clark, 2008; Table 4-1) studies were purchased from MWG Biotech, and used initially to amplify 12 to 24 cottonmouth DNA samples. We utilized the published primers that recorded the greatest variability, and did not examine any locus with fewer than 3 alleles in the original target species. A gradient thermocycler was used to optimize temperature and other parameters (DNA, Mg$^{2+}$, and primer concentrations) to give repeatable, clear results. Products were visualized on 2% agarose gels stained with ethidium bromide and examined for presence of two or more bands of different size. If optimization failed to yield a product after multiple attempts, or only one band was visible, the individual locus was dropped.

**Results**

Table 4-1 shows which loci amplified cottonmouth DNA and which gave variable-sized products. Overall, 71% (22/31) of tested loci amplified and 32% (10/31) yielded visual variable fragment sizes. Two other loci (6.5%) yielded what appeared to be visible differences; however, after fluorescent labeling, CH2D was found to be monotypic. Because of the low visible variation in *Scu05*, it was not utilized further. Forward primers for the ten variable loci were fluorescently labeled on the 5’ end with either HEX or 6-FAM, and PCR conditions were optimized for each locus (Table 4-2). Slight deviations in cycle number and/or temperature were found to not adversely affect amplification of products.

Genotypes were determined for all individuals at all ten loci by analyzing PCR products on either a MegaBACE 1000 or Applied Biosystems 3730 automated sequencer with internal standard ladders, and fragment sizes were determined using
was used to examine the data for large allele dropout, null alleles, and stuttering signals, and only the LS population showed a potential null allele and stuttering, at locus Scu26. CERVUS version 3.0 (Marshall et al., 1998) was used to determine observed and expected heterozygosities, and GENEPOP version 4.0 (Raymond and Rousset, 1995) was used to test for linkage disequilibrium and significant deviations from Hardy-Weinberg equilibrium. Following sequential Bonferroni tests, no linkage disequilibrium was found for any loci pair. One locus in the LS population (CH1A) and two loci in the PP population (CH2E and CH5A) were found to deviate significantly from Hardy-Weinberg equilibrium. Allele sizes fell within or overlapped those reported for the original target species.

Discussion

Development of species-specific microsatellite loci, while much easier than it once was, is a time-consuming and potentially costly endeavor. However, a relatively easy and inexpensive substitute is to attempt the use of markers developed in closely related species. For snake species in particular, some microsatellite loci have been found to amplify products in even distantly related species (Gibbs et al., 1998; Prosser et al., 1999; Bond et al., 2005; Hille et al., 2002). Although this wide-ranging cross-reactivity in snakes may be an exception, other studies have described the usefulness of non-target species loci for non-snake organisms (Donaldson and Vercoe, 2008).

The cottonmouth is a well-studied viperid snake, yet no microsatellite markers have been developed for the species. The ten loci reported here have been shown to be polymorphic in the Florida cottonmouth, and may be useful for future studies of
population genetics. Further, we have shown that there is potential for these and other loci, developed in closely (or even distantly) related species, to be useful for genetic studies of other crotaline snakes, although each must be examined in a case-by-case fashion.

While we tended to use primers with known polymorphism in their target species, it is possible that polymorphism may be even greater in non-target species. While some loci from snakes of the genus *Crotalus* and *Sistrurus* were polymorphic in *A. p. conanti*, two loci developed in *C. viridis concolor* (a North American rattlesnake) and two developed in *Vipera berus* (a European viperine snake) did not amplify regions in *A. p. conanti* DNA. This may be expected for *V. berus*, as it is distantly related, but is slightly surprising for *C. v. concolor*. It would be of interest to examine South American crotaline and/or Asian viperine snakes to see if microsatellite usefulness can be mapped phylogenetically. Additional recently published crotaline loci (Munguia-Vega et al., 2009) may also be useful for such studies.

As previously shown, published primers may be a useful starting point for population genetic studies in non-target species. By validating such primers for use in non-target species, researchers may save both time and money, and may be able to expand the set of useful microsatellites for a given species. Crotaline snakes appear to be a good example of a taxon with many published loci overall and with multiple studies indicating cross-reactivity. We have validated a set of 10 microsatellite loci developed in related species for use in the non-target cottonmouth snake.
Table 4-1. Utility of 31 microsatellite primers for use with cottonmouths (*Agkistrodon piscivorus*). Target species, accession number (where published), and number of alleles (N<sub>A</sub>) from original study are given. Results from the current study are abbreviated as follows: amp/no amp = relative ability to amplify PCR product; var/no var = size variation in amplified PCR product.

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<td>11</td>
<td>amp, no var</td>
</tr>
<tr>
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<td><em>Sistrurus catenatus</em></td>
<td>--</td>
<td>Gibbs et al., 1998</td>
<td>8</td>
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</table>

*Clark, 2006 data for Crotalus horridus.*
Table 4-2. Information for microsatellite loci used with *Agkistrodon piscivorus conanti* DNA. Optimal PCR conditions including ambient temperature ($T_a$), MgCl concentration and number of cycles are given. Also presented are overall allele size ranges, and number of alleles ($N_A$), observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$) for two populations.

<table>
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<th>Locus</th>
<th>Label</th>
<th>PCR</th>
<th>MgCl</th>
<th>Cycles</th>
<th>Size</th>
<th>Paynes Prairie</th>
<th>Lower Suwannee</th>
<th>Reference</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$T_a$ (°C)</td>
<td>(mM)</td>
<td>(bp)</td>
<td>$N_A$</td>
<td>$H_O$</td>
<td>$H_E$</td>
<td>$N_A$</td>
</tr>
<tr>
<td>CH1A</td>
<td>6-FAM</td>
<td>59.5</td>
<td>1.5</td>
<td>36</td>
<td>200-225</td>
<td>7</td>
<td>0.608</td>
<td>0.648</td>
</tr>
<tr>
<td>CH2E</td>
<td>HEX</td>
<td>59.5</td>
<td>2.0</td>
<td>36</td>
<td>167-203</td>
<td>11</td>
<td>0.588</td>
<td>0.651a</td>
</tr>
<tr>
<td>CH4B</td>
<td>6-FAM</td>
<td>57.5</td>
<td>2.0</td>
<td>36</td>
<td>146-166</td>
<td>6</td>
<td>0.745</td>
<td>0.748</td>
</tr>
<tr>
<td>CH5A</td>
<td>HEX</td>
<td>57.5</td>
<td>2.0</td>
<td>38</td>
<td>135-146</td>
<td>4</td>
<td>0.490</td>
<td>0.558a</td>
</tr>
<tr>
<td>Crti09</td>
<td>HEX</td>
<td>52.3</td>
<td>2.0</td>
<td>40</td>
<td>309-379</td>
<td>12</td>
<td>0.824</td>
<td>0.848</td>
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<td>Crti10</td>
<td>6-FAM</td>
<td>52.6</td>
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<td>17</td>
<td>0.882</td>
<td>0.915</td>
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<td>CwB23</td>
<td>6-FAM</td>
<td>52.6</td>
<td>2.0</td>
<td>36</td>
<td>200-238</td>
<td>11</td>
<td>0.725</td>
<td>0.684</td>
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<tr>
<td>CwC24</td>
<td>HEX</td>
<td>55.6</td>
<td>2.0</td>
<td>36</td>
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<td>21</td>
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<td>0.932</td>
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<tr>
<td>Scu11</td>
<td>6-FAM</td>
<td>52.9</td>
<td>2.0</td>
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<td>143-182</td>
<td>14</td>
<td>0.902</td>
<td>0.864</td>
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<tr>
<td>Scu26</td>
<td>HEX</td>
<td>57.5</td>
<td>2.0</td>
<td>38</td>
<td>128-175</td>
<td>11</td>
<td>0.725</td>
<td>0.831</td>
</tr>
</tbody>
</table>

*aSignificant deviation from Hardy-Weinberg equilibrium after Bonferroni correction.*
CHAPTER 5
POPULATION GENETIC ANALYSIS OF ONE INSULAR AND THREE MAINLAND POPULATIONS OF THE FLORIDA COTTONMOUTH

Introduction

The North American snake genus *Agkistrodon* is comprised of four species: *bilineatus* (the cantil), *contortrix* (the copperhead), *piscivorus* (the cottonmouth), and *taylori* (Taylor’s cantil). It has been hypothesized that the common *Agkistrodon* ancestor developed sometime in the late Oligocene (28 to 24 mya), and that *piscivorus* separated from the remainder of the species during the middle Miocene (~16 to 15 mya) (Van Devender and Conant, 1990). Contrary to this, Parkinson et al. (2000) utilized mitochondrial and transfer RNA sequences and indicated that *contortrix* separated earliest, with *piscivorus* second. Guiher and Burbrink (2008) used mitochondrial DNA sequences of the cytochrome b gene and found support for the same pattern of relationship as Parkinson et al. (2000). They also determined that the initial separation of *contortrix* occurred about 6.60 mya with *piscivorus* separating around 5.30 mya.

