Characterizing the Roles of Coevolution and Convergence in the Evolution of Venom Resistance in Mammals

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ABSTRACT

Though the study of coevolutionary relationships has been a focus of evolutionary biology, demonstrating true reciprocity at the molecular and functional level in a natural system has remained elusive. Convergent evolution, though a seemingly disjunct phenomenon, has been found to be a fundamental aspect of coevolution particularly in species-rich interactions. While theoretical frameworks have shown that these two processes are intertwined, few empirical examples exist which demonstrate how these processes correlate in a natural system, and none have demonstrated the mode of both molecular and functional evolution in a coevolving trait. In this work I comprehensively review venom resistance as a trait ideal for understanding the biophysical and evolutionary dynamics of coevolution. Subsequently, I use this framework to examine the roles of molecular and biophysical convergence in two such systems hypothesized to be coevolving. Finally, I develop an experimental system which explicitly maps the biophysical and molecular evolution of a trait via direct testing of ancestral phenotypes, to begin to unveil the true mode of evolution as well as the roll of convergent evolution in a trait hypothesized to be coevolving.

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INTRODUCTION

CHARACTERIZING THE ROLES OF COEVOLUTION AND CONVERGENCE IN THE EVOLUTION OF VENOM RESISTANCE IN MAMMALS

Since the 1970s, coevolutionary thought has been greatly influenced by the Red Queen Hypothesis (Van Valen 1973), which posits that interacting species are continuously adjusting to compensatory evolutionary changes to keep up with one another—the end result being no net advantage by any one partner within the coevolutionary interaction. This and other models of coevolution assume that either frequency dependent selection generates cyclic fluctuating polymorphisms, or that selective sweeps create an ever-escalating arms race (Thompson 1994, Jokela et al. 2000). However, other models suggest that coevolutionary cycling or gradual tit-for-tat arms races may be rare or nonexistent in empirical systems, and that chaotic, complex, and irregular cycling may be the norm (Seger 1988, Thompson 1994). More recent work modeling coevolutionary dynamics in the context of species-rich and disperse coevolutionary interactions demonstrates that convergent evolution plays a major role in trait evolution within coevolutionary interactions, especially in cyclic fluctuating polymorphism coevolution among generalists (Guimaraes et al. 2011). The association between these two phenomena has also been shown in canonical coevolutionary systems through phylogenetic comparative approaches (Thai et al. 2012, Jousselin et al. 2003).

Coevolution has also been shown to accelerate molecular evolution in genes under selection as well as increase evolutionary rates of divergence and polymorphism (Mitter et al. 1988, Wiegmann et al. 1993, Becerra 1997, Farrell 1998, Becerra and Venable 1999, Forde et al. 2008, Paterson et al. 2010, Bérénos et al. 2011, Marston et al. 2012, Brockhurst and Kostella 2013). However, the mode of this acceleration has been hotly debated since the birth of the field. Many have proposed that coevolution proceeds in a gradual manner, while others have pointed out that this type of change does not align with our knowledge of constraints of protein biochemistry and suggest that saltatorial evolution is likely to dominate even in quickly evolving coevolutionary arms-races

(Goldschmidt 1940, Gould 1977, Stenseth and Maynard-Smith 1984, Dawkins 1996). Still others have suggested that coevolution *sensu stricto*, may be a relatively rare phenomenon, and many seemingly tightly paired interactions may be evolving at unequal rates where one partner has long 'escaped' the arms race (Stewart-Oaten 1982, Abrams 1986, Thompson 2001, Hanfin et al. 2008).

Convergent evolution, where species evolve the same adaptation in response to a similar selection pressure, is well known in cases of mutualistic coevolution (Meyer 2006, Cote 2000, Jordano 1995), and is predicted to be a characteristic component of disperse coevolution (Guamaraes et al. 2012, Nuisimer et al. 2012). Detailed work examining the molecular and functional basis of adaptive convergence often reveals differing levels of convergence that range from phenotypes with vastly different genetic bases, to complete parallelisms at the amino acid level (Shi and Yokoyama 2003, Hoekstra et al. 2006, Dean and Thornton 2007, Stern 2013). The spread of these levels informs the degree to which constraints influence adaptive function, and can elucidate the degree to which adaptive evolution and coevolution may be working across diverse taxa.

Integrating evolutionary history with an understanding of the biochemical consequences of adaptive coevolutionary change will allow biologists to not only estimate the tempo of coevolutionary change, but begin to understand how biophysical mechanisms and molecular convergence influence this tempo. Several recent studies of adaptive evolution have bridged this gap and demonstrated that experimental data on ancestral functionality are critical to understanding the evolutionary and phenotypic consequences of molecular change (Hiebl et al. 1987, Jessen et al. 1991, Kornegay et al. 1994, Yokoyama 1998, Yokoyama and Tada 2000, Shi et al. 2001, Nachman et al. 2003, Shi and Yokoyama 2003, Hartley et al. 2006, Hoekstra et al. 2006, Zhang 2006, Dean and Thornton 2007, Yokoyama et al. 2008, Harms and Thornton 2013). While observing matched adaptive phenotypes has made it possible for biologists to postulate the functional and evolutionary significance of coevolutionary relationships, quantification of molecular and biochemical changes underlying phenotypic change allows biologists to

finally test the major underlying assumptions about coevolutionary tempo, mode, and mechanism.

In Chapter 1 of this work I comprehensively review the system which I focus on— mammalian resistance to snake venom—and outline it as a model system for understanding complex coevolutionary adaptations in natural systems (Holding et al. 2016b). This work was a collaborative effort which was developed extensively and equally with my co-first author. I contributed to the writing of all sections of this work, and was the primary contributor to the review of molecular mechanisms as well as the review of using longer (phylogenetic) time-scales to better understand modes of evolution.

In Chapter 2 I utilize a comparative phylogenetic approach combined with extensive background data on the biochemical basis of a seemingly highly constrained adaptation to reveal and assess convergent adaptation and potential coevolution. Specifically, I examine the evolution of a snake venom target, the nicotinic acetylcholine receptor (nACHr), in a mammalian species (*Mellivora capensis*) thought to be resistant to and prey upon snakes whose venoms target this receptor via α-neurotoxins (Drabeck et al. 2016). Using publicly available data, museum specimens, and phylogenetic comparative methods I showed that the evolution of resistance to α-neurotoxin has evolved a minimum of four times across mammals. Using extensive background data on site-directed mutagenesis of nACHr, and maximum likelihood tests of positive selection I identify two sites suspected to be responsible for loss of α-neurotoxin binding and distinguish at least two different biochemical mechanisms responsible for resistance at these sites. This chapter highlights that the combined approach of assessing biochemical and comparative phylogenetic evidence for trait function can reveal the prevalence and degree of convergence of an adaptive trait, and pinpoint the molecular basis of a trait hypothesized to be coevolving.

In Chapters 3 and 4 I focus on a second instance of mammalian resistance to snake venom, and use this system to fully elucidate the mode of evolution in a trait hypothesized to be coevolving. In both chapters I focus on opossums in the clade

Didelphini, which exhibit exceptional resistance to snake venom and are thought to be co-evolving with the venomous snakes which they are known to prey upon (genus *Bothrops*) (Jansa and Voss 2011). My work examines the functional basis and evolutionary mode of a single aspect of their resistance —a blood protein (von Willebrand Factor) — thought to protect against venom proteins which target it.

Using both *ex vivo* and *in-vitro* assays, I explicitly test the physiological and biophysical function of this interaction across several species of resistant and susceptible opossums. I utilize phylogenetic methods to reconstruct hypothesized ancestral states for this trait, and tests the biophysical function of ancestral proteins *in-vitro*. In so doing I molecularly and functionally characterized the evolution of an adaptive trait and reveal a complex and otherwise hidden evolutionary history.

Results suggest that the evolution of venom resistance has evolved many times (convergently) across opossums, suggesting the interaction between opossums and vipers may be much more ecologically and evolutionarily important than previous though. Results also reveal that the biochemical mechanisms and sites conferring resistance are quite diverse, suggesting either that the evolution of resistance on this protein is relatively unconstrained, or that it is evolving in response to diverse venom CTLs. A pattern of strict saltatorial tit-for-tat coevolution is apparent when examining ancestral node data. Though ancestral node data for partnered venom proteins are necessary to confirm reciprocity, this is the first work to explicitly reveal a an evolutionay mode consistant with tit-for-tat coevolution using empirical data from reconstructed ancestral phenotypes.

