

# *Occasional Papers of the Cape Fear Serpentarium*

## Ontogeny of the Bushmaster Shock Death\*

by Dean Ripa © 2007



**Figure 1.** At 9-months old this South American bushmaster (*Lachesis muta*) is still a fairly small snake, less than 1 meter long and a slender 450 grams in weight—about the size of an adult American copperhead (*Agkistrodon contortrix*). It has even less available venom than a copperhead of that size, about 0.2 cc (or 40 mg when dried). It would probably not inject more than a third of this supply even in a full bite. Yet its bite can produce grave effects in minutes! The lethal dose of bushmaster venom for man is therefore quite small (Chapters 25 - 26 review the probabilities). Note the enormous fangs of this little snake, already as long as those of an adult *Crotalus adamanteus* outweighing it by 10 times! Photo Regina Ripa. Cape Fear Serpentarium.

THE VISCOUS, YELLOWISH proteinaceous secretion of the relatively small serous glands of *Lachesis* species gives no hint in appearance of the dissimilar and perhaps much more dangerous properties it presents upon comparison with the also thick, yellowish, serous secretions of other vipers. Most viperine venoms kill or cause harm by disrupting normal hemostasis, and are lethal to man in several consistent ways: through interference with coagulation (e.g., hemorrhagic syndromes that produce free bleeding), vascular thrombosis (leading to cellular anoxia), tissue hydrolysis, and other predominately localized effects. These advance slowly, requiring a period of time before overwhelming a large animal like a human being.

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The story of bushmaster venom till now has been almost entirely of these, as it were, “digestive” components; being all those agents responsible for the breaking down of the blood and tissue of prey, to prepare it for gastric consumption. Hypotheses of lethality in man rest largely on the venom’s fibrinogen-clotting and hemorrhagic effects (though occasionally reported neurotoxicity; e.g., Magalhaes et al., 2003). Those agents in bushmaster venom that digest the prey are implicated as the thing that kills the human being. As noted in Estavao-Costa et al. (2000), “features of bushmaster envenomation are serious hemorrhage, blood coagulation disorders, and renal failure.” While no doubt these factors have occurred clinically (and geographic variation in these and all snake venoms makes broad statements incautious), they are not the first order of importance when treating bushmaster bite. For example, death from renal failure is a process of several days; and even bleeding

to death from hemorrhaged blood vessels and incoagulable blood is no quick development. In short, these are not rapid acting effects, and require time to work their destructive magic on the cardiovascular system. Bushmaster bite needs none of this—it can drop blood pressure to nothing in minutes.

It is true that a number of bushmaster fatalities have required such an appropriate time period as proteolytic effects can produce. Bolaños (1982) records 4 such deaths, and Hardy and Silva Haad (1997) yet another. These cases present the classically deranged coagulation of many other *Crotalid* bites. When these cases are actually examined, however, the cause of death cannot be ascribed to these factors specifically. Without exception the victims died from shock, not hemorrhage. It is given that the act of bleeding to death can, or should, produce shock; after all the means are not dissimilar; both involve vascular depressurization. However, these kinds of cases should usually exhibit some form of organ damage (from hemorrhage) as well, and this, apparently, was not the problem: shock was. Thus we see an etiological variation through means that are not primarily proteolytic in kind.

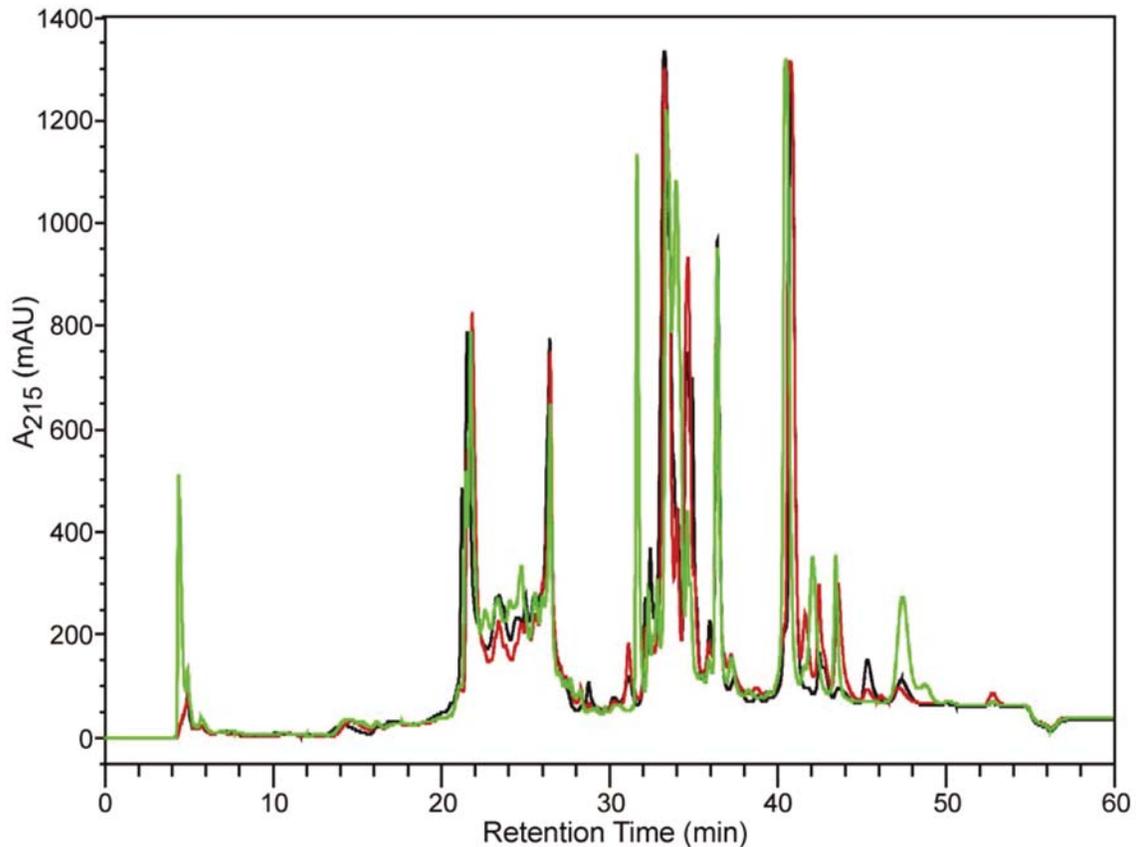
The lethal action of bushmaster venom in man is due to its ability to produce a rapid and fatal “shock syndrome” resulting in an irreversible hypotension. This shock-state (often called hypovolemia) proceeds from origins distinct, say, from those multifactorial processes seen in the *Bothrops* bite, where the patient may already have been severely bleeding for several days, and whose constitution is worn away by necrosis and infection, etc.; or from the *Crotalus* bite, where again, hemodynamic alterations affecting the integrity of the tissue produce a gradual degradation to the whole system that may end in a fatal shock state. This is not to say that bushmaster venom does not also do these things, but they appear to be less profound and are not the first order of emergency in treatment. To date, no bushmaster bite victim—at least no victim who has received enough antivenom—has succumbed to the bleeding disorders common to envenomings of conspecifics like *Bothrops*. On the contrary, bushmaster bite victims die unanimously from shock, and often in spite of prompt antivenom treatment.