The cottonmouth is generally held to contain three subspecies: *conanti* (Florida cottonmouth), *leucostoma* (western cottonmouth), and *piscivorus* (eastern cottonmouth) (Gloyd and Conant, 1990). Only *conanti* is located in peninsular Florida, although it may interbreed with *leucostoma* on the northwestern part of its range and with *piscivorus* on the northeastern portion of its range. Recent molecular data also indicate that *leucostoma* and *piscivorus* should be grouped and considered a sister taxon to *conanti* (Guiher and Burbrink, 2008).

In a recent study, Douglas et al. (2009) examined the genetic relationship among the species and subspecies of *Agkistrodon*. They analyzed mitochondrial DNA sequences, and their data supported both the late separation of *A. piscivorus*
(subsequent to A. contortrix) and the grouping of A. p. leucostoma and A. p. piscivorus
(with A. p. conanti being separate). They also reported support for the origin of A. p.
conanti from Mexico via rafting across the Gulf of Mexico. Interestingly, their data
indicated that A. p. conanti was limited in its range expansion due to 1) separation of
Peninsular Florida from the mainland by a mid- to late-Pleistocene incurrence of sea
water and 2) competition from established A. p. piscivorus after reunion of Peninsular
Florida and the mainland.

Among snake species, the cottonmouth has been extensively studied in terms of
its natural history and ecology (Gloyd and Conant, 1990). Within Florida, this snake is
usually found in or near freshwater habitats, and it is one of the most common snakes.
Possible reasons for the success of this species in Florida include the widespread
occurrence of freshwater (much habitat), the relative low altitudes (low barriers to
dispersal), and the ubiquitous nature of its dietary preferences (wide trophic niche
breadth; Lillywhite and McCleary, 2008). The relative abundance of individuals makes
this species suitable for population-level studies, especially baseline studies for later
comparison.

Although it is well known that insular populations of animals are under different
ecological constraints than mainland populations and may, therefore, evolve along
different trajectories, predicting the manner in which these populations evolve is
problematic. Among snakes, previous comparisons of insular populations with their
mainland counterparts have examined phenotypic and ecological characters such as
morphometrics (Shine, 1987), venom activity or composition (Mebs, 1970; Selistre and
Giglio, 1987; Williams and White, 1987) prey base (Wharton, 1966, 1969; Shine et al.,
2002; Wüster et al., 2005; Lillywhite and McCleary, 2008) and interspecies interactions (Bonnet et al., 1999; Shine et al., 2002).

There is one insular population of Florida cottonmouth—that on the island of Seahorse Key (SHK)—that is of special interest. This population was intensively studied by Wharton (1966, 1969), and was found to have a unique and interesting natural history. The SHK population feeds primarily on fish that are regurgitated or dropped by colonially-nesting birds, including Brown Pelicans (*Pelecanus occidentalis*), Double-crested Cormorants (*Phalacrocorax auritus*), and White Ibises (*Eudocimus albus*). This relatively easy source of high-quality food has apparently allowed the SHK population to be maintained in high density, with many very large individuals in the population.

SHK is a ~67 ha island located ~5 km off of the coast of mainland Florida and is separated from the mainland by salt water (Figure 5-1). This marine corridor may act as a dispersal barrier, reducing gene flow from the mainland to the island and vice versa. The island is thought to have been isolated from the mainland for about 3300 to 3900 years, based on consistent rates of sea level rise since the late Holocene (Roark, 2003). However, because of occasional heavy rains and large freshwater runoff via the Suwannee River, it is likely that water between the mainland and SHK is highly variable in salinity. The relative isolation and abundant food source leads to the question as to whether the SHK population of snakes is genetically isolated from mainland populations and is possibly inbred.

To examine possible genetic isolation of the SHK population, comparisons were made with snakes from three other geographically defined populations: Paynes Prairie
Preserve State Park (PP), Lower Suwannee National Wildlife Refuge (LS), and Big Cypress National Preserve (BC). LS is a 21450 ha refuge located on the mainland northeast of SHK. It contains a mixture of floodplain cypress swamps and estuarine habitats, and is closest to SHK of the areas studied. PP is located in north central Florida and is 8500 ha in area. Year-to-year rainfall amounts for the prairie range from times of relative dryness with small ponds to times in which much of the park is flooded, usually due to tropical storm action; it is surrounded by upland live oak forest and a series of interconnecting streams, swamps, and seasonally flooded forests. Finally, BC is located in south Florida, is ~291400 ha in area, and is part of the Everglades. It contains diverse habitats dominated by seasonally flooded sawgrass fields and includes cypress swamp and permanent ponds and ditches.

In order to examine the effects of local ecology on population evolution, it must first be shown that populations have separate genetic identities. In order to do this, and to examine whether the salt water corridor is an effective barrier for isolation of the insular population, the genetic relationships among the described populations were examined. This comparative genetic analysis was done utilizing microsatellite markers, which are useful for discerning fine population structure. Examination of population structure within a common species is of interest in areas like Florida because of potential habitat fragmentation due to human development.

**Materials and Methods**

**Animals**

Genetic samples from a total of 129 animals were used, spread amongst the four populations (N = 25, SHK; N = 51, PP; N = 38, LS; and N = 15, BC). Live animals were found by searching trails, stream banks, and ponds or by slow road cruising in an
automobile; road-killed animals were visually spotted by road cruising. For each
individual, spatial data were recorded as Global Positioning System (GPS) coordinates.
Live animals were transported to the laboratory for sample collection. Blood from live
animals or body wall from fresh road-killed animals were preserved either in lysis or
tissue buffer, respectively. DNA was extracted using a Qiagen DNEasy DNA extraction
kit, and initial concentrations of DNA were determined using a NanoDrop 8000.

Analysis

Previously published crotalid microsatellite loci primers were purchased from
MWG Biotech AG and the forward primers were fluorescently labeled either with HEX or
6-FAM (Table 5-1). These loci were developed using different crotalid species, so we
initially validated their use in cottonmouths. The loci used were as follows: CH1A,
CH2E, and CH4B (Clark, pers. comm.); CH5A (Villarreal et al., 1996); Crti09 and Crti10
(Goldberg et al., 2003); CwB23 and CwB24 (Holycross et al., 2002); and Scu11 and
Scu26 (Gibbs et al., 1998).

DNA fragments were amplified via PCR, with conditions dependent upon the
specific locus in question, with an annealing temperature of 72°C and elongation
temperatures between 52.3 and 59.5°C (Table 5-1). Fluorescently-labeled amplified
products were analyzed by capillary electrophoresis on either an Applied Biosystems
3730 or MegaBACE 1000, and fragment sizes were determined using GENEMARKER
software.

MICRO-CHECKER version 2.2.0.3 (van Oosterhout et al., 2004) was used to examine
the data for large allele dropout, null alleles, and stuttering signals. CERVUS version 3.0
(Marshall et al., 1998) was used to determine observed and expected heterozygosities,
and GENEPOP version 4.0 (Raymond and Rousset, 1995) was used to test for linkage
disequilibrium and significant deviations from Hardy-Weinberg equilibrium (HWE). GenAlEx 6.2 (Beck et al., 2008; Smouse et al., 2008) was also utilized to determine migration rates among the populations.

To determine the population identity of each individual, STRUCTURE 2.2 (Pritchard et al., 2000; Falush et al., 2003) was used. This program uses a Bayesian model clustering system to determine population structure and assign every individual to a specific population. It is based on overall allele frequencies and uses a Markov chain Monte Carlo (MCMC) simulation. The program further analyzes the data to determine the most likely number of populations being represented by the data set overall. The data were analyzed using a burn-in period of 50000 followed by 200000 MCMC repeats, and the admixture model was used. Initial, smaller analyses of $k = 1$ to 10 populations indicated that the data did not support models for $k = 1, 2, 7, 8, 9$ or $10$ population, so the final analysis was run 7 times for each of $k = 3$ through 6 independent populations.

The program TESS, which is a similar Bayesian model that utilizes a Hidden Markov Random Field simulation and incorporation spatial data (GPS), was also used to determine population identity for each individual animal.

**Results**

The LS population showed a potential null allele and stuttering at locus Scu26, and the SHK population showed a potential null allele and stuttering at locus CH2E. Following sequential Bonferroni tests, no linkage disequilibrium was found for any loci pair. However, because of high allelic diversity, each of the individuals from BC had a unique allelic identity for locus CwC24. Also, because loci CH4B and CH5A were monotypic within the SHK population, meaningful disequilibrium comparisons could not be performed. One locus in the LS population (CH1A) and two loci in the PP population
(CH2E and CH5A) were found to deviate significantly from HWE, but all BC loci were in HWE (Table 5-2). Three loci in the SHK population were monotypic (CH4B and CH5A) or nearly monotypic (CwB23), so tests for HWE could not be done.

The results of the STRUCTURE 2.2 analysis indicated that the most likely scenario includes k = 4 populations (lnP(D) = -4154.8). The grouping of individuals into these populations can be seen in Figure 5-2, and it shows clearly defined delineations among the four populations. With minor deviations, individuals collected from an area identified genetically with other individuals from that same population, indicating little mixing among the populations. F_{ST} values, which indicated likelihood of group segregation based on variation within each population and for all populations combined, indicated that all four populations are genetically separate. Using standard published numbers (Pritchard et al., 2000), the F_{ST} values were found to indicate moderate (0.051, BC; 0.085, LS), great (0.151, PP) and very great (0.581, SHK) genetic isolation.