CHAPTER 1. VENOM RESISTANCE AS A MODEL FOR UNDERSTANDING THE MOLECULAR BASIS OF COMPLEX COEVOLUTIONARY ADAPTATIONS[1]

Introduction

Coevolution between predators and prey is an important evolutionary force for the generation and maintenance of adaptive variation. Most studies of coevolving traits in nature have focused on accessible morphological variation (Benkman et al. 2003, Toju 2008). However, with recent advances in genomic and proteomic techniques, as well as an improving understanding of molecular function, we can now meaningfully examine coevolutionary dynamics at the molecular level (Hanifin et al. 2008, Nash et al. 2008, Zangerl et al. 2008, Jansa and Voss 2011, Scanlan et al. 2011, Feldman et al. 2012). Among coevolving systems, the interactions between venomous and venom-resistant animals hold exceptional promise for investigating molecular coevolution. In this review, we outline how population-level, phylogenetic, and biochemical approaches can be applied to these systems to study the molecular and functional basis of complex phenotypic interactions. Coevolution has been widely invoked to explain trait variation in venomous and venom-resistant animals, and is often discussed in terms of molecular "arms races" (Casewell et al. 2012b, Vonk et al. 2013). Studies of venom evolution have shown that molecular evolutionary processes such as positive selection, gene duplication, exon shuffling, and transcriptional splicing, among others play major roles in generating venom diversity (Fry et al. 2005, Doley et al. 2009, Casewell et al. 2012a, 2014, Vonk et al. 2013, Rokyta et al. 2015). In contrast, few studies have focused on the evolution of venom resistance, despite the fact that reciprocal evolution of weapons and defenses is required for an explanation based on an arms race or other coevolutionary dynamic to apply (Janzen 1980). The molecular basis of venom resistance has been investigated in only a few mammals and venomous snakes (Barchan

[1] Reproduced with permission from Drabeck D. H., Holding M. L., Gibbs L. H., and S. A. Jansa. 2016. Venom Resistance as a Model for Understandinf the Molecular Basis of Complex Coevolutionary Adaptations. Integrative and Comparative Biology 56(5): 1032-1043

et al. 1995, Sanchez and Rodriguez-Acosta 2008, Jansa and Voss 2011, Drabeck et al. 2015, Estevão-Costa et al. 2016), and rapid evolution and positive selection have been demonstrated for only two proteins that are targeted by venom toxins (e.g., Jansa and Voss 2011, Voss and Jansa 2012, Drabeck et al. 2015).

Diverse venoms span Animalia, with venom occurring in Cnidaria, Arthopoda, Annelida, Bryozoa, and Chordata (reviewed in Casewell et al. 2012b). While our review covers recent work focused on mammalian venom resistance due to its prevalence in the literature, studies of toxin resistance combined with other ecological and physiological data suggest that resistance is likely to have evolved in diverse groups of animals (Heatwole and Powell 1998, Heatwole et al. 1999, Voss and Jansa 2012). Thus, the mechanisms and approaches we discuss here have the potential to be applied across a diverse set of taxa beyond mammals. The diversity of venomous species and the broader network of species they interact with represent replicated opportunities to ask important questions about coevolution.

Here, we provide a brief review of what is known about the molecular basis of resistance to venoms, as well as outline research directions which would advance our understanding of coevolution between venom toxins and their targets and inhibitors. We first summarize the molecular mechanisms that underlie venom resistance, focusing on the mammalian systems in which resistance to venoms is best characterized. We then discuss conceptual approaches for studying the evolution of resistance within and between species. We focus on linking predictions from theory with empirical tests from specific systems, with an emphasis on how these approaches inform our understanding of coevolutionary interactions between venomous and resistant taxa. Finally, we describe gaps in our knowledge that should be addressed to fully pursue research on venom/resistance systems and suggest approaches to fill these gaps.

Molecular mechanisms of resistance

Venom toxins target a wide array of biologically important molecules and disrupt numerous physiological functions. As such, understanding the molecular basis of venom resistance requires knowledge not only about how venom molecules exert their toxic effects but also about how resistant animals cope with these toxins at a molecular level. Though we understand some resistance mechanisms, our knowledge of the molecular basis of venom resistance lags well behind our understanding of venom composition and toxicity (Fig. 1). Consequently, an integrated picture of venom resistance and its role in the evolution of venom diversity and toxicity has yet to be realized. An important step in understanding how these traits might coevolve is to identify additional molecular mechanisms of resistance, because the molecular phenotypic interface between venomous and resistant species involves complex interactions between the venom components of one species, and the target or inhibitor proteins of the envenomated species. The

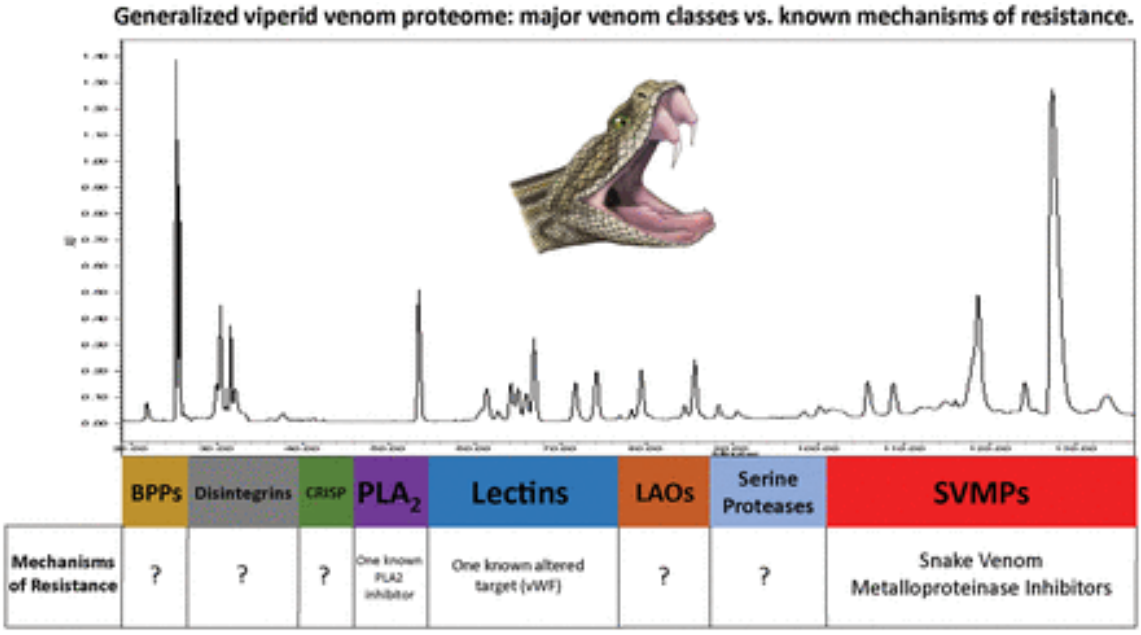

Figure 1.1- A representative HPLC venom chromatogram from a rattlesnake (*C. oreganus*) with elution regions roughly labeled according to the venom protein family based on peak identifications in Margres et al. (2014). Below the toxin names, the known mechanisms of resistance to these snake venom toxin classes are shown, highlighting that resistance is only known for less than half of the known toxin classes (Jansa and Voss 2011). BPP, Bradykinin potentiating peptides; PLA_2, phospholipase 2s; LAO, L-amino oxidases.

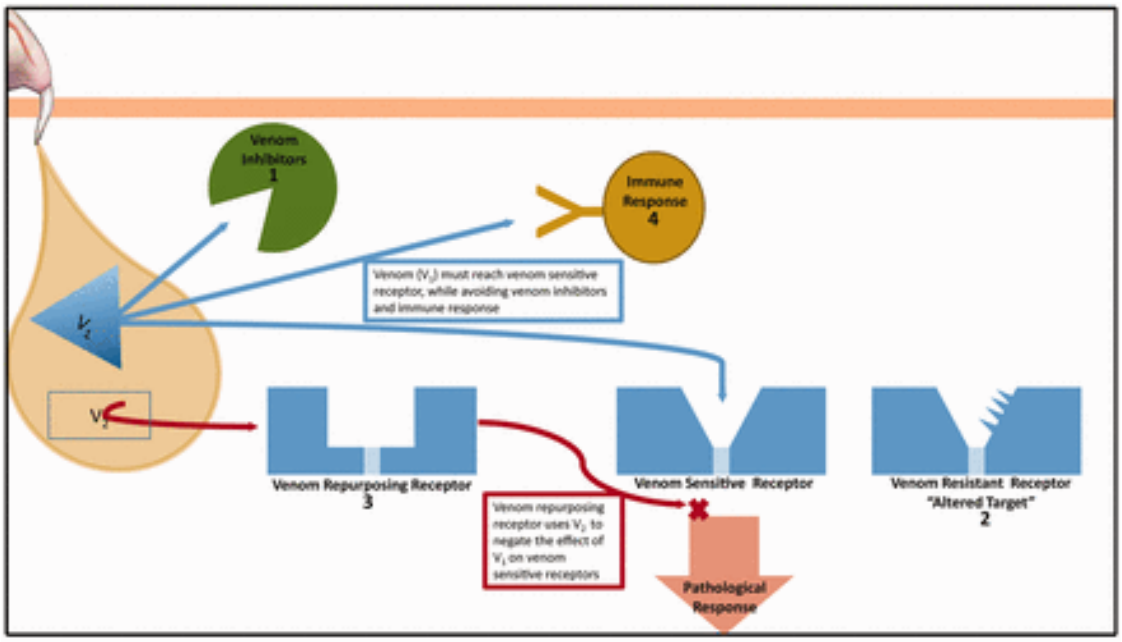

Figure 1.2- Conceptual presentation of the molecular mechanisms of venom resistance (ordered 1–4 to correspond to order presented in text) in relation to the actions of venom, emphasizing the diverse ways in which resistance can be achieved and the evolutionary pressures encountered by all proteins at the molecular phenotypic interface of interactions between a venomous and resistant species. Pathological response induced by venoms (depicted by the red arrow) is interrupted through both direct and indirect mechanisms.

interaction of these various molecules forms a complex phenotypic space where the outcome of envenomation is decided (Fig. 2).