Clinical reports of bushmaster envenoming describe edema (believed caused by plasminogen activation; e.g., Sanchez et al., 2002), inflammatory action (caused by numerous agents including serine proteinases, phospholipase A<sub>2</sub> [PLA<sub>2</sub>], metalloproteinases, serotonin, nitric oxide, histamine, etc., some of these by-products of the victim’s own metabolism and immune system; e.g., Silva et al., 1985); hemorrhage (due to metalloproteinases which degrade the capillaries; e.g., Rucavado et al., 1999); hemolysis (mediated by lectin, therefore disruption of the cell wall is not direct in kind; e.g., Silva Haad, 1981; Otero et al., 1998); myotoxicity (applicable to the muscle only in direct intramuscular inoculation and believed due to the action of specialized enzymes and proteinases in conjunction with leukocytes and macrophages infiltrated by the inflammatory response; e.g., Otero et al., 1998; Fuly et al., 2000); incoagulability (from defibrinating activity due to effects of thrombin on fibrinogen, with enzymatic activity like PLA<sub>2</sub> and proteinases preventing blood-clotting (e.g., Yarleque et al. 1989); coagulant activity (resulting in clots obstructing capillary blood flow; e.g., Magalhaes and Diniz, 1979; but I know of no directly attributable case of this happening); proteolytic activity (specifically the attack of thrombin-like proteases, metalloproteinases, cytolytic and myotoxic [e.g., Otero et al. 1998], rarely seen with prompt antivenom [Ripa, 1999]); neurotoxicity (affecting the Vagus, 10th cranial nerve, disrupting the muscles of speech and swallowing [dysphagia and dysphonia], interrupting transmissions to the heart and smooth muscles of the visceral organs (Fan and Cardoso, 1995); this, further exaggerated by shock, results in convulsive abdominal pain, projective vomiting and diarrhea; e.g., Ripa, 1999, 2002); and finally, most importantly, hypotensive shock itself (potentiated by kininogen and kallikrein-like proteinase, releasing bradykinin and kallikrein, inspiring rapid hypotension; e.g., Silva, 1981; Diniz and Oliveira, 1992; Felicori et al., 2003).

It is the latter, almost invariably, that kills the victim. This occurs in a quick fashion (within the first 24 hours) as a seemingly “autopharmacological response” (Ripa, 1999) or over the course of about four days, where its evolution would seem little mediated by antivenom. Thus I have posed two fatal outcomes in bushmaster envenoming, one *the rapid* (based on a sudden shock-syndrome) and the other *the delayed*, which may be a process of these, and other, multiplying factors.\*

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\* It may not be a coincidence that serum sickness also commences on about the 3rd or 4th day after antivenom, an additional burden on the patient. It is an intriguing premise, though not a necessary one, to think that two similar but distinct pathologies may be working on the patient simultaneously at this critical time!

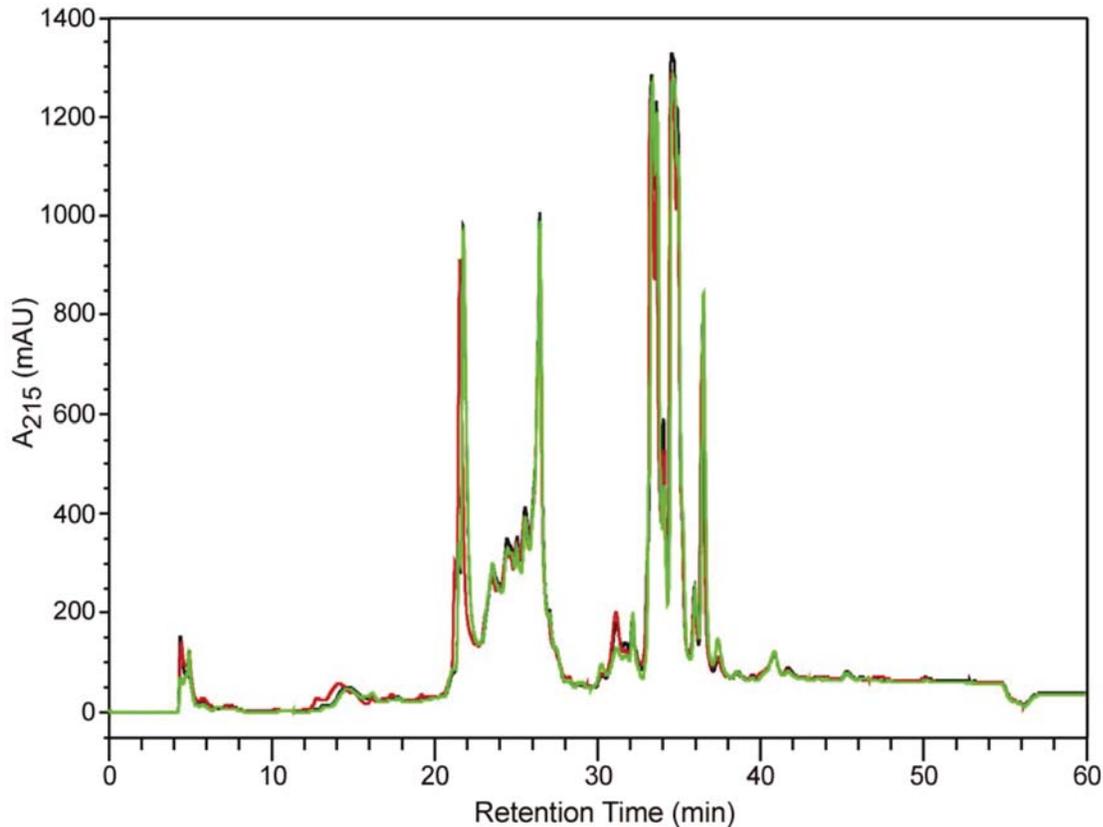


**Figure 2. Illus. 1.** HPLC chromatogram of venoms from different species of Bushmaster snakes. Venom from *Lachesis melanocephala* is in black. Venom from *Lachesis stenophrys* is in red. Venom from *Lachesis muta muta* is in green. One milligram of venom was loaded onto the column in each case. From Ripa, Boomershine and Ripa (in prep); HPLC by Will Boomershine.

Whether occurring “within minutes” as Ditmars reported, or within hours (see newspaper account in Chapter 23), or on the third and fourth day (as reported in Bolaños, 1982) these are most certainly shock-deaths, hypovolemic and/or vasodilative in kind. Abundant explanations can be proposed: diminished fluid volume resulting from excessive fluid demands in other parts of the body, inhibiting the heart’s ability to move the blood (e.g., due to the rapidly expanding edema of the bitten extremity; or from a less centralized vascular dilation also leeching blood from the heart), or the main arteries of the heart itself may be pooling blood. Hence the increased heart rate within the first few minutes of bushmaster bite (Ripa, 1999; Chapter 22) is probably attempt by the heart to compensate, with the subsequent slowing of the heart (as the pulse falls and grows faint) from blood deprivation, from having less and less blood to beat. The result is the “*Lachesis Syndrome*,” the fatal shock-state typical of bushmaster bite. There are other factors of relative significance, such as hemorrhage, also bringing blood loss, and hemolysis, which, of course, effects the very integrity of the blood itself. Finally (and this must be considered very significant in

the cases so far seen) we have the type of medical treatment, which may include surgery (fasciotomy). The latter is a sure recipe for a funeral. While common sense would tell you that the last thing you should do is operate on somebody who is bleeding to death, this has not stopped many doctors so far. In such cases (e.g., Bolaños, 1982) we have seen previously managed shock-states return and, with redoubled strength in the weakened victim, end fatally.