Results from the TESS run were similar to the STRUCTURE results. Once again, all individuals from one geographic population were genetically grouped with the other individuals from that region (Figure 5-3). The same minor exceptions were seen using the TESS program. TESS also gives a spatial visual representation of the populations by incorporating GPS data (Figure 5-4). GenAlEx results indicated low migration rates, with the greatest rate (4.44 individuals per generation) being found between PP and LS, and the lowest rate (0.96 individuals per generation) being between PP and SHK.

**Discussion**

Based on these results, the four studied populations are genetically different from one another, indicating at least partially different evolutionary trajectories. More importantly, F_{ST} values indicate that the insular population (SHK) is more genetically
isolated than the other three populations. This is of interest, because it indicates that
the marine corridor separating SHK from the mainland is an effective barrier to
migration of the animals and, therefore, gene flow between the populations. Despite the
proximity of the LS population to the SHK population, LS is more genetically close to
both PP and BC than it is to SHK.

Allelic diversity among the populations indicates potential inbreeding in the SHK
population, as it had the fewest number of alleles for all loci and the lowest average
number of alleles per individual for all but locus CH1A. The most extreme examples of
this are loci CH4B and CH5A, which are monoallelic in the SHK population. As reduced
allelic diversity is an indication of an inbred population (either through genetic bottleneck
or founder effects), this indicates inbreeding in the SHK population.

In order for populations of organisms to evolve independently, they must first be
isolated by some means, and geographic barriers can be highly effective. In the case of
the cottonmouth populations studied here, sea water appears to be involved in isolation
of the SHK population. Beside this, however, the other three mainland populations
showed levels of isolation, albeit less than the SHK population. This is understandable
in the case of BC, because it is separated from the northern populations by
approximately 400 km. However, the distance between SHK and LS (~30 km) is much
shorter than between LS and PP (~80 km).

The establishment of effect isolation is the first step in examining the evolution of
phenotypic characters in populations. With this knowledge, it is possible to examine
differences among populations meaningfully. The Florida cottonmouth, and especially
the SHK population, makes a great model for studying evolution of phenotypic
characters in a natural setting. Specifically, because of the rich history of research into its venom, replete with numerous conflicting reports in the literature, and the relative common occurrence of the species throughout its range, the Florida cottonmouth is a worthy study species.

The results of this study are also important because they give baseline data into the population genetics of an animal that is not currently considered threatened. As interest in many species occurs once that species has reached a critical level, baseline data is usually lacking or severely limited. In the state of Florida, where land development occurs at a very rapid pace, these data can serve as standards for future examinations of habitat fragmentation due to human land development.
Table 5-1. Specifics of microsatellite amplification. Elongation temperatures varied depending on the locus, as did the number of cycles.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Fluorescent Label</th>
<th>Primer Sequences</th>
<th>PCR Ta (ºC)</th>
<th>MgCl (mM)</th>
<th>Cycles</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH1A</td>
<td>6-FAM</td>
<td>F-GCAGGCACACTGTTGTCACCTGT R-TCAACCAAATTTTGCTCATGC</td>
<td>59.5</td>
<td>1.5</td>
<td>36</td>
<td>Clark, 2006</td>
</tr>
<tr>
<td>CH2E</td>
<td>HEX</td>
<td>F-TCTGATTGCAAGCTCTGGTTT R-ATTCCAGGAAGTCAAAGGAG</td>
<td>59.5</td>
<td>2.0</td>
<td>36</td>
<td>Clark, 2006</td>
</tr>
<tr>
<td>CH4B</td>
<td>6-FAM</td>
<td>F-CAGGTGACCTGAAAGGCACA R-ATCACTTTCTGGGAGCAGTT</td>
<td>57.5</td>
<td>2.0</td>
<td>36</td>
<td>Clark, 2006</td>
</tr>
<tr>
<td>CH5A</td>
<td>HEX</td>
<td>F-CCAGAGCCATCAAGGCCCTT R-TGCAGAGGACGACTTTTGTTA</td>
<td>57.5</td>
<td>2.0</td>
<td>38</td>
<td>Clark, 2006</td>
</tr>
<tr>
<td>Crti09</td>
<td>HEX</td>
<td>F-TAGGAATAGAAATGTCAGG R-TAATGTAATGTTAGCAGGA</td>
<td>52.3</td>
<td>2.0</td>
<td>40</td>
<td>Goldberg et al., 2003</td>
</tr>
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<td>6-FAM</td>
<td>F-ATGACCTCTGACTCTGGTT R-ACAGCTATCCTAGAGTGA</td>
<td>52.6</td>
<td>2.0</td>
<td>36</td>
<td>Goldberg et al., 2003</td>
</tr>
<tr>
<td>CwB23</td>
<td>6-FAM</td>
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<td>2.0</td>
<td>36</td>
<td>Holycross et al., 2002</td>
</tr>
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<td>2.0</td>
<td>36</td>
<td>Holycross et al., 2002</td>
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<td>57.5</td>
<td>2.0</td>
<td>38</td>
<td>Gibbs et al., 1998</td>
</tr>
</tbody>
</table>
Table 5-2. Locus- and population-specific data for microsatellite analysis of cottonmouths. A total of 129 individual snakes were used for the comparisons, and the number for each population is given. \(N_A\) is the number of alleles per locus for each population and in total. \(H_O\) is observed heterozygosity and \(H_E\) is expected heterozygosity.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Total Size (bp)</th>
<th>Total (N_A)</th>
<th>(H_O)</th>
<th>(H_E)</th>
<th>SHK (n = 25)</th>
<th>PP (n = 51)</th>
<th>LS (n = 38)</th>
<th>BC (n = 15)</th>
</tr>
</thead>
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<tr>
<td>CH1A</td>
<td>200-225</td>
<td>10</td>
<td>0.440</td>
<td>0.377</td>
<td>7</td>
<td>0.608</td>
<td>0.648</td>
<td>9</td>
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<tr>
<td>CH2E</td>
<td>167-203</td>
<td>13</td>
<td>0.400</td>
<td>0.598</td>
<td>11</td>
<td>0.588</td>
<td>0.651(^a)</td>
<td>10</td>
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<tr>
<td>CH4B</td>
<td>144-166</td>
<td>7</td>
<td>0.000</td>
<td>0.000</td>
<td>6</td>
<td>0.745</td>
<td>0.748</td>
<td>4</td>
</tr>
<tr>
<td>CH5A</td>
<td>135-146</td>
<td>4</td>
<td>0.000</td>
<td>0.000</td>
<td>4</td>
<td>0.490</td>
<td>0.558(^a)</td>
<td>4</td>
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<tr>
<td>Crti09</td>
<td>309-379</td>
<td>14</td>
<td>0.640</td>
<td>0.496</td>
<td>12</td>
<td>0.824</td>
<td>0.848</td>
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<tr>
<td>Crti10</td>
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<td>18</td>
<td>0.600</td>
<td>0.500</td>
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<td>0.882</td>
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<td>CwB23</td>
<td>200-238</td>
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<td>0.040</td>
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<td>0.684</td>
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<td>21</td>
<td>0.863</td>
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<td>Scu11</td>
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<td>0.600</td>
<td>0.571</td>
<td>14</td>
<td>0.902</td>
<td>0.864</td>
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<td>Scu26</td>
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<td>0.280</td>
<td>0.301</td>
<td>11</td>
<td>0.725</td>
<td>0.831</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\)Loci with significant deviations from Hardy-Weinberg equilibrium
Figure 5-1. Locations of cottonmouth populations studied. The image was created using Google Earth.
Figure 5-2. **STRUCTURE** population identity for individual cottonmouths in Florida. This image was generated using **STRUCTURE** 2.2. Each vertical bar represents one individual, and colors indicate likelihood that an individual genetically identifies with that population. With few exceptions, individuals identify genetically with the population in the region from which they were sampled. Specimens with multiple colors (such as the large maroon bar in the Paynes Prairie region) indicate mixed identity, and the amount of one color indicates the percentage with which the individual identifies with that population.

Figure 5-3. **TESS** Population identity for individual cottonmouths in Florida. This image was generated using **TESS** 2.2. Each vertical bar represents one individual, and colors indicate likelihood that an individual genetically identifies with that population. With few exceptions, individuals identify genetically with the population in the region from which they were sampled. Specimens with multiple colors (such as the large maroon bar in the Paynes Prairie region) indicate mixed identity, and the amount of one color indicates the percentage with which the individual identifies with that population.
Figure 5-4. Spatial and genetic relationships of individual Florida cottonmouths. This image was created using Tess 2.2. Each black dot represents one individual, and each polygon indicates the population with which that individual identifies genetically. Colors, in general, indicate the following: green—Big Cypress; blue—Paynes Prairie; maroon—Lower Suwannee; and orange—Seahorse Key. Distances between dots are to scale with GPS data.
CHAPTER 6
ENZYMATIC ACTIVITY IN THE VENOM OF THE FLORIDA COTTONMOUTH: A POPULATION-LEVEL COMPARISON

Introduction

The Florida cottonmouth snake, *Agkistrodon piscivorus conanti*, is a common snake associated with fresh water habitats throughout the Florida peninsula. It is known to be a dietary generalist (Lillywhite and McCleary, 2008), consuming various species of vertebrate prey. Its venom is complex, containing many different biologically active enzymes and functional non-enzymatic peptides. Although there may be accompanying local tissue damage, the major effect of cottonmouth envenomation is a disruption of hemostasis. This disruption includes hemolysis of red blood cells, perturbation of the blood clotting cascade, and hemorrhaging from blood vessels, all of which result in a precipitous drop in blood pressure. This fall in blood pressure leads to incapacitation and possible death.