In this review, we characterize known mechanisms of venom resistance into four categories: (1) venom inhibitors, molecules that deactivate venom toxins before they reach their targets and benefit from a match to venom; (2) altered targets, proteins that have evolved to no longer bind venom toxins but that still retain their original physiological function and benefit from a mismatch to venom; (3) repurposed toxins, where venom toxins are used by the victim to short-circuit the physiological effect of the toxin and the resistant animal benefits from a match; and (4) acquired immunity, where resistance is acquired through repeated sub-lethal exposure to toxins

Venom inhibitors

Many animal species have evolved at least partial resistance to venom in the form of circulating proteins that bind and inhibit venom proteins (Perez and Sanchez 1999,

Perales et al. 2005, Biardi 2008). These blood-serum factors neutralize snake venom metalloproteinases (SVMPs) and phospholipases (Perez et al. 1979, Catanese and Kress 1993, Perales et al. 2005). They have been identified in at least 30 mammal species from six orders (Perez et al. 1978, Biardi 2008, Voss and Jansa 2012), and in a number of squamate reptiles that are either resistant to their own venom or preyed on by venomous snakes (Perales et al. 2005).

Most of the known venom inhibitors function through direct interaction with venom proteins. For example, the α1β glycoproteins found in opossums, mongooses, and some rodents, and the inter-α trypsin inhibitor of ground squirrels irreversibly bind to a single venom protein to render it inactive (Biardi 2008). A related mechanism exists in the European hedgehog (Erinaceus europaeus), where a β-macroglobulin draws venom proteases into close proximity with a string of amino acids that changes conformation to enclose the venom, acting as a molecular cage (de Wit and Weström 1987). Both of these mechanisms rely on inhibitors recognizing toxic molecules, where affinity of one protein for another is a key part of the inhibitory process. Inhibition is also non-enzymatic, with inhibitors binding venom proteins in a 1:1 stoichiometry that inactivates both proteins (Perez and Sanchez 1999, Biardi et al. 2011).

Almost all of the known inhibitor proteins are associated with the Immunoglobulin (Ig) and Ficolin/Opsonin supergene family, for this reason, it has been suggested that these proteins are a derived part of the innate immune system (Perales et al. 2005, Sanchez and Rodriguez-Acosta 2008). As such, their evolution may differ from other classes of venom-resistant molecules described below. In particular, the serum inhibitors identified to date are all members of large, relatively old gene families, whereas the "altered targets" described below are typically members of small gene families (e.g., nAchR) or are encoded by single genes (e.g., vWF). Whereas positive selection may play an important role in the evolution of both toxin inhibitors and altered targets, the processes of gene duplication, gene turnover, and neofunctionalization may be the primary forces driving the evolution of circulating toxin inhibitors (for review see Taylor and Raes 2004).

Altered targets

Resistant mammals can also cope with venom by evolving venom-targeted receptors that no longer bind damaging venom proteins, while retaining their essential physiological functions (Barchan et al. 1992, Jansa and Voss 2011, Drabeck et al. 2015). While therapeutic potential has focused attention on serum inhibitors, few studies have examined the role that modified venom targets play in resistance, and the implications of these adaptive changes on coevolutionary dynamics remain largely unexplored. Target alteration is typically mediated by a small number of point mutations that change the protein so that it no longer binds the toxin but still recognizes its endogenous ligand. To date, only three venom-targeted proteins have demonstrated resistance to their toxic ligands: the muscular nicotinic acetylcholine receptor (nAChR), the blood coagulation protein von Willebrand Factor (vWF), and the alpha-l-proteinase inhibitor.

The muscular nAChR mediates synaptic transmission from nerves to muscles, and is targeted by alpha-neurotoxins present in the venom of elapid and hydrophiid snakes (Neumann et al. 1989, Barchan et al. 1995). In-vitro and in vivo research showed that the nAChR protein of hedgehogs, mongooses, and cobras—three taxa that survive envenomation by neurotoxic snakes—shows strong binding resistance for alpha nurotoxins (Barchan et al. 1992, 1995, Takacs et al. 2004). Site-directed mutagenesis revealed that this loss of toxin binding ability is explained by amino acid substitutions at two sites on the molecule's surface. A larger, comparative study subsequently showed that these same two amino acid sites have evolved independently under positive selection in four venom-resistant mammalian lineages: hedgehogs, mongooses, honey badgers, and pigs. This is the only known example of convergent adaptive evolution of a molecule involved in venom resistance; interestingly, the resistance seems to be mediated through two distinct biochemical mechanisms (Takacs et al. 2001, 2004). Cobras and mongooses have substitutions that confer resistance via glycosylation that leads to steric hindrance, whereas hedgehogs, honey badgers, and domestic pigs have substitutions that involve charge interference (Drabeck et al. 2015).

The second case of target evolution is the blood protein vWF, which mediates blood coagulation. The vWF protein is targeted by venom C-type-lectins (such as botrocetin), and has evolved under positive selection in a clade of didelphid opossums that prey upon venomous snakes and survive their bites (Jansa and Voss 2011). Although we do not yet know the functional significance of the observed amino acid changes in vWF in these taxa, eight sites are under strong positive selection in this lineage and seven of these are critical for botrocetin binding.

Finally, another altered target apparently exists in the Virginia opossum (*Didelphis virginiana*). The alpha-l-proteinase inhibitor (α1-antitrypsin) serves as an important inhibitor of endogenous proteases in the Virginia Opossum, but it is uniquely not deactivated by crotaline snake venoms, suggesting that it too has acquired functionally important amino acid changes (Catanese and Kress 1993). However, the evolution of this molecule has not been examined in any detail.

Repurposed toxins

A novel mechanism of resistance has been demonstrated in species of grasshopper mice (*Onychomys sp*.) that regularly attack and eat bark scorpions (*Centruroides sp*.) and sustain their stings (Rowe and Rowe 2006, 2008). Scorpion stings are extremely painful to most mammals, but grasshopper mice show a reduced pain response compared to laboratory mice (Rowe and Rowe 2008). This pain resistance works by binding a scorpion toxin to a previously untargeted pain receptor, which induces analgesia, blocking the effects of other pain-inducing venom components (Rowe et al. 2013). While one bark scorpion venom protein, CvIV4, induces pain by activating the sodium channel Nav1.7, it does not directly interact with Nav1.8, a downstream sodium channel which is an essential part of the pain signaling pathway (Rowe et al. 2013). However, rather than evolving a change to the direct target (Nav1.7), grasshopper mice have been shown to have amino acid changes on Nav1.8. These changes on Nav1.8 bind to another venom toxin, which induces numbness, negating painful effects of CvIV4, without altering its direct target, Nav1.7 (Rowe et al. 2013).

Similar to altered venom targets, this molecular adaptation seems to be associated largely with a small number of amino acid changes. Comparative work along with mutagenesis revealed that although a particular amino acid site determines most of the sensitivity to venom (resistance via analgesia) in grasshopper mice, this same residue is present across a diverse array of mammals, all with diverse natural histories that are not necessarily associated with venom exposure (Rowe et al. 2013). Thus, scorpion venom inhibition in grasshopper mice is likely an exaptation which perhaps has predisposed this species to exploit a toxic prey item (Rowe and Rowe 2006, 2008, Rowe et al. 2013).

Acquired immunity

Laboratory mice (Mus musculus) have been used to determine baseline toxicity of snake venoms for decades, but recent work suggests that mice may mount an immune response via mast cell (MC) activation when injected with venom from various snake species. Metz et al. (2006) found that MCs released carboxypeptidase A (CPA) that protected against systematic consequences of venom injections. Furthermore, MC-deficient mice have increased susceptibility to certain venoms (Schneider et al. 2007, Akahoshi et al. 2011, Marichal et al. 2013). Mouse IgE, FcεRI-expressing effector cells and MCs are all involved in acquired immunity to venom and can result in increased survival to lethal doses of venom (Starkl et al. 2016). However, this effect varies between strains of mice, suggesting that potential for acquired resistance via type 2 immunity is a trait that may be inherited, and as such subject to natural selection. These studies represent a mechanism for mitigation of venom morbidity and mortality previously undescribed for any species, including those for which venom resistance is well documented.