Most snake envenomings respond well enough to immunotherapy that you start to see some good results soon after administering it. The complex processes of hemorrhage, hemolysis, neurotoxicity, etc., cease and in little while the body begins restoring itself. Not so with the “L-syndrome,” which, like an exploded bomb keeps on spreading shrapnel from its own inertial thrust. The fact of defusing “the bomb” (with immunotherapy) does not soon reverse the effects of the explosion which continues its cascade. Other mediations must be attempted. As with catastrophic necrosis (where the most minute sums of certain venoms, though early neutralized, tricks the



**Figure 3. Illus. 2.** HPLC chromatogram of venoms obtained from specimens of *Lachesis melanocephala* of different ages. Venom from 2 week old specimens is in black. Venom from 4 week old specimens is in red. Venom from 3 month old specimens is in green. One milligram of venom was loaded onto the column in each case. From Ripa, Boomershine and Ripa, (in prep); HPLC by Will Boomershine.

immune system into mimicking the chemical invader in its effort to repel it and ends up murdering its own cells), the L-syndrome is self-perpetuating, because the body itself has been recruited to enact the deadly program. The venom, which originated the process, has ceased being the active agent and neutralization of its chemistry will not certainly alter the paradigm. If surgery is attempted during this critical period the effects will be enhanced, and even if having abated, will probably return full on and the patient will die. In modern cases of bushmaster bite, most fatalities that have been recorded have involved surgery (Chapter 23).

The envenomings of Gaboon viper (*Bitis gabonica*) are brought to mind. This is another species whose venom produces a quick and deadly shock effect in human beings. Mallow et al. (2003) summarizes from Grasset and Goldstein (1947), and Gatullo et al. (1983): “Abrupt hypotension develops *within seconds* in i.v.-injected anesthetized dogs. Profound peripheral vasodilation and reduced aortic impedance

cause an increase in stroke volume, thereby increasing ventricular discharge.”

In bushmaster bite I have insisted that the first course of treatment should be to address the “shock effect,” even to the detriment of restoring normal coagulation if these means are not available; and by all means to avoid invasive means to correct edema (e.g., fasciotomy) and necrosis, as these efforts will only enhance and prolong the shock state. If the patient is going to perish from a bushmaster bite, it is the shock that is going to do it, and hemostatic changes, while occurring in consort, are by comparison a less urgent problem. This is quite unlike the sympatric *Bothrops* with which bushmaster bite is often confused.

What is the chemical action of the L-syndrome? Previous studies (e.g., Felicori et al, 2003) reveal significant levels of kallikrein-like proteinase and kininogenin, both bradykinin releasers, in bushmaster venom. Bradykinin has been linked repeatedly to hypotension in other snakebites, and Silva, on the basis of symptoms, made the correlation with *Lachesis* more

than twenty-five years ago (Silva, 1981). My analysis (from Ripa, Boomershine and Ripa, in prep) confirms the presence of these releasers in three species of bushmaster; however, it reveals something entirely new. The development of these toxins is ontogenic, their concentration and proportion overwhelmingly concurrent with the snake's age.

What is most lethal in bushmaster venom has so far been misinterpreted in test animals as a proteolytic (digestive) component, agents that are not very potent in bushmaster venom. On this basis, neonate bushmasters were previously considered by some authorities (e.g., Gutiérrez et al., 1990) to be "not very dangerous." Indeed, my analysis adjourns with quite the opposite conclusion. What is most surprising is the disproportion of the shock-producing agents in the venom of the neonate, more than double that of the adult.

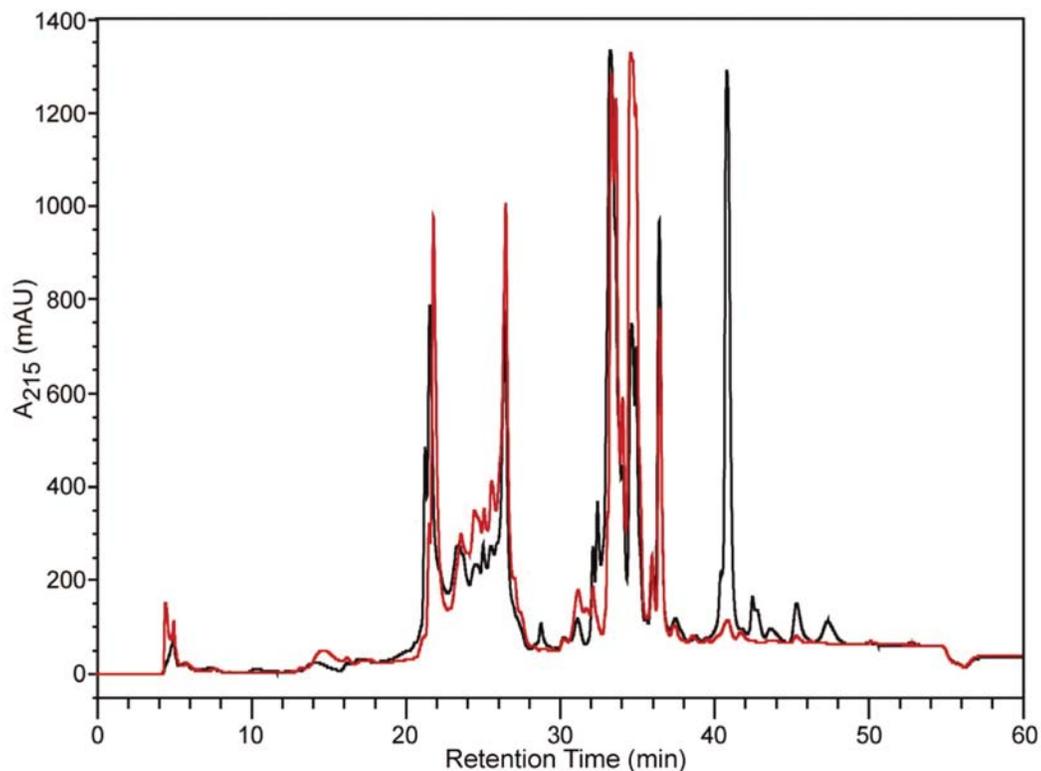
Gutiérrez et al. (1990) noted a curious absence of "toxic" agents in the venom neonate *Lachesis stenophrys*, emphasizing the low hemorrhagic, myonecrotic, and inflammatory effects of that venom (Chapter 21 discusses this data further). Believing

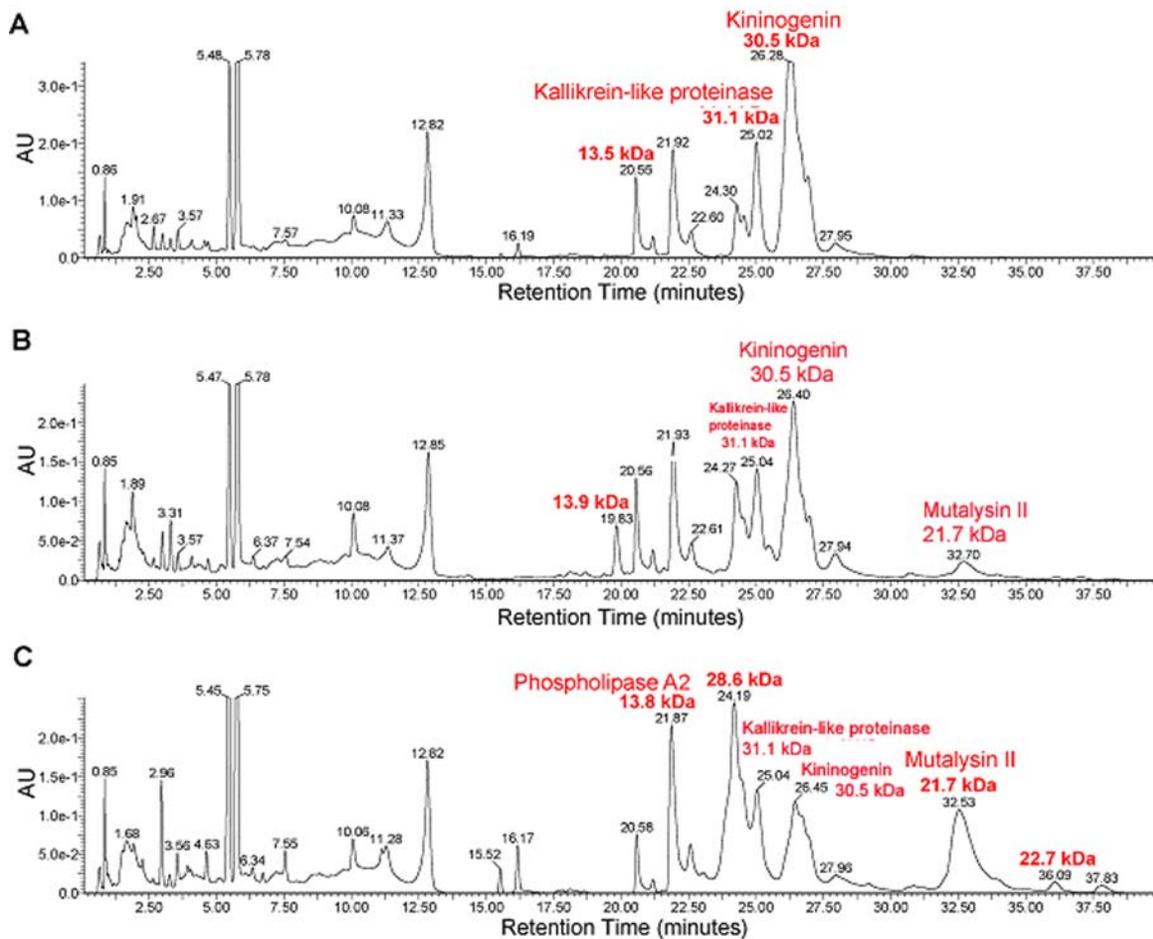
these actions to be the explicitly lethal effects of the venom ensemble, and noting their greater lack in the neonate, they concluded that the neonate needed to mechanically overpower its prey (e.g., restraint and constriction in the jaws) and that its venom must not be very dangerous to man. The problem with their model was in the definition of toxicity, or rather the kind of toxicity they analyzed, which did not account for an L-syndrome-like effect. In their model, bushmaster venom gained in toxicity as the snakes reached maturity, but when the snake was a baby it was hardly venomous at all.