The majority of active components of cottonmouth venom are enzymes. Among these, cottonmouths are known to have specific phosphodiesterase (Mebs, 1970), 5'-nucleotidase (Richards et al., 1965), alkaline phosphomonoesterase (Tan and Ponnudurai, 1990), hyaluronidase (Powlick and Geren, 1981), L-amino acid oxidase (Ahn et al., 1997), metalloproteinase (Jia et al., 2009), serine proteases (thrombin-like—Kress and Catanesi, 1980; kallikrein-like—Bailey et al., 1991; and arginine esterase—Kocholaty et al., 1971), and phospholipase A2 (Ownby et al., 1999). There are also general enzymatic activities, based on their effects on substrate, and these include aminopeptidase (Prescott and Wagner, 1976), ATPase (Mebs, 1970), fibrinogenase (Nikai et al., 1988b), non-specific esterase (Bernick and Simpson, 1976), and non-specific protease (Wagner and Prescott, 1966a).
Previous studies using cottonmouth venom have noted large variation in activity or effects when different individuals or commercially-produced lots were utilized (Tan and Ponnudurai, 1990). Venom variation is of interest because it has implications for snakebite treatment including antivenom therapy, and cottonmouths are traditionally responsible for human envenomations (Watt, 1978). Further, as venom is likely important for prey capture and appears to evolve quite rapidly (Lynch, 2007), it is a useful phenotypic trait for examinations of evolution.

Several previous studies have examined potential correlations among venom characteristics and other factors such as geographic location, inter- and intra-populational phylogeny, and diet. Daltry et al. (1996b) found individual Calloselasma rhodostoma to have venom correlated with local prey species, indicating that natural selection for prey is very important in influencing venom evolution. Williams et al. (1988) found that insular populations of Notechis ater niger had venom more tightly correlated with distance from the mainland, indicating vicariance, and therefore genetic drift, was important for venom evolution. In a study of Trimeresurus stejneger, Creer et al. (2003) postulated that natural selection was likely the most important factor influencing temporal changes in venom composition. More recently, Barlow et al. (2009) found venom composition in four species of Echis to correlate with prey species, again indicating natural selection.

There is some evidence that venom production in snakes is energetically costly (McCue, 2006), so it may be possible that optimizing venom for certain prey is necessary for overall fitness. Indeed, although most hydrophiine sea snakes are highly
venomous, one species that is a dietary specialist of fish eggs, *Aipysurus edouxii*, has effectively lost its main neurotoxin (Li et al., 2005).

To examine potential differences in venom composition within the Florida cottonmouth, three geographically isolated populations were selected. The focal population was that found on the island of Seahorse Key (SHK; Figure 6-1). Snakes on this island have a unique ecology in that they feed primarily on marine fishes that are dropped or regurgitated by colonially nesting water birds. The other population locations were Paynes Prairie Preserve State Park (PP), which is located in north central Florida and is a seasonally flooded prairie with associated fresh water ponds and streams, and Lower Suwannee National Wildlife Refuge (LS), which is located on the Gulf coast of Florida (near SHK) and has fresh and brackish water habitats. The two mainland populations appear to have the diverse diets normally attributed to the species. Because of this, it was of interest to determine whether the three populations showed any differences in venom enzymatic activities.

Although Tan and Ponnudurai (1990) examined the activities of many different venom enzymes, they utilized venom provided by commercial sources. These venoms were pools of a number of individual snakes from the respective sources, so no levels of among-population variation could be determined. They did, however, find variation among the venoms from different sources. Among the enzymes showing the greatest amount of variation were protease, hyaluronidase, L-amino acid oxidase (LAAO), and phospholipase A₂ (PLA₂). The protease assay utilizes casein as substrate and is an indicator of the overall effects of possibly many different proteolytic enzymes. Proteases are generally responsible for hydrolysis of structural proteins, may cause
local and systemic tissue damage, and may be useful for prey digestion. Hyaluronidase, causes hydrolysis of hyaluronic acid between cells, thereby allowing other venom components to travel through cell barriers. Hyaluronidase is sometimes considered a “spreading factor” because of this activity. LAAO causes oxidation of L-amino acids via a two-step deamination process. This catalysis may lead to cell death via apoptosis, but the overall biological effects have not been determined. PLA2 is generally responsible for degradation of cell membranes, as it catalyzes break down of phospholipids.

**Materials and Methods**

**Animals**

Snakes were collected from the three different regions by either active searching on foot along trails and waterways or by road cruising. A total of 76 animals (SHK = 25; PP = 25; LS = 26) were captured and transported to the laboratory for venom extraction. Animals were anesthetized using intravascular or intracardiac injections of Propofol (Rapinovet, Schering-Plough Animal Health Corporation; 10 mg/ml, 8 to 10 mg/kg body mass), and venom was extracted from anesthetized animals using a human constant current peripheral nerve stimulator (10 to 30 mA; Fisher & Paykel Health Care) applied across each venom gland (McCleary and Heard, 2009). For each individual, venom from both glands was pooled. Morphometric and demographic data were also collected for all individuals, and these included mass, snout-to-vent length (SVL), total length, and sex. Sex was determined using stainless steel sexing probes.

**Venom Preparation**

Venom was collected in polypropylene beakers and aliquoted into microcentrifuge tubes for quantification and lyophilization. All venoms were reconstituted in 0.85% NaCl
to approximately 0.5 mg protein/ml venom prior to use in enzyme assays, and venom was utilized within 24 hours of reconstitution. Select individual venom samples (N = 64) were visualized by SDS-PAGE gel electrophoresis to examine them for obvious differences. For electrophoresis, samples of 25 µl (pre-lyophilization) were reconstituted and run on 12% acrylamide gels with 2-(N-morpholino)ethanesulfonic acid running buffer.

**Enzyme Assays**

Enzyme assays were conducted as previously described (Tan and Ponnudurai 1988, 1990), with slight modifications, and all assays were validated using pooled venom samples to verify linear kinetic reactions. For the protease assay, venom (20 µl) was added to 150 µl 2% casein (sodium salt, Sigma) solution in 0.25 M phosphate buffer, pH 7.75, and mixed in a shaking incubator at 37ºC and 125 rpm. After 120 min, all reactions were stopped by addition of 150 µl 5% trichloroacetic acid. Samples were then centrifuged for 5 min at 10,000 rpm to precipitate unreacted casein. Aliquots (200 µl) of supernatant were transferred to a UV microplate for spectrophotometric analysis at 280 nm. Tyrosine dilutions (0 to 1.25 mM) were used to create a standard curve by which tyrosine production could be determined, and results are calculated as nmol tyrosine/mg venom protein/h. Negative controls were done utilizing 0.85% saline without venom, and these blank values were subtracted from all experimental values.

Hyaluronidase activity was examined utilizing hyaluronic acid as substrate. In a microplate, 20 µl venom samples were added to 50 µl of 0.75 mg/ml human umbilical cord hyaluronic acid (Sigma) in 0.2 M sodium acetate buffer with 0.15 M NaCl. These samples were incubated at 37ºC and shaken every 60 s. After 60 min, all reactions were halted using 100 µl 2.5% cetyltrimethylammonium bromide in 2% NaOH. All
samples were then read spectrophotometrically on the plate reader at 400 nm. A standard curve containing hyaluronic acid dilutions (0 to 0.75 mg/ml) was used to determine activity. Bovine testes hyaluronidase (Sigma) was used as a positive control, and 0.85% NaCl without venom was used as a negative control. Saline blank values were subtracted from all others, and the results are reported as µg hyaluronic acid used/mg venom protein/h.

LAAO activity was determined using L-leucine as substrate. Briefly, 10 µl of 0.0075% horseradish peroxidase was mixed with 180 µl of 0.2 M triethanolamine buffer with 0.007% o-dianisidine and 0.1% L-leucine in a microplate. These mixtures were pre-incubated at 37°C in the plate reader for 5 min. At that time, 10 µl of venom sample were added to each well to initiate the reaction. Absorbances were read at 436 nm every 15 s for 45 min, and samples were shaken prior to each reading. Reaction velocities were calculated over the linear portion of each reaction and compared to a standard curve of hydrogen peroxide concentrations. *Crotalus atrox* LAAO (Sigma) was used as a positive control, 0.85% saline was used as a negative control, and results are reported as nM o-dianisidine reduced/ng venom protein/min.