Though little is known about acquired immunity as a mechanism of venom resistance in free-living mammals, it may play a substantial role for both predators and prey that survive bites from venomous snakes. Additionally, ophiophagous mammals are known to eat the whole snake, including the venom glands (Almeida-Santos et al. 2000, Begg et al. 2003). This suggests that venom proteins come into contact with mucus

membranes before digestive enzymes are able to degrade them, thus providing an additional route of sub-lethal exposure which may subsequently serve to bolster immunity to venoms. Whether via mucosal or subdural (injected) exposure, this mechanism may serve to supplement general innate mechanisms of resistance described above (Mowat and Weiner 1999, Ogra et al. 2001).

Coevolution of resistance and venom at different evolutionary timescales

Coevolution operates and can be studied at different evolutionary timescales (Thompson 2005). At short timescales, coevolution plays out among geographically structured populations that are connected by gene flow, creating a selection mosaic across the landscape (Thompson 2005). This has been termed the Geographic Mosaic Theory of Coevolution, and its predictions have been supported in a variety of predator–prey systems (Hanifin et al. 2008, Nash et al. 2008, Toju 2008), where hot-spots of reciprocal section and cold-spots without it exist for all pairs of enemy species. Studies have documented population-level variation in venom resistance (Poran et al. 1987, Biardi 2008, Rowe and Rowe 2008, Biardi and Coss 2011, Holding et al. 2016, Pomento et al. 2016), but the possibility that this variation reflects geographically variable coevolutionary selection pressures requires parallel assessments of variation in both the venom and venom-resistance phenotypes to determine whether local venom variability selects for variable resistance, and vice versa (Janzen 1980, Gomulkiewicz et al. 2007).

At longer evolutionary timescales, phylogenetic analyses allow us to investigate the long-term dynamics of coevolution. Integrating phylogenetic predictions with molecular biology techniques for the expression and *in-vitro* testing of mutations and ancestral protein states permits assessment of the evolution of adaptive function. Thus, we can empirically test hypotheses about the mode and tempo of adaptive coevolution. Below we review the evolutionary and coevolutionary insights that can be gained through comparisons made at both timescales for species of venomous and resistant animals.

Short timescales—population-level variation

Population-level variation in venom composition is common (Chippaux et al. 1991, Daltry et al. 1996, Alape-Girón et al. 2008, Gibbs and Chiucchi 2011, Rokyta et al. 2015) but only a few studies have quantified intra-specific variation in levels of venom resistance (Poran et al. 1987, Poran and Coss 1990, Biardi et al. 2006, Biardi 2008, Rowe and Rowe 2008, Biardi and Coss 2011, Pomento et al. 2016). These few studies demonstrate a general pattern where the frequency of encounters with venomous enemies plays an important role in maintaining resistance (Fig. 3). For example, in the southern grasshopper mouse, one population that is sympatric with the Arizona bark scorpion was more resistant to the venom of this scorpion than a different population living without scorpions (Rowe and Rowe 2008). Similarly, squirrels seem to be more resistant when sympatric with rattlesnake predators. In the California ground squirrel (*Otospermophilus beecheyi*), LD_{50} and overall serum-to-venom binding scores are higher, and serum-based inhibition of metalloproteinase and hemolytic activity are more effective in areas with many snakes, compared to sites where snakes are absent or rare (Poran et al. 1987, Biardi et al. 2000, 2006, Biardi 2008). The closely related rock squirrel (*O. variegatus*) shows a similar pattern in its ability to limit adverse effects of venom fibrinolytic activity (Biardi and Coss 2011). Finally, serum from the eastern gray squirrel (*Sciurus carolinensis*) is more effective at inhibiting timber rattlesnake (*Crotalus horridus*) metalloproteinase activity in a population where the snakes occur, than in a population where the snakes are absent (Pomento et al. 2016).

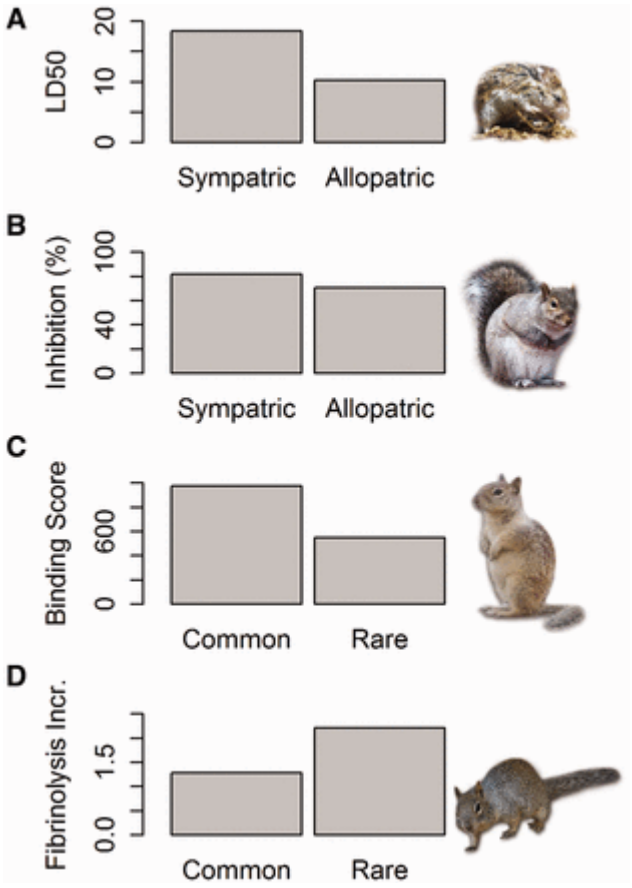

Figure 1.3- Various measures of venom resistance from the four prey species where resistance has been analyzed for variation at the population level, where populations were assayed that vary in terms of presence/absence or density of specific venomous predators. Each species shows a pattern of local adaptation where there is higher venom resistance in locations where selection from a specific venomous predator is expected to be stronger due to greater abundance of snake predators (A) southern grasshopper mice (*O. torridus*) require higher lethal doses of bark scorpion (*C. exilicauda*) venom when sympatric with the scorpions (Rowe and Rowe 2008); (B) eastern gray squirrels (S. carolinensis) are more effective at inhibiting SVMPs when sympatric with timber rattlesnakes (*C. horridus*, Pomento et al. 2016) (C) California ground squirrels (*O. beecheyi*) have higher serum to venom binding scores when in areas where northern pacific rattlesnakes (*C. oreganus*) are more dense (Biardi 2008); and (D) the serum of rock squirrels (*O. variegatus*) limits the increase in venom fibrinolytic activities observed when venom and serum are mixed when taken from areas where western diamondback rattlesnakes (*C. atrox*) are more common (Biardi and Coss 2011).

These population-level patterns suggest a significant role for local selection from venomous enemies in maintaining high resistance in the face of potential costs; however, the role of coevolution in these systems is less certain. For example, the encounter rate with scorpions may select for grasshopper mouse resistance, with no reciprocal evolutionary response in the scorpion population. Coevolution requires an evolutionarily

response in the venomous species to variation in some aspect of the resistance phenotype, and vice versa (Janzen 1980). Analysis of selection gradients and reciprocal selection (Lande and Arnold 1983, Brodie and Ridenhour 2003), tests for trait correlations among populations in each species (Hanifin et al. 2008), and analysis of whether trait variation reflects local adaptation to variation in the other species (Blanquart et al. 2013) can provide such support.

Case Study

To provide an example of using measures of local adaptation in venom and resistance to study coevolution, we describe our recent work on venomous rattlesnakes and their resistant squirrel prey (Holding et al. 2016). Local adaptation was measured using reciprocal crosses of venom from northern pacific rattlesnakes (C. oreganus) and blood serum from California ground squirrels collected among 12 populations where these species interact. The SVMP activity of venoms was measured twice, once on the venom alone, and again after incubation with squirrel serum containing inhibitors. This allowed venom inhibition to be scored as a measure of the snake–squirrel interaction in sympatric and allopatric combinations of venom and serum. The fully reciprocal cross of all 12 populations showed that the snakes possessed venom that is locally adapted to overcoming inhibition of metalloproteinase activity by squirrel serum factors, while the sign and magnitude of local adaptation varied across the sites in a way that is partly predicted by environmental variation. Whereas all rattlesnakes maintained some venom metalloproteinase activity following treatment with ground squirrel serum, populations of snakes retained more SVMP activity with local than with foreign ground squirrels (Fig. 4).