The data assembled in Chapter 21 show how untrue this idea is. Newborn bushmasters efficiently dispatched their prey through the use of venom alone, and in a rapid time-frame. Mechanical restraint of the prey was unnecessary. The prey died almost equally rapidly when strike-released.

The dichotomy is this: Gutiérrez et al. (1990) correctly adduces the low blood/tissue destructive effects (e.g., the hemorrhage, necrosis, etc., of classic viperine envenomation) of the baby venom in prey; but did not recognize that these are not the effects that actually kill the prey. Nor are these the effects

**Figure 4. Illus 3.** HPLC chromatogram of venoms from specimens of *Lachesis melanocephala* of different ages. Venom from adult specimens is in black. Venom from two-week old specimens is in red. One milligram of venom was loaded onto the column in each case. From Ripa, Boomershine and Ripa (in prep); HPLC by Will Boomershine.



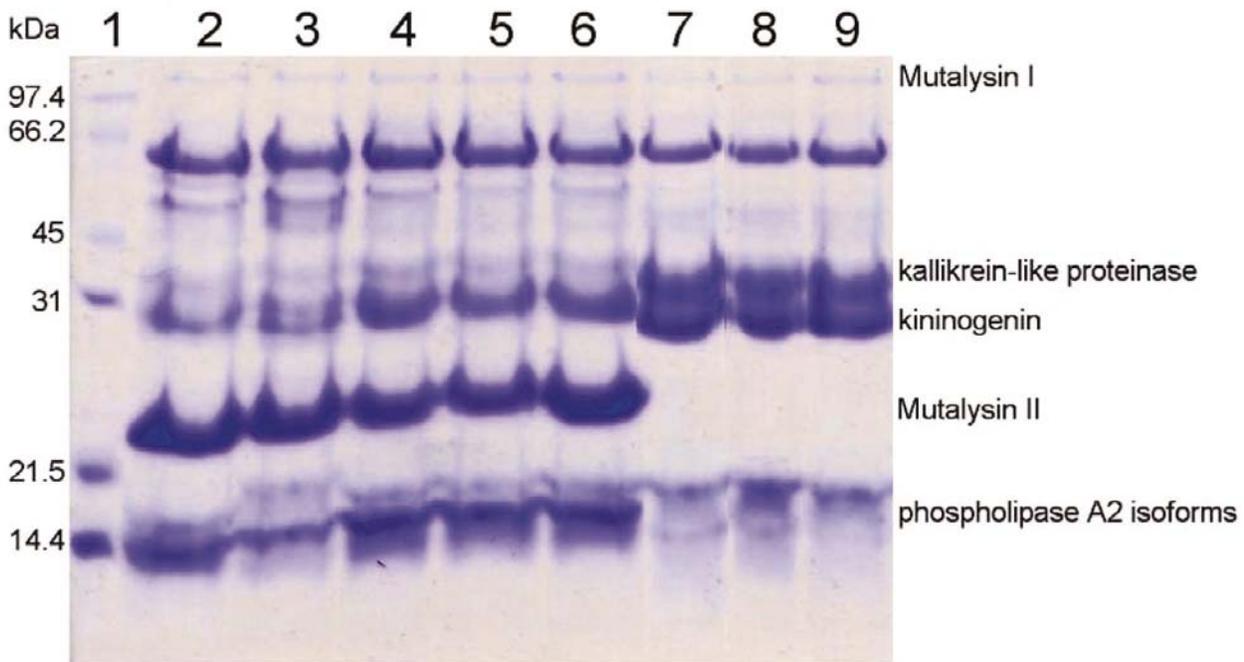


**Figure 5. Illus. 4.** UPLC chromatograms of venom from *L. melanocephala* of various ages. A is neonate, B is 10 month old juvenile, and C is mature adult. Small black numbers indicate retention time in minutes. Red labels indicate the molecular weight of protein in that peak as determined by electrospray-time-of-flight (ToF) mass spectrometry and in some cases indicate the identity of the protein based on molecular weight. Peaks labeled in red indicate that this age of snake contains more of this component of venom than other ages. Note preponderance of “K-complex” in neonate and juvenile (however decreasing somewhat in juvenile), and dramatic reduction in adult. Note dramatic increase of Mutalysin 2 (absent in neonate, nearly absent in juvenile) to adult. The venom is shifting from almost total “shock-syndrome” production toward greater proteolytic (exodigestive) activity. The peak represented by 28.6kDa is an unidentified protein typical of *L. melanocephala* and less abundant in *L. stenophrys*. From Ripa, Boomershine and Ripa (in prep); UPLC by Will Boomershine.

that most seriously endanger the lives of human beings in a modern world where antivenom is, or should be, readily available in time to treat most pitviper bite. Where antivenom and other support treatment is readily available, there is simply no reason to die from proteolytic factors in a bushmaster bite. Indeed, it may be impossible to do so even without antivenom, for proposing such an intense envenoming as would kill a human being from hemorrhagic effects is to pose a concomitant L-syndrome reaction of such magnitude that the shock-death would surely precede them. This sounds incautious, but, contradicting presumptive assertions in the literature till now, no such fatality has ever been shown (at least we cannot say positively that the culprit was not really a *Bothrops*).

The fatality rate from bushmaster bite remains one of the highest of any snake. Chapters 22 - 24 provide a range of venomous effects and symptoms, and shows which are most explicitly dangerous in the bushmaster bite. None of them are “proteolytic” in the classic sense (as blood and tissue destructive), except insofar as vasodilation could enhance tissue destructive effects through increasing venous permeability.