PLA$_2$ activity was determined using a commercial kit (Cayman Chemical) in which activity is detected spectrophotometrically. In general, 5 µl of assay buffer, 10 µl of diluted venom (reconstituted in assay buffer), 10 µl of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), and 200 µl of diheptanoyl thio-phosphatidylcholine substrate were combined in a microplate well, and the rate of change was determined from 0.5 to 3 min at 414 nm. In the assay, PLA$_2$ from the venom removes a fatty acid chain from the substrate and half of the DTNB (now 5-thio-2-nitrobenzoic acid) takes its place. The remaining 5-thio-
2-nitrobenzoic acid is detected via spectrophotometer. The substrate provided in the kit was further diluted 1:3 in assay buffer (25 mM Tris-HCl, pH 7.5, with 10 mM CaCl₂, 100 mM KCl, and 0.3 mM Triton X-100), and venom was diluted to approximately 0.001 to 0.005 mg/ml prior to use in the assay. Positive controls were conducted using provided bee venom PLA₂, and negative controls used assay buffer instead of diluted venom.

For each enzyme assay, every individual animal’s venom was run in quadruplicate (except the PLA₂ assay, which was run in duplicate or triplicate), and the average of the replicates was used for data analysis. Protein concentration of each venom sample was determined using the Pierce bicinchoninic acid method with bovine serum albumin standards (Thermo Scientific). Differences in enzyme activities among populations were determined using analysis of variance (ANOVA) with Tukey’s post-hoc test and between sexes using two-tailed, unpaired t-tests. Examinations of correlation among different venom activities and demographic data were done utilizing regression analysis. To examine combined effects among the enzyme activities, a multivariate analysis of variance (MANOVA) was conducted with population as the independent variable.

Results

Electrophoresis

The samples run through sodium dodecyl sulfate polyacrylamide gel electrophoresis showed much similarity in protein banding pattern, at least in terms of the number of bands present (Figure 6-2). There is some obvious individual variation, both in terms of numbers of bands and intensity of specific bands. However, there is no readily apparent population-level pattern among these differences.
Protease

There was a significant difference in mean protease activity between the LS and PP populations, with the SHK population intermediate and not different from either of the other two (Figure 6-3; P < 0.001; one-way ANOVA with Tukey post-hoc test). There was no significant difference in protease activity between male and female snakes (P = 0.57), and there was no correlation between protease activity and either SVL (P = 0.46), mass (P = 0.79), or venom volume (P = 0.57).

Hyaluronidase

There were no significant differences in mean hyaluronidase activity among the three populations (Figure 6-4; P = 0.43). There was no significant difference in activity between the sexes (P = 0.06), although the data are approaching significance. Further, there was no correlation between hyaluronidase activity and either SVL (P = 0.47), mass (P = 0.40), or venom volume (P = 0.27).

L-Amino Acid Oxidase

SHK snakes showed elevated mean LAAO activity over both LS and PP snakes (Figure 6-5; P < 0.001; one-way ANOVA with Tukey post-hoc test). There was no significant difference in LAAO activity between male and female snakes (P = 0.87), and there was no correlation between LAAO activity and either SVL (P = 0.87), mass (P = 0.79), or venom volume (P = 0.15).

Phospholipase A₂

LS snakes showed a significantly elevated PLA₂ activity over PP snakes, with SHK snakes being intermediate (Figure 6-6; P < 0.001; one-way ANOVA with Tukey post-hoc test). There was no significant difference in PLA₂ activity between males and females.
(P = 0.92), and no correlation between PLA2 activity and either SVL (P = 0.13), mass (P = 0.12), or venom volume (P = 0.30).

**Venom Correlations**

To examine any potential correlations among the enzymes examined, pairwise regression analyses were done. For all pairs (protease vs. hyaluronidase, protease vs. LAAO, protease vs. PLA2, hyaluronidase vs. LAAO, hyaluronidase vs. PLA2, and LAAO vs. PLA2), there were no significant correlations (P = 0.16, 0.96, 0.28, 0.67, 0.77, and 1.0, respectively). Preliminary assumption testing for MANOVA analysis examined normality, linearity, outliers, homogeneity, and multicollinearity, with no serious violations found. There was a statistically significant difference among populations on the combined dependent variables (F(8,140) = 5.44; P < 0.001; Wilks’ Lambda = 0.58; partial $\eta^2 = 0.237$). When considered separately utilizing sequential Bonferroni corrections, the same patterns were found as with the ANOVA examinations described. Statistically significant differences among the populations were seen in protease (F(2,73) = 9.23; P < 0.001; partial $\eta^2 = 0.202$), LAAO (F(2,73) = 7.84; P = 0.001; partial $\eta^2 = 0.177$), and PLA2 (F(2,73) = 4.50; P = 0.014; partial $\eta^2 = 0.110$).

**Discussion**

The results indicate that there are some differences in venom composition among the different populations studied. In particular, the SHK population has a greater activity of LAAO than the other two, and intermediate activity for both protease and PLA2. This population is separated from the mainland by a salt water corridor that may effectively reduce migration to and from the island. Such separation may lead to differential rates of evolution when compared with snakes on the mainland, and this could be manifested as a phenotypic change in venom composition or activity. However, the results
presented here show an unclear picture of venom evolution and indicate that more than isolation may be involved.

The SHK population seems to be a good candidate for differences in venom not simply because of its insular habitat, but also because of its unique diet on the island. It would be logical to expect, if venom production is costly as indicated in previous studies (McCue, 2006), that snakes feeding on a ready supply of easy prey would eventually lose venom toxicity. Under such a scenario the cost of venom production would have to create a large enough drain on resources to make a difference in fitness. If, however, the abundance of prey offsets the energetic costs of venom production, venom would be less likely to take a directional evolutionary trajectory and may instead evolve simply due to stochastic factors (i.e. genetic drift).

One very interesting result of this study is the wide variation in activity found for all enzymes assayed. Not only is there a major overall variation, but there is also so much variation that any population-level differences may be obscured. This is interesting in that natural variation is important for natural selection to affect evolution. It is possible, however, that the wide diet seen normally in mainland snakes is subject to selection so that cottonmouths may switch prey species under suboptimal conditions. This might make even the island snakes evolutionarily predisposed to maintaining venom variation.

A further confounding factor for the SHK population is in their capacity as carrion feeders. While many snake species are known to consume dead prey opportunistically, the majority of the diet of SHK snakes is made up of carrion. Although individual snakes on this island have been seen to bite dead fish before consuming them, they also often simply swallow fish without envenomation. This is important because
besides incapacitation of prey, venom is likely used for aiding in digestion, especially of large prey items. If venom is not being used for either incapacitation or digestion, then it is even less likely to be under the influence of natural selection.

It is possible that venom toxicity could be maintained in SHK snakes due to long-term fluctuations in local climate. During periods of prolonged drought, when birds are less likely to nest successfully on the island, the snakes may have to rely on alternative prey such as introduced rats. Likewise, alternate prey such as lizards may be important for young snakes on the island, making toxicity useful.

This is the first known report examining individual variation within and among natural populations of the cottonmouth. Overall, it indicates that natural levels of venom variation are high. Although some differences in population-level venom enzyme activity were seen, caution must be used when interpreting these results, as many other ecological factors may be involved in shaping venom composition. This is a first step in examining venom evolution at the level at which evolution acts the most—the population.
Figure 6-1. Map of Florida with population locations indicated. The inset is an enlargement of the northern part of the state, and the populations are as follows: LS, Lower Suwannee National Wildlife Refuge; PP, Paynes Prairie Preserve State Park; and SHK, Seahorse Key. Maps were generated using Google Earth.
Figure 6-2. Electrophoresis banding patterns for individual cottonmouth venom samples. Individual samples were run on 12% acrylamide gels with 2-(N-morpholino)ethanesulfonic acid running buffer. Multiple individual samples from each population were used for comparison, including six animals from Big Cypress National Preserve. Relative molecular masses are given on the left side of each gel and are calculated from molecular ladders of known sizes. Possible venom components are bracketed and described on the right side of each gel. LS = Lower Suwannee National Wildlife Refuge and PP = Paynes Prairie Preserve State Park.
Figure 6-3. Comparison of mean protease activities for three populations of Florida cottonmouth. Enzyme assays utilized venom samples from individual snakes from three different geographic locations. SHK is Seahorse Key, LS is Lower Suwannee National Wildlife Refuge, and PP is Paynes Prairie Preserve State Park. Letters indicate significant groupings (P < 0.001) using a one-way ANOVA with Tukey post-hoc test. Activities are reported as nmol tyrosine produced per mg venom protein per hour, and error bars indicated ± 1 standard error.
Figure 6-4. Comparison of mean hyaluronidase activities for three populations of Florida cottonmouth. Enzyme assays utilized venom samples from individual snakes from three different geographic locations. SHK is Seahorse Key, LS is Lower Suwannee National Wildlife Refuge, and PP is Paynes Prairie Preserve State Park. No significant differences were seen among the populations using a one-way ANOVA with Tukey post-hoc test. Activities are reported as \( \mu g \) hyaluronic acid consumed per mg venom protein per hour, and error bars indicated ± 1 standard error.
Figure 6-5. Comparison of mean L-amino acid oxidase (LAAO) activities for three populations of Florida cottonmouth. Enzyme assays utilized venom samples from individual snakes from three different geographic locations. SHK is Seahorse Key, LS is Lower Suwannee National Wildlife Refuge, and PP is Paynes Prairie Preserve State Park. Letters indicate significant groupings (P < 0.001) using a one-way ANOVA with Tukey post-hoc test. Activities are reported as nM o-dianisidine reduced per mg venom protein per min, and error bars indicated ± 1 standard error.
Figure 6-6. Comparison of mean phospholipase A₂ activities for three populations of Florida cottonmouth. Enzyme assays utilized venom samples from individual snakes from three different geographic locations. SHK is Seahorse Key, LS is Lower Suwannee National Wildlife Refuge, and PP is Paynes Prairie Preserve State Park. Letters indicate significant groupings (P < 0.001) using a one-way ANOVA with Tukey post-hoc test. Activities are reported as nM o-dianisidine reduced per mg venom protein per min, and error bars indicated ± 1 standard error.
CHAPTER 7
A POPULATION-LEVEL COMPARISON OF THE DIET OF THE FLORIDA COTTONMOUTH, AGKISTRODON PISCIVORUS CONANTI