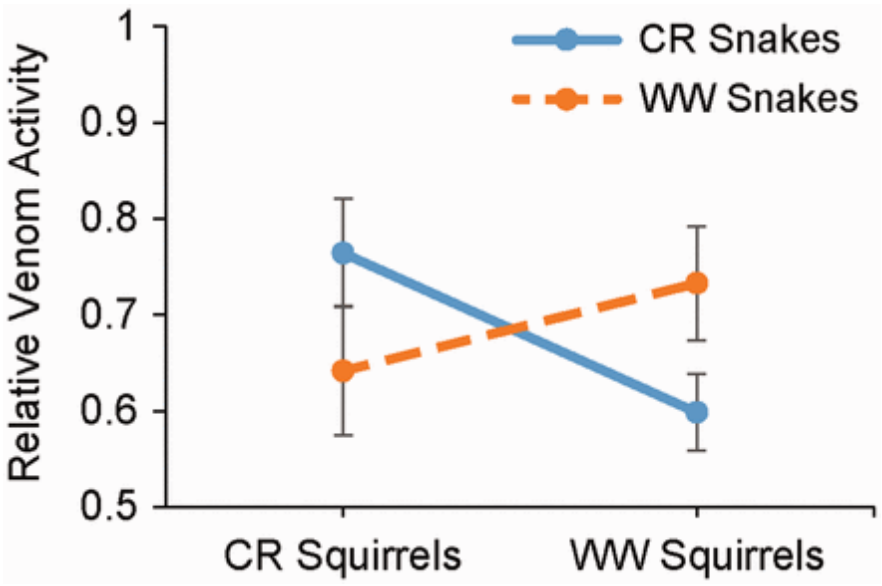

Figure 1.4- A pattern of rattlesnake local adaptation to avoiding metalloproteinase inhibitors in mammal serum from a reciprocal crossing study of two California populations of northern pacific rattlesnakes (*C. o. oreganus*) when paired with sympatric and allopatric California ground squirrel (*O. beecheyi*) serum. Shown is the percentage of venom metalloproteinase activity maintained after incubation with inhibitory serum relative to venom-only trials (mean ± SE). The venom of 10 snakes and 10 squirrels from each site was used, with snake venom and squirrel serum were randomly paired. Animals were collected from Wind Wolves Preserve (WW) and Chimineas Ranch Ecological Reserve (CR).

This work provides evidence that venom resistance does not just vary as a function of location, but that different venom and resistance phenotypes can show complex interactions in their effect on the outcome of envenomation. The predator-genotype × prey-genotype (G × G) interactions necessary for local adaptation, and G × G × environment interactions characteristic of geographic selection mosaics (Gomulkiewicz et al. 2007), seem to exist for venom and resistance in this system. Furthermore, the snake was the locally adapted species in this interaction, suggesting that the prey are not always ahead in coevolutionary interactions involving venom and resistance as suggested by the general Life-Dinner Principle (Dawkins and Krebs 1979). Finally, the existence of local adaptation merits a reappraisal of the idea that escalatory arms races generate much of the variation in venom and resistance, as such dynamics are not predicted to lead to local adaptation. Instead, the outcome of envenomation in this system appears to be determined by how effectively inhibitor X binds to venom protein Y. In the context of Fig. 2, the venomous species is hypothesized to benefit from a match to target and a

mismatch to inhibitors, antibodies, and repurposed venom receptors, while the opposite is true for the resistant species. These molecular matching mechanisms, which pit venom phenotypes against resistance phenotypes in a binding-avoidance-binding-seeking fashion (Dybdahl et al. 2014, Cagliani et al. 2016) are distinctly different in action from a quantity-based escalatory arms race. The discovery of a role for phenotype matching (as in phenotype matching versus phenotype differences: Ridenhour and Nuismer 2007) in coevolving venom and venom resistance proteins does not exclude a parallel role for arms race dynamics, which could govern the speed of enzymatic action or the overall concentration of each protein (Holding et al. 2016). Future work to characterize the role alternative coevolutionary dynamics in shaping the overall complex venom phenotype will be valuable.

Longer time-scales—phylogenetic and functional reconstruction

Understanding the evolution of the molecules involved in venom resistance can also benefit from the application of phylogenetic approaches at deeper evolutionary timescales. For example, the identification of multiple instances of convergent acquisition of a venom-resistant nAchR molecule was only revealed through a comparative phylogenetic study that included species of resistant and non-resistant mammals (Drabeck et al. 2015). Similarly, powerful phylogeny-based tests of positive selection (Yang et al. 2005, Yang 2007) have revealed instances of positive selection on particular amino acid sites in particular venom-resistant lineages (Jansa and Voss 2011, Drabeck et al. 2015). Robust phylogenies also provide the essential framework for reconstructing ancestral character states, including ancestral protein sequences (Pauling and Zuckerkandl 1963). Integrating these types of phylogenetic approaches with laboratory studies of ancestral protein function has been coined the “functional synthesis” (Dean and Thornton 2007), and provides much promise for understanding the evolution of protein function across diverse systems (for review see Hartley et al. 2006, Hoekstra et al. 2006, Dean and Thornton 2007, Harms and Thornton 2013)

While the phylogenetic approaches mentioned above have laid the groundwork in this field of research, the promise of the functional synthesis has yet to be applied to understanding the evolution of proteins involved in venom resistance. Golding and Dean (1998) suggest the ideal candidates for this empirical functional approach are those in which there is a clear and measurable physiological shift, strong evidence for selection, and robust phylogenetic histories. With the growing number of identified instances of venom resistance in mammals (reviewed in Voss and Jansa 2012), case studies of adaptive evolution of venom-resistant molecules (Jansa and Voss 2011, Drabeck et al. 2015), the explosive growth of phylogenetic knowledge for mammals, and the development of heterologous expression systems for these proteins, we are now poised to apply the functional synthetic research program to understanding how resistance evolves (Appendix figure 1.1). Although much of this research is in its infancy, below we highlight a case approach which shows how the functional synthesis could be applied to understanding the evolution of molecules involved in venom toxicity and resistance.

Case study

Using an existing system as an example, we will expand on one case outlined in Appendix figure 1.1 for which the functional synthetic approach is tractable. Specifically, molecular models suggest that vWF can no longer bind botrocetin, a venom C-type lectin (CTL), in resistant opossums (Jansa and Voss 2011), and recent physiological assays provide strong evidence that opossum vWF in fact does not respond to very high doses of botrocetin (D. H. Drabeck, unpublished data). Of the nine species of didelphid marsupials in the clade Didelphini, seven are either known to eat venomous snakes and/or be resistant to vWF-binding venom CTLs. Though members of this clade show accelerated adaptive evolution at the CTL binding site (vWF A1), sequence variability at these sites is present between species, suggesting that there may be functional variability in vWF resistance across Didelphini (Jansa and Voss 2011). A robust species-level phylogeny for New World opossums makes it possible to employ powerful modern phylogenetic tools to infer ancestral protein sequences of vWF for all members of this clade. Biochemical assays that quantify binding affinities of opossum vWF for venom CTLs can provide functional data for each

amino acid site of this protein, including its ancestral states, across the clade on which it has evolved. These data can illuminate the molecular and functional tempo and mode of evolution of adaptive traits. For example, Fig. 5 illustrates two competing hypotheses for the evolution of a resistant target to a venom toxin. In the first hypothesis evolution of resistance is gradual, and mutations appear progressively (and perhaps convergently) along the lineages leading to the resistant phenotype. In the alternative scenario, evolution is saltational—the mutations responsible for resistance all arise at the base of the resistant clade, and subsequent mutations (if any) have no effect on the resistance phenotype. Importantly, the only way to distinguish between these two hypotheses is through functional laboratory studies of reconstructed ancestral proteins. If resistance evolved suddenly at the base of the clade, then ancestral proteins will not vary in their binding ability and will have similar binding ability as the modern proteins, all of which exhibit the resistant phenotype. Alternatively, if acquisition of resistance is a gradual process, then ancestral proteins should vary in their ability to bind the toxin protein.