In this chapter I explore why bushmaster venom is quickly lethal to prey, and conclude—quite the reverse of Gutiérrez et al. (1990)—that not only is the neonate venom fully toxic, but drop for drop it is significantly more toxic to prey animals and human beings than the venom of the adult snakes. The differ-



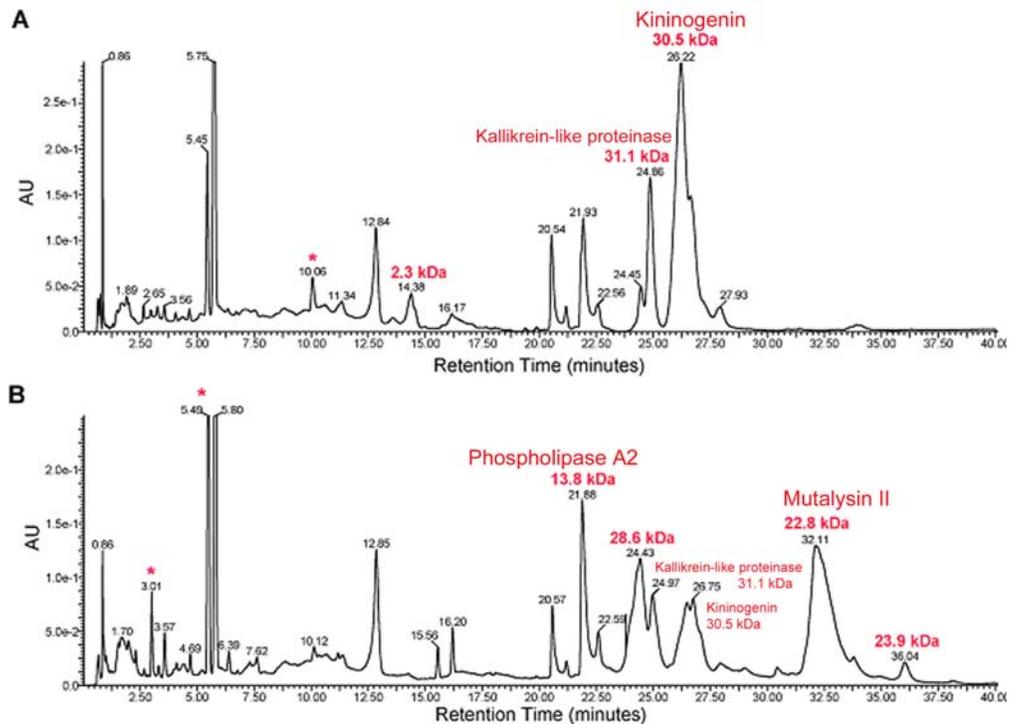
**Figure 6. Illus. 5.** SDS-polyacrylamide gel electrophoresis (15%) of venoms obtained from specimens of bushmaster snakes of different species and different ages. (1) Molecular weight standards: a, 97,400; b, 66,200; c, 45,000; d, 31,000; e, 21,500; f, 14,400. (2) Venom from adult *Lachesis stenophrys* specimen. (3) Venom from adult *Lachesis muta muta* specimen. (4) Venom from adult *Lachesis melanocephala* specimen #1. (5) Venom from adult *Lachesis melanocephala* specimen #2. (6) Venom from adult *Lachesis melanocephala* specimen #3. (7) Venom from two-week old *Lachesis melanocephala* specimen. (8) Venom from four-week old *Lachesis melanocephala* specimen. (9) Venom from three-month old *Lachesis melanocephala* specimen. For each sample, 45 mcg of venom was loaded. All samples were reduced with 2-mercaptoethanol. The identity of the proteins is indicated on the right. From Ripa, Boomershine and Ripa (in prep); electrophoresis by Will Boomershine.

**Phospholipase A<sub>2</sub>** – are calcium dependent enzymes that hydrolyze the two-ester bonds of 1,2-diacyl-3sn-phosphoglycerides. They display a wide variety of activities including presynaptic/postsynaptic, neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, antiplatelet, convulsant, hypotensive, haemolytic, hemorrhagic and edema-inducing effects (Huang et al., 1997). These belong to group II secretory phospholipase A<sub>2</sub>s, formed by venoms from Crotalidae and Viperidae families.

**Mutalysin I & II** – are hemorrhagic metalloproteinases that are thought to cause local and systemic hemorrhage (Estevao-Costa et al., 2000). Mutalysin I (100 kDa) is a member of the class P-IV reprolysins (Bjarnason and Fox, 1994; Kini and Evans, 1992). Mutalysin II (22.5 kDa) is a member of the class P-I reprolysins (Bjarnason and Fox, 1994; Kini and Evans, 1992). Mutalysin I has 35X higher hemorrhagic activity than mutalysin II while mutalysin II has 27X higher proteolytic activity towards dimethylcasein than mutalysin I (Estevao-Costa et al., 2000). Both mutalysin I & II degrade the α-chain of fibrinogen over the β-chain (Estevao-Costa et al., 2000). Mutalysin II will also degrade the β- and α-β-chains of fibrin (Estevao-Costa et al., 2000). Mutalysin II interacts with the human plasma glycoprotein α<sub>2</sub>-M (725 kDa) in a 1:1 ratio (Estevao-Costa et al., 2000). Binding of α<sub>2</sub>-M to mutalysin II inhibits proteinase activity (Estevao-Costa et al., 2000; Starkey and Barrett, 1982). Mutalysin I is unaffected by α<sub>2</sub>-M binding indicating that it may be involved in systemic bleeding (Estevao-Costa et al., 2000). Mutalysin I also inhibits collagen induced, but not ADP induced, platelet aggregation (Estevao-Costa et al., 2000).

**Kininogenin** – is a serine protease (28 kDa) that releases bradykinin from kininogen (Diniz and Oliveira, 1992). It also induces a hypotensive effect (Diniz and Oliveira, 1992).

**Kallikrein-like proteinase** – is a 33 kDa glycoprotein that has considerable homology to serine proteases from other snake venoms (Felicori et al., 2003). It too releases bradykinin from kininogen and induces hypotensive effects (Felicori et al., 2003). This protease also activates plasminogen (Felicori et al., 2003).



**Figure 7. Illus. 6.** UPLC chromatograms of venom from *L. stenophrys* of various ages. A is neonate (2-weeks-old) and B is adult. Small black numbers indicate retention time in minutes. Red labels indicate the molecular weight of protein in that peak as determined by electrospray-time-of-flight (ToF) mass spectrometry. Peaks labeled in red indicate that this age of snake contains more of this component of venom than other ages. The \* indicates peaks with different intensities between ages but no molecular weight was determined. Again, as in *L. melanocephala* neonates, note dramatic increase of “K-complex” and deficit of typical proteolytic factors (PLA2 and Mutalysin 2) in the neonate. The venom is shifting from direct lethality to delayed lethality with exodigestive effects. From Ripa, Boomershine, and Ripa (in prep); UPLC by Will Boomershine.

ence is in the action of the lethality, which in other writing has been variously misunderstood as cytologically destructive in kind.

### What’s the toxicity?

The venom of adult bushmasters shows a significantly higher proteolytic action than that of neonates in mice (Gutiérrez et al., 1990), and this can be demonstrated clinically in the record of damage in adult vis-à-vis neonate bites on human beings (e.g., Ripa, 1999; Chapter 22). These effects, while potentially dangerous if left untreated, should not be confused with a much more dangerous action, one serving no explicitly digestive function, and which constitutes the venom’s single most lethal agent: the ability to instigate rapid shock.