Introduction

The Florida cottonmouth (*Agkistrodon piscivorus conanti*) is known to consume various vertebrate prey species including fishes, amphibians, reptiles, birds, and mammals in the wild (Lillywhite and McCleary, 2008). It is also known to be a scavenger of both natural prey and human refuse (Wharton, 1969), and will consume many species in captivity that would not normally be experienced in the wild (Allen and Swindell, 1948). Many reports of ingested prey have focused on individual snakes, rather than on overall population-level prey base, with only one published study detailing the specific diet of a population from one location (Wharton, 1966, 1969). This study focused on the snakes of the island of Seahorse Key (SHK), located ~5 km from the western Florida coast and found that this population greatly utilized dead fish scavenged under colonies of nesting birds. No other cottonmouth population has been reported to have such a carrion-based diet.

Previous studies examining the relationship between diet and venom composition have indicated that venom is more tightly correlated with local prey type than with other parameters (Daltry et al., 1996; Creer et al., 2003). One other study found a different pattern, in which venom composition in snakes on islands was more closely associated with the time the island had been isolated from the mainland, rather than with diet (Williams et al., 1988). The relationships of the first two studies indicate that venom composition may be evolving via natural selection, but the last study indicates that genetic drift may be more important.
It is understandable that prey could be a major factor in the evolution of animals, especially venomous snakes. The venom must be efficacious enough to facilitate prey capture, so alterations in its composition could greatly enhance or hinder the ability of the species to feed. This, in turn, could lead to alterations in reproduction and abundance of the species.

In order to determine whether prey base may have an influence on phenotypic characters relating to venom composition, it is necessary to characterize diets of defined populations. As an attempt at this, cottonmouths from three different geographic regions were examined for their dietary preference. These populations included SHK and two mainland sites, Paynes Prairie Preserve State Park (PP) and Lower Suwannee National Wildlife Refuge (LS). PP is located in north central Florida and includes a grassland area with multi-year flooding cycles, numerous fresh water ponds and streams, and patchy forest. Lower Suwannee is located on the western coast of Florida, separated from SHK by salt water, and contains both fresh and salt water habitats. The differences in habitats may lead to differences in prey base, although both PP and LS are very heterogeneous in their habitat composition.

Prey determination can be difficult for many reasons. First, many animals are encountered because they are actively seeking food (Daltry et al., 1998), whereas recently fed animals would be more likely to take shelter to digest. Because of this, many collected snakes are likely to lack stomach and intestinal prey items. Second, in animals where food items are present, they are often found in a highly degraded state, making identification to any level difficult and to the species level impossible. Third, some animals are digested more quickly than others. This could lead to their reduced
presence in the intestines and, especially, their feces. The only way to realistically
determine all food items is through dissection of each individual. This practice is not
tenable in endangered species and is often not practical for non-endangered species. It
is, however, viable in dead animals.

With these limitations in mind, useful data on diet can be collected from animals
recently killed in the wild (by automobiles) or preserved in museum collections. Many
vertebrate body parts either are not digestible by snakes (mammalian hair, etc.) or are
resistant to digestion (large bones). In many cases, identification to the class level is
relatively easy, and for some well-preserved or unique features it may be possible to
identify the dietary organism to the species level. In some cases it may even be
possible to determine the number of prey ingested by the snake (such as when two
copies of the same bone are found). By utilizing metrics collected for museum research
collections, it may even be possible to determine the size of the prey in life. Overall, the
less destructive use of road-killed animals can be very informative.

Stable isotope analysis is often used to examine the flow of energy through
ecosystems (Post 2002) or the specific diets of certain species (Dalerum and
Angerbjörn, 2005). In order to determine the importance of specific prey species to a
predator, however, the specific isotopic signature must be determined for all potential
diet items. These can then be compared to the isotopic signatures of the predator to
look for similarities. Although not specific enough to completely determine diet, simple
analysis of stable isotope signatures of predators from different regions can give an
indication of their similarities in diet.
Materials and Methods

Diet items were identified from snakes from each of the populations by a combination of three different methods. First, fresh road-killed animals from the areas of interest were collected when possible and dissected (N = 33). Stomach and intestinal contents were collected and sorted in 70% ethanol. After isolating fragments of bone, scales, hair, feathers, or other identifiable anatomical parts (such as otoliths and lenses of fishes), each was identified to the lowest taxonomic level utilizing the zooarcheological research collection at the Florida Museum of Natural History (FLMNH) and through the resources of the Herpetology, Ichthyology, Ornithology, and Mammalogy sections of FLMNH. Second, the same basic procedure was used to identify fragments located in the gastrointestinal tract of specimens from the American Museum of Natural History (N = 1) and FLMNH (N = 13). Third, the feces of short-term captive live animals were analyzed in a similar fashion (N = 35). Of these animals, many (N = 17) did not contain diet items and so were removed from analysis.

Snakes were categorized in terms of type of prey from gut or fecal contents. Prey items were all identified to order, as this was the deepest taxonomic level possible in many cases due to degradation of the sample. The number of animals containing each order of vertebrate prey was determined for each population. These tallies were then compared against each other. As an example, a snake containing three fish would be included in the fish category once, and a snake containing a fish, a snake, and a lizard would be included in both the fish and squamate categories (once each). To boost the overall sample size, vertebrate prey data from Wharton (1969) were added to the analysis. Although Wharton (1969) reports the presence of invertebrates in the gut contents of cottonmouths, these were excluded from analysis. The reason for this is
that there have been no direct reports of cottonmouths consuming invertebrate prey, and many of the vertebrate prey they do consume (frogs, fish, lizards, etc.) tend to have their own gut contents when consumed by the snake. This means that undigested fragments of invertebrate exoskeletons may appear in the gut and fecal contents. In many cases, the invertebrate fragments are accompanied by parts of a potential prey species. Further, some of the invertebrates are very small, and would not be expected to be ingested by a cottonmouth.

To determine whether there were any significant differences in dietary composition, a $\chi^2$ test was performed. Expected values were obtained by adding all dietary numbers from all three populations together and determining a common proportion. Observed values used were from tabulated diet numbers from each population. These tests were performed on multiple data sets using different criteria including: 1) all new diet items from this study combined with data from Wharton (1969) ($N_{TOTAL} = 158; N_{SHK} = 110; N_{PP} = 27, N_{LS} = 21$); and 2) diet items only new to this study, from snakes over 500 mm snout to vent length (SVL) and 300 g ($N_{TOTAL} = 58, N_{SHK} = 16, N_{PP} = 23, N_{LS} = 19$). For all analyses, a post-hoc Monte Carlo simulation using 10000 random draws of N samples (dependent on population size for each analysis) was conducted using specific sample sizes, and the resulting P-values are indicated as $P_{MC}$.

A further preliminary analysis was done as part of a more extensive study (Lillywhite, unpublished) to compare the stable isotope signatures of animals from the three regions. This was done by using tissue from either scale clips or tail tips. These samples were prepared in a drying oven and analyzed by mass spectrometry. Samples
loaded into tin capsules were placed in a Carlo Erba NA1500 CNS elemental analyzer. They were combusted under oxygen-rich air in a quartz column at 1000°C, and the sample gas was transported in a helium carrier stream through a hot reduction column (650°C) consisting of elemental copper for oxygen removal. To remove water from the samples, the effluent stream was passed through a chemical trap. It was then passed into a ConFlo II preparation system and finally into a Finnigan-MAT 252 mass spectrometer running in continuous flow mode to measure sample gas relative to a laboratory reference gas. For this portion of the study, animals from SHK (N = 127) were of all sizes, while snakes from PP (N = 18) and LS (N = 20) were at least 300 g and 500 mm SVL. The stable isotopes examined were nitrogen-15 and carbon-13.