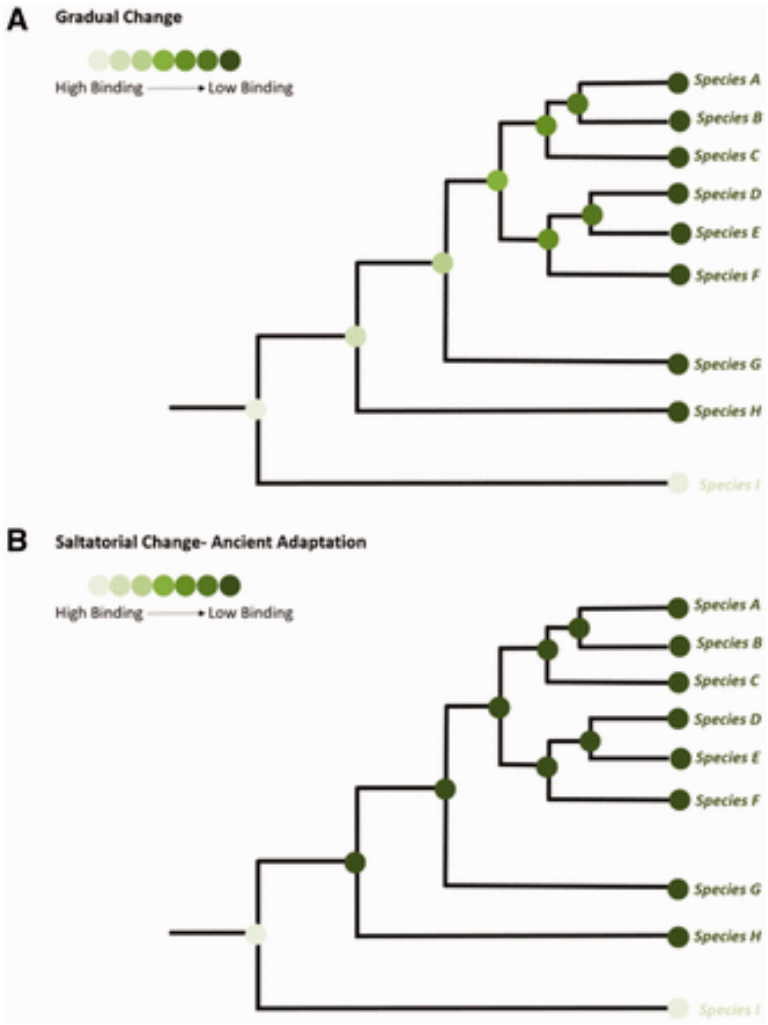

Figure 1.5- Alternative hypotheses for the evolution of a venom-resistant protein. Under the gradual-change hypothesis (A), venom resistance is acquired gradually during the evolution of the venom-resistant clade. Under the saltatorial-change hypothesis (B), venom resistance (measured as ability to bind a venom toxin) is acquired once at the base of the venom-resistant clade. Under both scenarios, extant taxa have equivalently low binding affinities to the venom toxin (indicated with dark green dots at the tips of the tree). The only way to distinguish between the two hypotheses is to assess binding affinity for reconstructed ancestral proteins, which will vary in their binding affinity under hypothesis (B), but not hypothesis (A).

Applying these same methods to the evolution of interacting snake venom proteins, such as botrocetin, would allow us to examine the functional evolution of a potentially co-evolving molecule. Work like this would provide the first example of a hypothesized coevolutionary interaction in which both interacting partners have been functionally characterized at the molecular level, and would serve to test assumptions about how evolutionary and ecological mechanisms shape functional changes in

ostensibly coevolving proteins (Gomulkiewicz et al. 2007, Ridenhour and Nuismer 2007). In particular, the simplest but most important assumption to test is whether there is evidence of reciprocal evolutionary change in molecular function in a natural system, which has long been an assumption of many canonical studies of coevolution, but has yet to be demonstrated (Bull and Molineux 1992, Brockhurst et al. 2003, Mizoguchi et al. 2003, Jessup et al. 2004, Hanifin et al. 2008, Jansa and Voss 2011, Scanlan et al. 2011). Venom resistance is a system which is plentiful with opportunities such as this for evolutionary biochemists, as there are several other putative protein–protein coevolutionary interactions (Supplementary Table 1), and likely many more to come as this field develops.

Future directions

Integrated phenotypes and the evolving resistome

The "resistome" can be considered an "integrated phenotype" in the same sense that the term has been applied to a wide variety of morphological traits (Murren 2012). Specifically, an integrated phenotype is defined as a set of functionally related traits that interact with each other in a way that affects their overall function (Murren 2012). The "resistome" then is the collection of molecules that confer venom resistance through the mechanisms outlined above. Methodologically, the use of "antivenomics" (Calvete et al. 2011) to isolate individual molecular components of resistance and then use them in *in-vitro* tests of function involving single versus multiple components allows us to address how these parts of the "resistome" might interact to confer organismal resistance. Are these interactions functionally synergistic or simply additive (Yeh et al. 2006)? Can information on functional interactions between proteins be used to organize individual molecular components of resistance into functionally defined modules (Yeh et al. 2006)? Do components of resistance show negative tradeoffs with other fitness-related traits in prey that could limit the evolution of overall resistance? Direct estimates of function are difficult to conduct for components of morphological traits, and so we see a special role for resistance (and venom) for assessing phenotypic integration in complex phenotypes from a functional perspective.

This “antivenomics” approach is a specific example of the general approach of using high-throughput sequencing, modern bioinformatics, and comparative analysis to identify the molecular basis of adaptive traits. Such an approach can also provide a guide for researchers to identify venom targets and inhibitors that are currently undetected. Subsequent comparative studies of these newly identified molecules holds enormous potential for understanding the molecular evolutionary processes involved in generating and maintaining variation in venoms as well as in venom resistance. The application of these and other integrated methods requires robust species and gene phylogenies, which are becoming increasingly common. Additionally, for both venom and resistance proteins which are members of large protein families and purported to be rife with gene duplication and neofunctionalization, it is vital to understand complete gene histories to apply methods such as ancestral reconstruction of phenotypes and tests of positive selection. Though only a few mechanisms resistance are known and even fewer examined in detail, applying these methods will doubtless illuminate the way forward in this field.

Summary

The hypothesis that venom and resistance are coevolved traits has been invoked because of the matched nature of these traits, yet evidence for reciprocal selection leading to evolutionary change in both venom *and* resistance traits has yet to be convincingly demonstrated. Here, we suggest several approaches which aim to provide strong evidence for reciprocal molecular coevolution in the context of venom and venom resistance evolution. Population level approaches which utilize reciprocal pairwise testing, analyses of selection gradients, and tests for trait correlations, can identify sources and strength of selection, potential costs and trade-offs, and examine statistical support for correlated adaptive variation. Evolutionary biochemical approaches allows the examination of the ancestral functional changes of proteins involved in venom and resistance, elucidating the mode and tempo of (co)adaptive change. Expanding the examination of single venom-target interactions to understanding the full complexity of venom resistance is necessary to examine this trait as an integrated molecular phenotype, and is now feasible with

modern high-throughput approaches. Given these insights, we stress the value of understanding venom resistance as a coevolving adaptive trait, and its potential for providing insights as to how coevolution occurs at a molecular level.

Acknowledgements

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CHAPTER 2. WHY THE HONEY BADGER DON'T CARE: CONVERGENT EVOLUTION OF VENOM-TARGETED NICOTINIC ACETYLCHOLINE RECEPTORS IN MAMMALS THAT SURVIVE VENOMOUS SNAKE BITES[2]

Introduction

Among the handful of mammals known to be resistant to venomous snake bites, the honey badger (*Mellivora capensis*) has a near legendary ability to attack venomous snakes (Elapdiae and Viperidae) and survive their toxic bites (Begg et al., 2003, Hughes et al., 2013, Waxman, 2014). However, the mechanisms by which resistant species, including honey badgers, are rendered invulnerable to snake bites are largely unknown. Most research into mammalian venom resistance has focused on blood-serum factors that neutralize venom metalloproteinases and phospholipases (e.g., Catanese and Kress, 1992, Lovo-Farah et al., 1996, Melo and Suarez-Kurtz, 1988, Menchaca and Perez, 1981, Neves-Ferreira et al., 2010, Perez et al., 1979, Tarng et al., 1986). Comparatively few studies have focused on the role that modified venom targets play in conferring toxin resistance. These venom targets are physiologically important protein receptors that no longer bind venom toxins, yet retain the ability to bind their endogenous ligands (Barchan et al., 1992, Barchan et al., 1995, Jansa and Voss, 2011).

The muscular nicotinic acetylcholine receptor (nAChR), a well-characterized transmembrane receptor that mediates synaptic transmission from nerves to muscles, is targeted by α-neurotoxins present in the venom of elapid and hydrophid snakes (Barchan et al., 1995, Neumann et al., 1986). Two mammals known to survive elapid bites, the Egyptian mongoose (Herpestes ichneumon) and the hedgehog (Erinaceus concolor), have mutations in the α1-subunit of their nAChR proteins that eliminate binding of the krait (Bungarus multicinctus) venom toxin α-bungarotoxin (Fig. 1B) (Asher et al., 1997, Asher et al., 1998, Barchan et al., 1992, Barchan et al., 1995, Haggerty and Froehner, 1981, Kao et al., 1984, Takacs et al., 2001, Takacs et al., 2004). Cobra-nAChR receptors (*Naja spp*.)

[2] Reproduced with permission from Drabeck, D. H., Dean, A.M., and S. A. Jansa. 2015. Why the honey badger don't care: Convergent evolution of venom-targeted nicotinic acetylcholine receptors in mammals that survive venomous snake bites. Toxicon 99(1): 68-72

also fail to bind α-neurotoxins, are immune to their own venom, and have convergently acquired similar amino acid replacements (Takacs et al., 2001, Takacs et al., 2004).