In my model, “lethality” is equated with those actions that are most directly toxic to the organism, that is, the agents that actually kill the prey. The classically proteolytic agents, such as those that demolish and

“digest” tissue, while surely toxic, are not the actions that kill the prey that bushmasters eat. They are not “first-kill” toxins in this sense, but secondary ones: their usefully digestive effects do not have time to develop before the prey is already dead. This is evidenced by the very low concentrations of these agents in the neonate venom (illus. 1 - 8 provides an overview of these proportions). Rather, the prey is immobilized swiftly—by shock. Other agents may go to work on the prey’s system both during and after this process, but this is not what brings about immobilization. Thus we must rate the toxicity of bushmaster venom according to the disproportion of its shock-producing agents and temporarily forget its proteolytic effects if we are to predict its first-lethal effects in man. It should be mentioned before we go further that the Gutiérrez et al. (1990) analysis was limited to the blood-tissue destructive elements of the venom, and at no point were the K-complex of fractions described or even mentioned. The concept of what bushmaster *should be*, according to contemporary knowledge of other venoms, dominated their model for lethality. In Gutiérrez et al. (1990), the expectations are of a viper that kills by the conven-

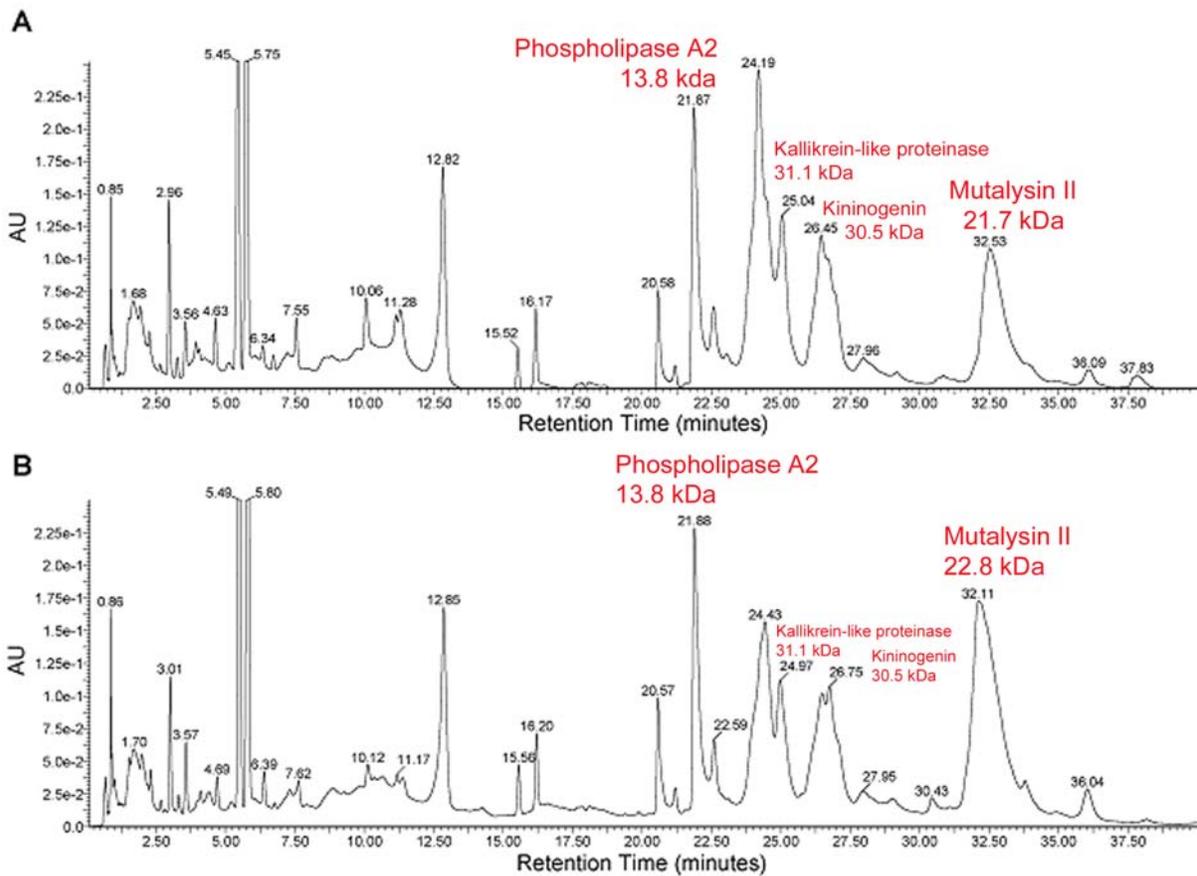
tional processes, and these processes all leave a visible fingerprint upon the cells. What the Gutiérrez et al. (1990) analysis told us then, was that these conventionally understood processes were very low in the venoms of neonates and juveniles, compared to the venoms of adult specimens.

Figure 6 (Illustration 6) shows an electrophoresis of three species of bushmaster venom, at early and mature stages of the snake's lives. Abundant components are seen at some ages and conspicuous absences in others. For example, in three samples of neonate venom from *Lachesis melanocephala*, taken at 2 weeks of age, 4 weeks of age, and 12 weeks of age, we see a preponderance of kallikrein-like proteinase and kininogenin. These are both bradykinin releasers, powerful vasodilators whose principle job in venom is to produce hypotension. These proportions are significantly higher than in adults. As Chipaux (2002) notes of bradykinin and prostaglandins increase levels in other kinds of snakebite: "The consequence of these mechanisms is a severe drop of

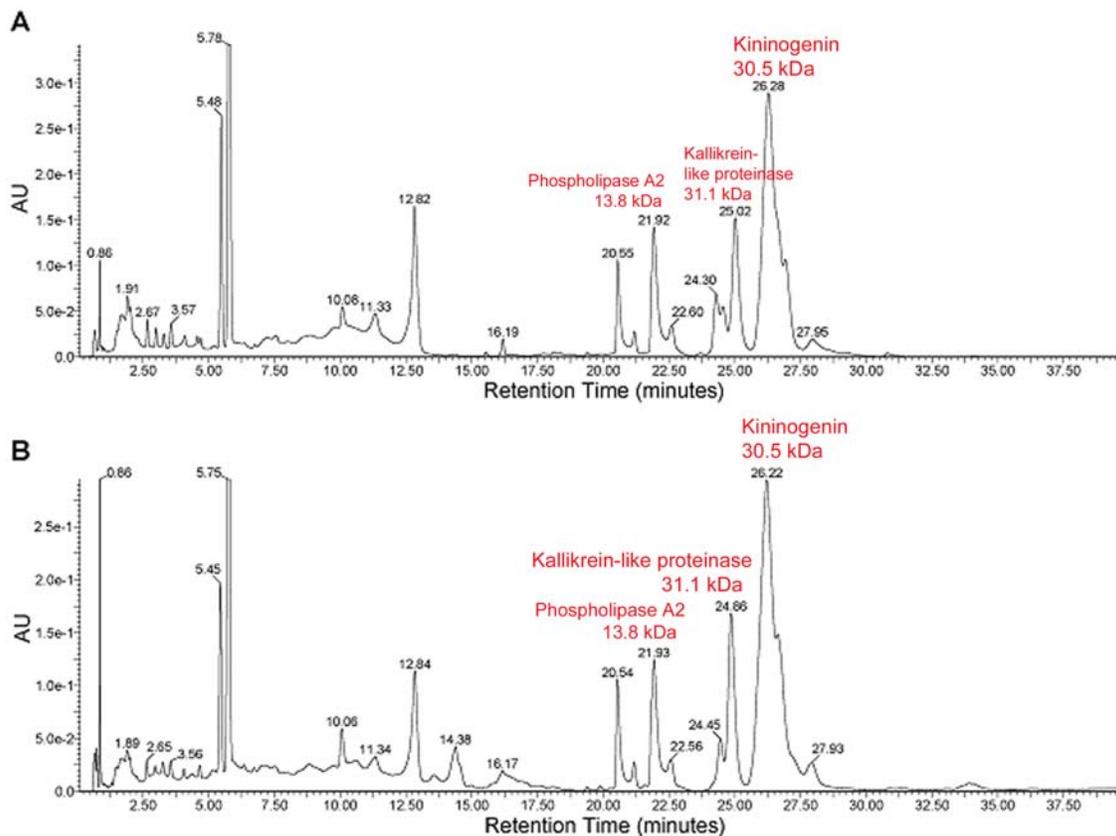
peripheral resistance causing the arterial pressure to drop as well. This drop is early on (a few minutes after the inoculation of the venom), fast and severe. The heart rhythm is not modified and this excludes a direct cardiac toxicity." In a few words the dynamic systemic changes seen in the bushmaster bites recorded in Chapter 22.