**Results**

The three main categories of vertebrate prey found were fish, squamates (lizards and snakes), and mammals, with birds being represented only in the SHK population. Because of this, analyses were run with the first three categories and a fourth “other” category that combined remaining items or with all four of these categories and a fifth “other” category. Besides these categories, amphibians (both urodele and anuran) and turtles were identified in gut contents. The relative proportions of the four-category totals can be seen in Figure 7-1, and of the five-category totals in Figure 7-2. These are both for the combined data set.

Using diet items determined from this study alone that met mass and SVL restrictions, there were no significant differences among the populations if the data were separated into 4 categories. However, when birds were considered as their own category, the SHK population was significantly different from expected dietary proportions ($\chi^2 = 11.51, df = 4, P = 0.0214, P_{MC} = 0.0234$). When the data from
Wharton (1969) and animals outside the strict criteria were used, there were significant differences among all three populations. This was true when the data was analyzed as four categories (SHK, $\chi^2 = 10.6$, df = 3, $P = 0.0141$, $P_{MC} = 0.0147$; PP, $\chi^2 = 15.78$, df = 3, $P = 0.0013$, $P_{MC} = 0.0021$; LS, $\chi^2 = 11.22$, df = 3, $P = 0.0106$, $P_{MC} = 0.0115$) and as five categories (SHK, $\chi^2 = 334.66$, df = 4, $P < 0.0001$, $P_{MC} < 0.0001$; PP, $\chi^2 = 30.67$, df = 4, $P < 0.0001$, $P_{MC} = 0.0001$; LS, $\chi^2 = 13.96$, df = 4, $P = 0.0074$, $P_{MC} = 0.0087$).

The preliminary stable isotope analysis can be seen in Figure 7-3. For these data, the two analyzed isotopes are plotted against each other. The figure indicates a difference in the SHK population isotopic signature compared to the other two sites.

**Discussion**

Although there was an apparent difference among the diets of the adult snakes analyzed specifically for this study, there were no statistical differences between observed and expected values (with the exception of SHK when utilizing 5 prey categories). It is likely that this is due to the small sample size caused by strict limitations on included samples, as inclusion of smaller individuals and data from Wharton (1969) yielded significant differences in dietary structure among all populations, whether using 4 or 5 categories of data.

The significant differences found when Wharton’s data was included must be viewed carefully, because it is possible that the large sample size for one population skews the overall expected values of the other populations. At the same time, however, the majority of prey items from SHK were fish, whereas they accounted for 38% of items from LS and only 10.3% of items from PP. These values indicate a real difference in fish consumption among the populations. Other prey categories, specifically
squamates, also are widely divergent among the populations, indicating a real
difference in either availability or utilization of prey in the different regions.

The stable isotope analysis indicates that these two particular isotopes are being
incorporated from different sources, with SHK being distinct from the two mainland
populations. This does not necessarily mean different prey species, however, as
isotopes are transferred up trophic levels and may be altered by both lower level
consumer and primary producer species (Post, 2002). The clear delineation between
the SHK and mainland animals is interesting nonetheless.

On SHK, snakes consume dead fish dropped by seabirds, but scavenging was
also seen in the mainland populations. This scavenging was indicated by both the
presence of larvae of carrion-consuming invertebrates and the general condition of
some prey items. As an example, two road-killed snakes from the PP population were
found to contain whole frogs that appeared to have first also been killed on the road, as
they were in a mangled condition. Further, a specimen from the LS population
contained a lizard that was in multiple pieces but only slightly digested. As
cottonmouths do not tear apart their prey, this indicated the lizard was dead prior to
ingestion by the snake. All of this points to the general opportunistic nature of the
cottonmouth.

Although it has previously been indicated that amphibians may be
underrepresented in snake gut and fecal contents due to their ease of digestion (Daltry
et al., 1998), it is notable that many amphibian fragments were identified in fecal
samples from live snakes. It is possible that the stress from manipulation of live snakes
decreases their gut passage time, allowing for less degraded samples in the feces.
However, this does indicate that amphibian structures can be found in feces of the snakes that consume them.

Road-killed animals were useful in examining population-specific diets of the cottonmouth. Although they may be less likely to contain prey in their gastrointestinal tracts, they did yield useful information. It would be of interest to examine the relative digestibility of various prey species in the laboratory to determine whether different prey items are consistently detectable.

Overall, this study adds to the understanding of the dietary habits of the Florida cottonmouth and supports the rather unique feeding style of the population on SHK. It supports the idea that cottonmouths are opportunistic feeders, as evidenced by scavenging and a wide array of prey species. It further indicates that these characteristics may allow the cottonmouth to adapt quickly to different ecosystems. This, in turn, may help explain the presence and relative abundance of the Florida cottonmouth throughout its range.
Figure 7-1. Four-category diet composition comparison for three populations of Florida cottonmouth. The “other” category includes amphibians, turtles, and birds. Charts made only from new data from snakes over 300 g are on the left, while previously published data and smaller animals are included on the right. SHK indicates the Seahorse Key population, PP is the Paynes Prairie population, and LS is the Lower Suwannee population. The populations in the “All” column were significantly different from expected values using a $\chi^2$ test ($P < 0.02$ for all).
Figure 7-2. Five-category diet composition comparison for three populations of Florida cottonmouth. The “other” category includes amphibians and turtles. Charts made only from new data from snakes over 300 g are on the left, while previously published data and smaller animals are included on the right. SHK indicates the Seahorse Key population, PP is the Paynes Prairie population, and LS is the Lower Suwannee population. SHK from the “New only” column (P < 0.03) and all populations in the “All” column (P < 0.01 for all) were significantly different from expected values using a \( \chi^2 \) test.
Figure 7-3. Stable isotope analysis of scale tissue from Florida cottonmouths. Samples were collected from three different populations (SHK = Seahorse Key, PP = Paynes Prairie, and LS = Lower Suwannee).
CHAPTER 8
EVOLUTIONARY IMPLICATIONS AND SUMMARY

Introduction

Previous studies have concluded that the phenotypic character of venom composition is evolving under the influence of natural selection (Daltry et al., 1996b; Creer et al., 2003) or genetic drift (Williams et al., 1988). In an attempt to test these hypotheses, I examined the genetic relationships among four populations of the Florida cottonmouth, *Agkistrodon piscivorus conanti*, and further compared venom enzyme activities in three of those populations.

As a first step, it was necessary to select venom characteristics that were known to have fairly high variation, as lack of variation would be more likely to simply lead to similar results among populations. Although much work has been done on the effects of cottonmouth envenomation and the composition and activity of cottonmouth venom, a review of the literature showed some differing results. Among the studies examining enzymatic activities, Tan and Ponnudurai (1988, 1990) specifically studied the effects of cottonmouth venom, and they found great variation in a number of enzymes. However, they did not examine individuals, but rather pools of venom. In order to get at differences among populations, it is necessary to look at phenotypic characters of individuals within those populations.

It was also necessary to devise a technique by which venom could be collected in a standard fashion. Previous published techniques of venom extraction were developed either to produce as much venom as possible or a sample of an arbitrary size for quick studies, and many either did not work with cottonmouths specifically or were not practical. Using a combination of previously utilized techniques, but with more updated
methods of anesthesia and electrical stimulation, we were able to increase safety for humans involved in the study, reduce stress on the animals, and collect venom in a standard and repeatable fashion.

After determining the manner of venom collection and the specific components of venom to be studied, we needed to find a method by which the relationships among the snake populations could be determined. Previous work utilizing mitochondrial DNA (Parkinson et al., 2000; Guiher and Burbrink, 2008) or assorted fragment length polymorphisms either focused on subspecies-level relationships or were unsuccessful at verifying population-level identity (Roark, 2006). Although no microsatellite loci had been developed specifically for use in the cottonmouth, they are powerful markers for discerning fine-scale population structure. We screened microsatellite loci developed in closely related genera of snakes, and although many either did not amplify a product in cottonmouths or did not show any variation, we found ten useful markers that did both.

After collecting 129 DNA samples from both live and road-killed snakes, we genotyped them using the ten microsatellite loci. Multiple analyses of these samples indicate that our four populations of interest are genetically isolated from each other to a significant extent. Our insular population, found on the island of Seahorse Key (SHK), was found to be the most isolated of the populations, regardless of its proximity to the Lower Suwannee National Wildlife Refuge (LS) population. SHK was also found to have indications of inbreeding within its population. Further, the LS population was less related to SHK than it was to the distant Paynes Prairie Preserve State Park (PP) population or the far distant Big Cypress National Preserve (BC) population.
For three of these populations, we collected venom samples. Unfortunately, the BC population was much more scattered than the others, and several searching trips yielded too few live animals (N = 6) to make a statistically sound comparison. Using the other three populations, however, we did find significant differences in three of the four venom enzymes studied. Protease and phospholipases A₂ (PLA₂) activities were found to be significantly different between the LS (highest) and PP (lowest) populations, with SHK being intermediate; hyaluronidase activity was not found to be significantly different among any of the populations; and L-amino acid oxidase (LAAO) activity was found to be significantly elevated in the SHK population.