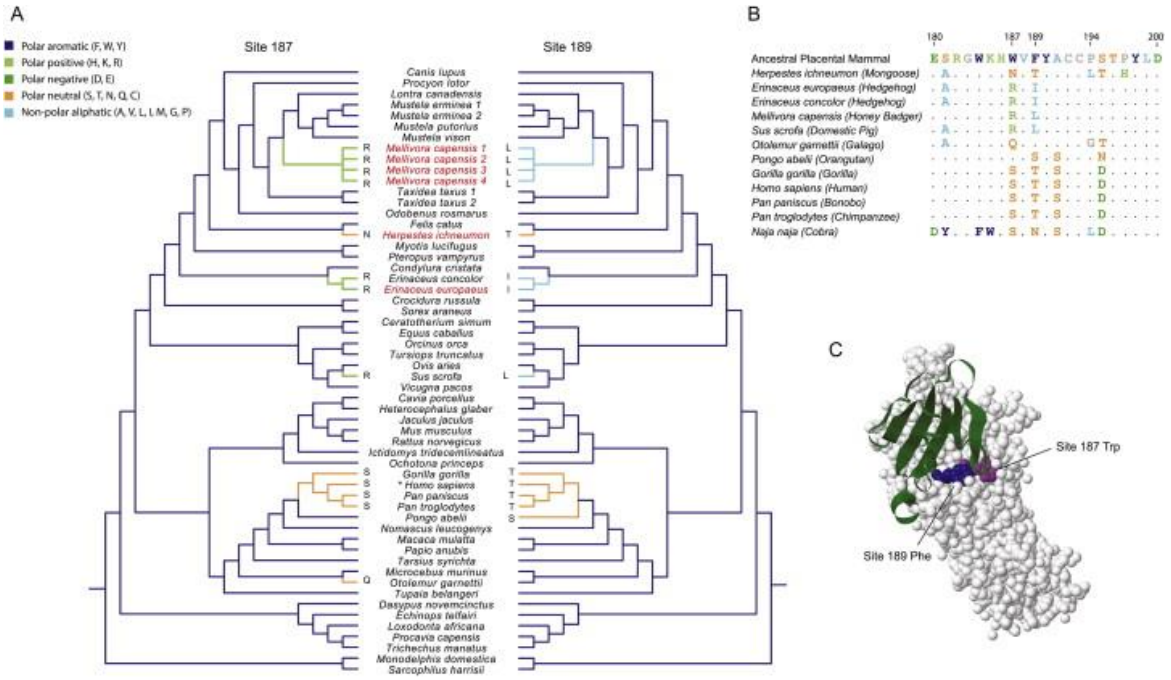

Figure 2.1- A) The evolutionary tree of mammals depicting relationships among species that have been sequenced for the α1 subunit of the nicotinic acetylcholine receptor gene (CHRNA1). The two trees are topologically identical, ancestral-state reconstructions of amino-acid sites 187 (left-hand tree) and 189 (right-hand tree) are indicated with colored branches (legend upper left). The derived amino acid is given at the branch terminus for species that differ from the ancestral condition (tryptophan and phenylalanine for sites 187 and 189, respectively). Species that are known to survive envenomation by elapid snakes (honey badger, mongoose, hedgehog) or have been shown experimentally to have lost (mongoose, hedgehog) binding between α-bungarotoxin and the nAChR receptor are shown in red, the reduced binding ability of human is indicated with an asterisk. (B) Alignment of the α-bungarotoxin-binding region of nAChR (Barchan et al., 1995) for the 11 mammal species that have lost one or both ancestral aromatic residues at sites 187 and 189. The reconstructed ancestral placental sequence is shown for reference as is the sequence from cobra. Dots indicate sequence identity with this ancestral sequence. (C) Molecular model (Dellisanti et al., 2007a, Dellisanti et al., 2007b, UniProt Accession 2QC1) of the α1 subunit of the nicotinic acetylcholine receptor (spacefill model) shown binding with α-bungarotoxin (green ribbon model). Sites 187 and 189 are shown as the ancestral amino acid for placental mammals.

Honey badgers have been observed to survive bites from puff adders (*Bitis spp*.) (Colleen Begg, pers. comm.), have been anecdotally reported to survive arterial injections of black mamba venom (Rousseau, 1982), and have a diet comprising up to 25% venomous snakes, including puff adders (*Bitis arietans*) and cape cobras (*Naja nivea*)

(Begg et al., 2003). However, the biochemical basis of their resistance to neurotoxic venom has not been examined.

To investigate the molecular basis of resistance to neurotoxic venoms in honey badgers, we examined the region of the nAChR receptor that contains the α-neurotoxin binding site and which has been previously implicated in venom resistance (Haggerty and Froehner, 1981, Kao et al., 1984). We asked whether the honey badger has independently acquired the same amino acid changes that alter toxin binding in other neurotoxin-resistant species, specifically, the presence of non-aromatic residues at position 187 and 189 of the nAChR α1 subunit. We sequenced this region of the nAChR receptor from honey badgers and other closely related but venom-susceptible mustelids. To extend the comparative context for the study, we retrieved 45 mammalian DNA sequences of the α1 subunit of the muscle cholinergic receptor gene (CHRNA1) from publically available sequence databases. We then employed a comparative phylogenetic approach to infer the molecular evolution of this receptor across a wide range of mammalian species, including those with known resistance and susceptibility to elapid venom.

Materials and Methods

Whole blood samples from four individuals of M. capensis were provided by the San Diego Zoo Institute for Conservation Research (2 samples) and the Fort Wayne Zoo (2 samples) (Fort Wayne, IN). We also sequenced five additional carnivores that are not known to be resistant to any snake venoms. Tissue samples from Lontra canadensis (J.F. Bell Museum tissue collection number MP137), Procyon lotor (MP444), Mustela erminae (MP409, MP410), and Mustela vison (MP0083) were obtained from the Bell Museum of Natural History (University of Minnesota), tissue samples of Taxidea taxus, were kindly gifted by Dr. Emily Latch (University of Wisconsin, Milwakee). Genomic DNA was extracted from these samples using a QIAGEN DNeasy kit according to the standard protocols for blood and animal tissue (Qiagen, Inc., Valencia, CA, USA). We designed primers to amplify an 850 bp piece of the alpha subunit of the muscular nicotinic

acetylcholine receptor gene (CHRNA1) that included the ligand binding site corresponding to residues 122–205 of the protein sequence. Polymerase chain reactions (PCRs) were carried out in 25 uL reactions using 1.0 µl of 10 mM ACH_F1 (5′-TGCAGATGGTGACTTTGCCATTGTCAAG-3′) primer solution, 1.0 µl of 10 mM ACH_R1 (5′AGTCTGTGGGCAGGTAGAACACC-3′) primer solution, 0.125 uL GoTaq polymerase (Promega Inc.), and recommended concentrations of GoTaq Green Buffer, MgCl2, and dNTPs. Reactions were performed for thirty cycles of melting at 94 °C for 30 s, followed by annealing at 58 °C for 15 s, and extension at 72 °C for 90 s. Reactions were preceded by a 2 min denaturation at 94 °C and included a final extension at 72 °C for 7 min. Amplified PCR products were sequenced by Beckman Coulter Genomics on an ABI 3730XL DNA Analyzer using BigDye Terminator v3.1 chemistry (Applied Biosystems, USA). Resulting sequences were assembled, edited, and aligned using Geneious version 5 (Drummond et al., 2010). Sequences generated for this report have been submitted to GenBank.

We searched GenBank and Ensembl for all available mammalian CHRNA1 sequences (Appendix Table 2.1). We aligned our mustelid DNA sequences along with these sequences using MUSCLE (Edgar, 2004) with default parameters specified by Geneious (v 5.5) and translated the resulting aligned DNA sequences to amino acids (Appendix Figure 2.1). We used recently published mammalian phylogenies (Meredith et al., 2011, Koepfli et al., 2008) to construct a tree describing well-established evolutionary relationships among the taxa that had CHRNA1 sequences. We used this phylogeny to infer ancestral amino acids using parsimony analysis as implemented in MESQUITE (Maddison and Maddison, 2011) and as the basis for tests of positive selection on the CHRNA1 gene. We tested for selection on the CHRNA1 gene in the three lineages that are known to be resistant to snakebite (H. ichneumon, Erinaceus sp., and M. capensis) using the branch-site tests for selection in the codeml program of PAML 4.8 (Yang, 2007). We identified these three lineages on the mammalian phylogeny as “foreground” branches that could have sites under selection and designated the remainder of the tree as “background” branches. The selection test compares the difference in ln-likelihood

values of a model that allows a proportion of positively selected sites on foreground branches ($\omega 2 > 1$) with one that does not allow positive selection on those branches by fixing $\omega 2 = 1$ (Zhang et al., 2005). We also used a Bayes-Empirical-Bayes (BEB) method to identify sites in the protein that had a high posterior probability of being under positive selection (Yang et al., 2005).

Results and Discussion

Phylogenetic analysis reveals that most placental mammals have aromatic residues at sites W187 and F189, and that the ancestral state for both of these sites optimizes as an aromatic amino acid (Fig. 1A). Selection tests strongly support a model that allows for a proportion of positively selected sites for CHRNA1 in Herpestes, Mellivora, and Erinaceus, the three lineages that are known to be resistant to snake venoms and to eat venomous snakes ($2\Delta\ell = 12.9$, $df = 1$, $p << 0.01$, Table 1). Approximately 3% of sites are identified under this model as being under positive selection in these lineages ($\omega 2 = 25.95$, Table 1), and the Bayes-Emprical-Bayes approach identifed sites 187 and 189 as under positive selection with posterior probabilities of 0.995 and 1.000, respectively.