Whether or to what extent kallikrein and kininogenin (here called "K-complex") also participate in the enzymatic or proteolytic digestion of the prey is not essential to this discussion. Death will be brought about by the shock-activators long before these actions can be appreciated, in an animal that will have already expired several minutes before it is swallowed, and more than 24 hours before it is gastrally digested.

The K-complex of bradykinin-releasers is in much greater proportion in the venom of baby and young bushmasters than in the venom of adults (Illus. 1 - 8). The shock effects observed in Bites 3 and 4 (Chapter 22), involving 1 year old and 2 month old bush-



**Figure 8. Illus. 7.** UPLC chromatograms of venom from neonate *L. melanocephala* (A) and *L. stenophrys* (B). In the neonate stages these two venoms are much the same, showing the same inequalities compared to adult snakes. From Ripa, Boomershine and Ripa (in prep); UPLC by Will Boomershine.



**Figure 9. Illus. 8.** UPLC chromatograms of venom from adult snake of *L. melanocephala* (A) and *L. stenophrys* (B). Note greater PLA2 in *L. melanocephala* and somewhat greater “K-complex,” with greater Mutalysin 2 in *L. stenophrys*. From Ripa, Boomershine and Ripa (in prep); UPLC by Will Boomershine.

masters, are thus explainable. There is simply much more “shock-agent” in the neonate venom than any other component, so we should expect these agents, both known to be fast acting, to strike first before the typically slower blood derangements are appreciated as local tissue damage.

For example, neonate venom showed only traces of phospholipase A<sub>2</sub> isoforms and were completely lacking in Mutalysin 2. The former enzyme is credited with a wide variety of actions, ranging from neurotoxicity to haemolysis, so it is too early to predict symptomatology here; however, Mutalysin 2, a metalloproteinase, appears to be a definite hemorrhagin. The dearth of both components could account for the low hemolytic and hemorrhagic effects seen in Bites 1, 2, 3, 4 and 5.

While Mutalysin 2 is completely absent in the neonate venom, Mutalysin 1 is amply represented, just as in adults. Clinical edema and inflammation in the neonate bite seems no less great than in the adult, so at least in Mutalysin 1 we have a probable culprit. Mutalysin 1 is then a powerful producer of edema

and inflammation, but its role in hemorrhage is much less significant. The effects seen in Bites 1, 3, and 4, all from young snakes, confirm this idea.

Phospholipase A<sub>2</sub> is found in so many disparate snake venoms that it is difficult to pinpoint its meaning in bushmasters. This, nevertheless, deadly enzyme is most abundant in adult *L. melanocephala*. PLA<sub>2</sub> may produce structural changes in the central nervous system (Russel, 1983), increasing presynaptic density, widening of the extracellular spaces, distension of nerve terminals and mitochondria, decreased numbers of synaptic vesicles, and rupture of the plasma membrane (Cedergren et al, 1973).

The rapid dynamics of the L-syndrome have been observed repeatedly. Jorge et al. (1997) recorded some 20 cases, and more have appeared since then. To date, however, only the shock-effect (with its severe hypotensive action) has proved significant in loss of human life in bushmaster envenoming. This does not exclude sequelae similar to other Crotaline envenomings, involving a slower degradation of the cardiovascular system that could invite a second fatal

shock later on. But we have no way to measure this, since all known fatalities have involved shock, even when death was delayed by several days. Without doubt, systemic degradations through haemostatic alterations, including hemorrhage and necrosis inviting secondary infection, could also enhance or alter (to sepsis) the shock-producing pharmacology, clinically appreciated as a second kind of shock (e.g., septic shock). And yet this is not a necessary hypothesis. The K-complex is present in enough quantity in all bushmaster venoms, whether adult or neonate, to produce shock without resorting to a theory of a secondary reaction (e.g., shock from bacterial infection). Further study as new bite cases come in will no doubt determine this feature more exactly.

Neonate bushmasters kill their prey very rapidly, whether performing strike-hold or strike-release tactics (Chapter 21). There is no appreciable deficit, vis-à-vis adults, in their ability to kill prey. If anything, the smaller food animal dies more quickly than the adult's larger prey. The disproportion of kallikrein-like proteinase and kiningenin in the neonate venom, and the lack of other haemostatic agents, suggests that the neonate venom kills the prey by inducing a shock effect. The hemorrhage producing enzymes and proteins in the venom have a long-term digestive purpose, but perhaps they could not kill the prey quickly enough for the snake to be able to recover it if strike-released. Hence, rating the venom of neonates according to classic proteolytic-type properties alone (as did Gutiérrez et al., 1990) will always produce a misleading picture of "low toxicity." The digestive components are not the explicitly lethal properties to begin with. They are in too short a supply. In the neonate venom we have a pharmacology almost entirely predicated on the use of shock as a quick-kill strategy. This bradykinin-releasing K-complex is a necessary component for an otherwise slow acting venom, benefitting neonates by making sure they get their vital first meals in life. While the K-complex is retained in the adult, it is not retained in such disproportion, being replaced by greater concentrations of Mutalysin 2 and PLA<sub>2</sub>. The venom becomes more proteolytic in the larger, more difficult to digest prey of adult snakes.

While in adult snakes we find less concentration of the shock-producing agents, the lack is certainly compensated by the greater amount of venom these larger snakes can deliver. In the adult snakes, uniformly, the shift appears toward the hemorrhagic productions of Mutalysin 2 and Phospholipase A<sub>2</sub>; that is, the venom is becoming more blood and tissue destructive. At some point in the snakes' lives a shift occurs toward greater proteolytic activity and less directly lethal action, proportionate to

the available K-complex. This change does not occur early on. At 10 months the venom is still relatively similar to the neonate's. Further analysis should tell us as what age this change occurs (e.g., and whether it corresponds with size, prey-size shifts [to relatively smaller prey], or sexual maturity). Perhaps the proportion of the K-complex is balanced toward the critical minimum necessary to insure that shock-instigators will always be in enough supply to do their job.

This study brings an unexpected new insight into the effects of bushmaster venom in prey and in human beings. Contrary to previous assertion, juvenile bushmasters are formidably armed. Drop for drop, their venom is significantly more dangerous to man than the venom of adults. Based on the effects seen in Bites 3 and 4 in Chapter 22, and the proportions of venom injected by other neonates of similar size when strike-holding prey (Chapter 21), I propose that as little as 20 - 25 mg (dry weight) of the neonate venom injected intramuscularly could be the relative lethal dose for an adult human being. This potency will be less in the mature snake, where the K-complex is almost halved (although surely made up for by the greater venom delivery possible in the adult snake). Nevertheless, the shift toward greater proteolytic action, as Mutalysin 2 and PLA<sub>2</sub>, may to some extent provide the needed synergy to compensate for the deficit.

The agonizing abdominal seizures experienced in consort to the sudden loss of blood pressure (and rapid heart rate accompanying this, being a good indicator of the onset of shock) can be explained at least partially by the effects of bradykinin on the smooth muscles of the abdomen and intestines, which cause spasmodic contractions in test animals (sensu Feres et al., 1994). These symptoms, along with dysphagia, dysphonia, numbness of the lips and face, etc., have probably been confused with an apparent neurotoxicity. As these data show, the expected instigator of neurotoxic action (PLA<sub>2</sub>) is very low in the venom of the neonate and sub-1-year-old juvenile; the phospholipase A<sub>2</sub> isoforms usually associated with neurotoxic action are but spottily represented. They are far outweighed by the K-complex, which, indeed, but for the presence of Mutalysin 1, we might say is really almost all the juvenile venom is composed of. The clinical data provided by Bites 3 and 4 in this book (Chapter 22) strongly suggest that the L-syndrome is alive and well in the juvenile snake, and is not dependent on PLA<sub>2</sub>.