There are significant differences in the activities of the venom of the three populations studied, but it is difficult to determine whether these are biologically meaningful. Although it seems possible that the SHK snakes are feeding upon a prey type that is more susceptible to a high LAAO activity, the dependence of these snakes upon carrion fish makes that seem unlikely. One previous study indicated that LAAO might help potentiate proteases, which could be useful with a high-protein fish diet. It may be just as possible that snakes on SHK are not under the same types of survival pressures as the mainland populations. SHK has a seasonal abundance of food and few potential snake predators outside of some diurnal birds (cannibalism notwithstanding). It is possible that LAAO is energetically expensive to make (but highly useful) so that the island snakes are not affected to the same extent that mainland snakes would be.

The instance of higher protease activity in the venom of LS snakes could be related to the specifics of their environment. Unlike SHK, LS has very diverse
substructure within its overall environment. This leads to a more diverse range of potential prey, so it may be more useful for snakes to have varied venom. Because protease activity is a general assay of multiple proteolytic enzymes, it is impossible to say exactly which proteases are increased in LS venom, or whether it may be all of them. Proteases are responsible for overall degradation of protein structures in the prey, so it is possible that LS snakes are consuming high-protein food items, such as fish, and that the elevated protease is simply an evolutionary response to enhance digestion. The presence of brackish water in this system makes the prevalence of fish prey seem a little greater, although there are many other potential prey species in this habitat.

It is difficult to draw conclusions concerning the PP population and its lower protease and LAAO activities. However, this is the only population studied that is in a purely fresh water environment. It could be that none of the enzymes studied are necessary in high amounts for incapacitation and digestion of prey in the prairie and fresh water swamp setting.

The similarity in hyaluronidase activity among the populations indicates that it is likely not a major factor in the biological activity of the venom, at least in terms of differential reproductive success. Hyaluronidase acts by degrading hyaluronic acid between cells to decrease their function as barriers. This allows the other venom components to slip through tissue layers and possibly become systemic. It also allows enzymes to spread, potentiating digestive enzyme effects. It may be that this enzyme functions more in a digestive capacity after the death of a prey animal than in aiding in
incapacitation. It could be that higher activities do not yield greater results, so elevation of this component costs energy without any added benefits.

Differences in PLA$_2$ activity among the populations also do not indicate a clear pattern. The same pattern found with protease was found with PLA$_2$, in that LS animals had higher activity than PP animals, with SHK snakes intermediate. PLA$_2$ is used, in general, to degrade cell membranes, but it does have very different functions with minor structural changes. Any changes in function would not be detected with the assay utilized in this study. It is possible that the prey being consumed by LS snakes is more susceptible to PLA$_2$ activity.

The difference in prey base is likely at the root of the differences in venom composition among the populations studied. Dietary analysis indicated that the prey base was different for each population, so venom composition may be evolving activity toward certain prey species. However, unlike the Daltry et al. (1996b) study, it may not be the specific prey items, but rather the overall amount of food energy available that is important. If food is limited, venom is expected to evolve toward greater incapacitation of available prey. If, however, food is not the limiting factor, venom may simply evolve in a random fashion (as was indicated by Williams et al., 1988). This leads to the question of whether insular populations are generally under different evolutionary constraints than mainland populations.

**Quantitative Trait Loci**

Although there is seemingly no clear-cut answer to the question of how venom is evolving in these populations, there is evidence that prey may be important. The populations of interest are genetically distinct, have overall venom activity that is different, and have differences in diet composition. It can not be stated definitively that
prey base differences are causing the observed venom activity differences, but it is possible to compare venom differences with genetic differences.

Quantitative traits are those phenotypic characters that are influenced by multiple genes and have a continuous distribution over their range of potential values, such as the venom activities examined in this study. They differ from discrete traits that are controlled by one gene, and are instead influenced by a suite of genes called quantitative trait loci (QTLs). These loci may be strongly influenced by environment. Recently, quantitative traits have been used to determine quantitative genetic differentiation, and these data have been compared to neutral marker gene differentiation. Quantitative genetic differentiation can be calculated as a $Q_{ST}$ value that considers the both within and between population variances in a phenotypic character. The $Q_{ST}$ is analogous to the $F_{ST}$ statistic calculated for neutral genetic traits. In the case of the $F_{ST}$, heterozygosities are calculated to determine the relative importance of variance between populations compared to the species as a whole.

Comparisons of $Q_{ST}$s and $F_{ST}$s values have been popular recently for inferring the effect of natural selection on quantitative traits (Leinonen et al., 2008). Lande (1992) considered that if $Q_{ST}$ and $F_{ST}$ values were similar, then there was no selection on the quantitative trait in question, and Yang et al. (1996) indicated that in these cases genetic drift can explain any differentiation. Merilä and Crnokrak (2001) published a model of $Q_{ST}/F_{ST}$ proportions that considered values near 1.0 to be indicative of drift alone, values above 1.0 to be indicative of diversifying selection, and values below 1.0 to indicate stabilizing selection. Although there is an assumption of heritability when using $Q_{ST}$s, it is often not possible to determine whether traits truly are heritable. The
Florida cottonmouth is not a model organism for these types of studies because of their relatively late reproductive age and two- to three-year reproductive cycles. However, it is still worthwhile to compare $Q_{ST}$s and $F_{ST}$s to get an indication of the potential importance of natural selection on enzyme characteristics.

Pairwise $F_{ST}$ values were determined for each pair of populations using Structure 2.2, and $Q_{ST}$ values were determined by calculating within and between population variances in enzyme activity for each venom enzyme and each pair of populations. The $Q_{ST}/F_{ST}$ proportions indicate that most of the enzyme activities are under diversifying selection among all population comparisons (Figure 8-1). The exceptions to this trend are two comparisons (SHK to LS and SHK to PP) of hyaluronidase activity. These values indicate that selection has less of an impact on values than does genetic drift.

Interpretation of these data must be considered carefully, however. Whitlock (1999) stated that diversifying selection in newly formed populations may not be detectable simply because too little time may have passed to increase $Q_{ST}$ values, even when populations truly are diversifying. This indicates that these calculations are too conservative to detect new species. On the other hand, Hendry (2002) indicated that it is more difficult to detect diversifying selection in populations that have been isolated for long periods of time. As populations diverge, the $F_{ST}$ value increases towards 1.0, which would obscure any large values of $Q_{ST}$. In these cases, diversifying selection would not be detected. At that point, however, it could be considered that the populations belong to different, distinct species.

**Conclusion**

The three populations of Florida cottonmouth utilized in this study were found to be genetically distinct from one another using microsatellite loci. These same populations
were found to have differences in activities of three of four venom enzymes. Further, the populations were found to have differences in prey type found in gut and fecal contents. Correlative comparisons of enzyme activity and neutral genetic markers indicate that the enzymatic activities are (for the most part) undergoing diversifying selection. Together, these data point to a divergence of venom activity and genetic relatedness that is at least correlated with prey differences, although diet can not definitively be considered to be driving this divergence.

Since we now have the means to collect cottonmouth venom in a standard fashion, and also can determine genetic relationships among these snakes using a powerful set of microsatellite loci, getting at the essence of the relative importance of natural selection in venom evolution should be easier. Without fully understanding the components present in the venom and their specific physiological action on prey, it is more difficult, so further work is needed in these areas. Although it is also difficult to catalogue the relative importance of different prey species in such a generalist feeder, diet breadth will also be important for future work. We now also have an understanding of the natural variation in activity of four enzymes, and this may lead to discovery of novel venom components that may be useful not only for studies of evolution, but also for practical use in antivenom therapy or pharmaceutical development.
Figure 8-1. $Q_{ST}/F_{ST}$ comparison of enzyme activities for each of three populations of Florida cottonmouths. Pairwise $Q_{ST}$ values were calculated as described in the text and pairwise $F_{ST}$ values were determined using STRUCTURE 2.2. A 1:1 line is included for reference. Using the model of Merilä and Crnokrak (2001), values above the line indicate characters undergoing diversifying selection, values on or near the line indicate characters not undergoing selection (but rather drift), and values below the line indicate characters undergoing uniform or stabilizing selection.
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BIOGRAPHICAL SKETCH

Ryan J. R. McCleary began a fascination with reptiles and amphibians at an early age when he lived near herpetologist Dr. James Gillingham. As a high school student, he was greatly influenced by his biology teacher, Mr. Steve Robbins, who encouraged him to pursue his reptilian interests. He graduated from Western Michigan University in 1993 with an undergraduate degree in biology and from Virginia Tech in 2001 with a master’s degree in the same field. At Virginia Tech, he worked with Dr. F. M. Anne McNabb examining the effects of pollutant chemicals on endocrine function in birds. During his tenure as a student, he has completed a summer natural history internship at the National Museum of Natural History (Smithsonian Institution) in Washington, DC, under the supervisor of Dr. Roy W. McDiarmid and a summer fellowship at the Lincoln Park Zoo in Chicago, IL. He has also conducted research in the Bahamas, Tobago, and Taiwan as well as parts of Florida. Recently, he completed the National Science Foundation East Asia Pacific Summer Institute program in Singapore.