There is more good evidence against neurotoxicity. Signs of neurotoxicity, even in strongly neurotoxic enve-

nomings such as those of cobras and kraits, usually do not appear earlier than several hours; while in the bushmaster, dysphagia, convulsive pain of the abdominal muscles (probably from contractions of the smooth muscles), projectile vomiting and diarrhea are experienced within 20 minutes of the envenoming; in other words, at the very start of the hypotensive crisis. From this I infer that true neurotoxicity (e.g., Vagal stimulation) is not responsible for these most conspicuous alterations seen in the L-syndrome (but this deserves further study). Bushmaster venom kills by producing a swift autopharmacological reaction due to a preponderance of kininogen-like substances that release bradykinin and kallikrein, in effect triggering the body to kill itself. The onslaught is more sudden than that seen in typical “neurotoxic” snakebite, and in a venom that is not, or should not, be more than mildly neurotoxic, it is difficult to implicate nerve poisoning in this effect.\* The sudden death syndrome (< 45 minutes) in bushmaster bite can be directly attributed to the abundance of K-complex in the venom. If these effects are not completely neutralized, the L-syndrome, although managed initially, can return and become irreversible within 1 - 4 days, advancing on the victim in combination with other proteolytic effects in an already weakened patient. While the delayed death sequence is surely exacerbated by the proteolytic degradations of blood and tissue, one wonders if these are really so essential to the formula. After all, neonate venom lacks almost all the major cell-destructive agents and is still capable of producing a significant shock-effect in man.

The till-now unexplained syndrome of “bushmaster bite death despite immunotherapy” can probably be blamed on the failure of treatment to control the activating factors of K-complex poisoning.

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\* Damico et al. (2005b) attempt to show neurotoxicity in *L. muta muta* venom in vitro, in mouse phrenic nerve diaphragm and chick bicenter cervicis preparations; however, the massive quantities they used (20 µg/ml, 50 µg/ml and 100 µg/ml, &c.), injected into the tiny mouse and chick parts, equivalent to as many as 100 lethal doses for an adult human being, would rather disprove an argument, than otherwise, that bushmaster venom inspires a significant neurotoxicity in man. No bushmaster can administer this much venom: 2 - 6 grams required to produce a neurotoxic effect in an animal weighing as much as the average adult person! The total yield for most bushmasters is not more than 333 mg and the amount injected in a bite is typically much smaller, in the range of 50 - 120 mg. Short of being bitten by more than a dozen bushmasters at once, we should detect no such effect in human beings; nor should we assume such an effect to be fatal, in any case. Even granting that, at these massive dosages, certain neurotoxic effects can be detected in mice and poultry, such results cannot be applied across species. To date, no human fatality from a bushmaster bite can be attributed to neurotoxicity, and such effects as have been portrayed as nerve-affecting (e.g., oropharyngeal dysphagia, often mistaken for paralysis) are rather symptoms of the onset of the bushmaster shock-syndrome, than any true neurotoxic presence in the venom.

## Materials and methods for HPLC

### High performance liquid chromatography

The venom was analyzed using C<sub>18</sub> reversed-phase high performance liquid chromatography (HPLC) on a Vydac 218TP510 column (10 x 250 mm). Components were resolved using a 120 mL linear gradient from 5% to 75% acetonitrile, 3% isopropanol, 0.1% trifluoroacetic acid (TFA) over 40 minutes. The absorbance was monitored at 215 nm.

### Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 15% gel (Laemmli, 1970). Samples were reduced with 2-mercaptoethanol. The gel was run at 150 volts for approximately 1.5 hours with 45 mg of each sample. Gels were stained with Coomassie Brilliant blue. The number and approximate molecular weight of the bands were estimated for comparative analysis.

## Methods and materials for UPLC

Ultra Performance Liquid Chromatography (UPLC) –the snake venom was analyzed by UPLC. Approximately 5 mg of lyophilized snake venom was dissolved in enough mobile phase A to bring the concentration to 5 mg/mL. 50 µg of venom was placed onto an Acquity UPLC BEH C18 1.7 µm 2.1 mm X 100 mm column and eluted with a linear gradient from 10 % mobile phase B to 50 % mobile phase B over 40 minutes at 0.2 mL/minute. Mobile phase compositions were as follows: mobile phase A, 0.1 % formic acid, 0.01 % trifluoroacetic acid, 2 % acetonitrile, 98 % water; mobile phase B, 0.1 % formic acid, 0.01 % trifluoroacetic acid, 2 % water, 98 % acetonitrile. Proteins were monitored by UV absorbance at 280 nm and analyzed by mass spectrometry using a Waters MALDI Q-ToF Premier mass spectrometer using electrospray ionization.

## Results

Comparison of venom from *L. melanocephala* of various ages (Figure 5; Illus. 4): The venom of adult and baby snakes is very similar in protein composition. However, there are some dramatic differences in the amounts of individual proteins between the two age groups. Baby snakes lack the 21.7 kDa protein with a retention time of 32.5 minutes. Adult snakes also appear to have a greater quantity of the 28.6 kDa protein with a retention time of 24.19 minutes. While baby snakes lack a major component of the venom, they do have higher quantities of several other proteins including the 13.5 kDa protein at retention time 20.55 minutes, the 31.1 kDa protein at retention time 25.02 minutes and the 30.5 kDa protein at retention time 26.28 minutes. The venom from a juvenile snake appears to fall in between baby and adult venom in terms of composition. The juvenile venom has increased amounts of the proteins at 24.27 minutes and 32.70 minutes versus the baby venom, while having decreased amounts of the proteins at 26.40 minutes, 25.04 minutes and 20.56 minutes. The peak at 19.83 minutes (13.9 kDa) could be a protein that only appears during this time of development or is due to individual variation, since this peak is not present in either the baby or adult venom samples.

Composition of venom from *L. stenophrys* of various ages (Figure 7; Illus. 6). The venom of adult and baby snakes is very similar in protein composition. However, there are some dramatic differences in the amounts of individual proteins between the two age groups. Baby snakes lack the 22.8 kDa protein with a retention time of 32.11 minutes. Adult snakes also appear to have a greater quantity of the 28.6 kDa protein with a retention time of 24.43 minutes, the 13.8 kDa protein with retention time of 21.88 minutes and the 23.9 kDa protein with a retention time of 36.04 minutes. While baby snakes lack a major component of the venom, they do have higher quantities of several other proteins including the 2.3 kDa protein at retention time 14.38 minutes, the 31.1 kDa protein at retention time 24.86 minutes and the 30.5 kDa protein at retention time 26.22 minutes. The differences in venom between adult and baby snakes for both *L. melanocephala* and *L. stenophrys* were very similar.

Comparison of venom between baby *L. melanocephala* and *L. stenophrys* (Figure 8; Illus. 7): The venoms from baby snakes from different species are

quite similar in both the quantity of proteins present and the identity of proteins present. There are some minor differences in retention times between peaks. However, these differences are probably due to sequence differences within the same functional protein rather than two different functional proteins.

Comparison of venom from adult *L. melanocephala* and *L. stenophrys* (Figure 9; Illus. 8): The venoms from adult snakes from different species are quite similar in both the quantity of proteins present and the identity of proteins present. There are some minor differences in retention times between peaks (such as 32.53 minutes for *L. melanocephala* and 32.11 minutes for *L. stenophrys*). These differences, however, are probably due to sequence differences within the same functional protein rather than two different functional proteins.

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