Table 2.1- Results of branch-site tests for positive selection on the CHRNA1 gene.

Model and log-likelihood	site class[a]	proportion of sites	ω background	ω foreground[b]
$\omega_2 = 1$	0	0.840	0.0196	0.00196
lnL = -1958.65	1	0.070	1.0	1.0
	2a	0.083	0.0196	1.0
	2b	0.007	1.0	1.0
$\omega_2 > 1$	site class	proportion of sites	ω background	ω foreground[b]
lnL = -1952.19	0	0.898	0.0218	0.0218
	1	0.074	1.0	1.0
	2a	0.026	0.0218	25.95
	2b	0.002	1.0	25.95

[a] Site class 0 and 1 apply to foreground and background lineages and include sites under purifying selection ($0 < \omega < 1$) and neutral sites ($\omega = 1$), respectively. Site class 2 allows a proportion of positively selected sites ($\omega > 1$) in the foreground lineages, where 2a includes sites under purifying selection ($0 < \omega < 1$) in the background lineages and 2b includes neutral sites ($\omega = 1$) in the background lineages.

[b] Lineages known to eat snakes and/or to be resistant to snake venom (i.e. *Herpestes ichneumon, Erinaceus* sp., and *Mellivora capensis)* were included in the foreground class.

Honey badgers, hedgehogs, and pigs

Ancestral state reconstruction shows that the honey badger (M. capensis) and the two hedgehog species (*Erinaceus concolor* and *Erinaceus europaeus*) have independently replaced both ancestral aromatic residues with an arginine and a leucine or isoleucine (W187R, F189L/I, Fig. 1A). Intriguingly, the pig lineage (*Sus scrofa*) has the same replacements as the honey badger (W187R, F189L, Fig. 1A). Experimental work on nAchR function has not included pigs, and it is unknown whether the molecule is capable of binding alpha-neurotxins in this species or not. For this reason we did not identify the

pig lineage as one of the potential lineages under selection in the positive selection tests. Nevertheless, anecdotal evidence suggests that domestic pigs will prey on a variety of venomous snakes and survive snakebite, wild pigs and neurotoxic elapid snakes also share an historical distribution (Calmette, 1908, Gifford-Gonzalez and Hanotte, 2011, Giuffra et al., 2000, Klauber, 1972, McDougall, 1997). While most accounts of venom resistance in pigs attribute this trait to their thick skin and a large layer of subcutaneous fat, our results support early accounts that pigs have some endogenous immunity to neurotoxic snake venom (Calmette, 1908, Grasset et al., 1935).

Prior experimental work has attributed the hedgehog's ability to survive high doses of α-bungarotoxin —four times the LD50 for mice — to replacements of ancestral aromatic residues at these sites (Barchan et al., 1992, Barchan et al., 1995). Supporting this, cloned α1 nAChRs and muscle extracts from hedgehogs fail to bind to α-bungarotoxin (Barchan et al., 1992, Barchan et al., 1995, Domont et al., 1991, Herter, 1965, Reeve, 1994). Experimental studies further demonstrate that replacements at sites 187 and 189 reduce affinities for long and short chain α-neurotoxins (Asher et al., 1997, Asher et al., 1998, Barchan et al., 1992, Barchan et al., 1995, Dellisanti et al., 2007a, Dellisanti et al., 2007b, Dimitropoulos et al., 2011, Neumann et al., 1986, Takacs et al., 2001, Takacs et al., 2004). Loss of both aromatic residues is seen in all cases of reduced α-neurotoxin binding (Neumann et al., 1986, Tzartos and Remoundos, 1990), although the degree to which binding is reduced may depend on the particular replacement and the genetic background on which it arises (Dellisanti et al., 2007a, Dellisanti et al., 2007b, Dimitropoulos et al., 2011, Takacs et al., 2001). In particular, structural analyses suggest replacing the uncharged tryptophan residue at site 187 with the positive guanidinium side chain of arginine is likely to abolish binding through charge interference (Fig. 1C) (Dellisanti et al., 2007a, Dellisanti et al., 2007b, Takacs et al., 2004). Thus, it appears that these three mammalian lineages (honey badgers, hedgehogs, and pigs) have convergently evolved the same mechanism to disrupt neurotoxin binding at their nAChR receptors.

Mongooses and cobra

Mongooses survive α-bungarotoxin at ten times the LD50 for mice, prey upon venomous snakes, and have α1 nAChR receptors that fail to bind to α-bungarotoxin from the krait (*B. multicinctus*) (Barchan et al., 1992, Barchan et al., 1995). However, mongooses do not share the same amino-acid replacements at sites 187 and 189 that are present in hedgehogs, honey badgers, and pigs (Fig. 1A). Rather, the W187N substitution in mongooses is thought to abolish α-neurotoxin binding through steric hindrance, caused by glycosylation of the asparagine residue (Barchan et al., 1995, Dellisanti et al., 2007a, Dellisanti et al., 2007b, Takacs et al., 2001). The same mechanism has been well documented in the cobra sequence (F189N), where elimination of glycosylation rendered mutants venom-sensitive (Takacs et al., 2004). Thus, it seems that resistance to α-neurotoxins at the nAChR has convergently evolved via steric hindrance in the mongoose and cobra, distinct from the charge disruption observed in hedgehogs, honey badgers, and pigs.

Primates

African great apes (members of Homininae) have non-aromatic replacements W187S and F189T, the galago (Otolemur) has W187Q only, and the orangutan (Pongo) has F189S only (Fig. 1A). The threonine residue at position 189 and the presence of a polar neutral residue at position 187 is convergent between African great apes and mongooses (*H. ichneumon*). However, great apes do not share the derived glycosylation site present in mongooses and are not immune to α-neurotoxins. Though cloned human nAChR has reduced affinity for α-bungarotoxin (intermediate between mongoose and mouse), putatively due to loss of these aromatic amino acids, it is unlikely that these specific replacements confer any selectively relevant toxin resistance (Barchan et al., 1995). Little is known about venom resistance in galago or the orangutan, or how replacements F189S or W189Q alone on these genetic backgrounds might affect neurotoxin binding.

Conclusions

Our results extend the known cases of convergent evolution at venom-targeted nAChR receptors to include the honey badger and domestic pig. The honey badger and pig lineages independently acquired replacements that are biochemically similar to those seen in the hedgehog: W187R (all three species) and F189L, (L in honey badger and pig, I in hedgehog). In mongoose, substitutions at these same sites also occur, but they eliminate α-neurotoxin binding through steric hindrance via glycosylation rather than charge interference via introduction of a positively charged amino acid. Hence, evolution of resistance in venom-targeted nAChR receptors shows that convergence in function at the same sites can be mediated through distinct biophysical mechanisms.

The ecological consequence of snake-venom resistance in mammalian predators is not trivial as hedgehogs, honey badgers, mongooses, and pigs are able to prey upon and survive bites from venomous snakes (Barchan et al., 1995, Domont et al., 1991, Herter, 1965, Reeve, 1994). Notably, resistance to venom may present these species with opportunities to exploit a valuable prey resource that is unavailable to most (venom-susceptible) mammals (Voss and Jansa, 2012).

This study adds to examples of convergent molecular evolution revealed by phylogenetic analyses, and is suggestive of a convergent adaptive function that has arisen in the face of similar ecological selection pressures (Hartley et al., 2006, Hiebl et al., 1987, Hoekstra et al., 2006, Jessen et al., 1991, Kornegay et al., 1994, Nachman et al., 2003, Shi et al., 2001, Shi and Yokoyama, 2003, Yokoyama, 1998, Yokoyama and Tada, 2000, Zhang, 2006). Convergent substitutions at loci known to alter protein phenotype implicate the predator–prey relationship between the honey badger and venomous snakes in driving selection for adaptations to cope with snake venom α-neurotoxins. These and other recent works (Jansa and Voss, 2011, Voss and Jansa, 2012) suggest that venomous snakes and resistant mammalian predators may be engaged in an important coevolutionary arms race. However, further research is merited to confirm the function of these modified receptors, and to elucidate if snake venom evolution has reciprocated.

Acknowledgments

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CHAPTER 3. EXAMINING THE RESISTANCE OF SOUTH AMERICAN OPOSSUMS TO VWF-BINDING VENOM C-TYPE LECTINS.

Introduction

Naturalists have long known that certain opossum species (in Tribe Didelphini of the marsupial family Didelphidae, Jansa and Voss 2011), are not only resistant to snake venom but also attack and eat pit-vipers with impunity (Oliveira and Santori 1999, Jared et al. 1998, Perez et al. 1978). This tribe includes the South American opossum genera *Didelphis*, *Philander*, *Lutreolina*, and *Chironectes* (Figure 3.1). Although venom resistance has not been broadly surveyed across opossum species, observational (Vellard 1945, Wood 1954, Fitch 1960, Perales et al. 1986, Melo and Suarez-Kurtz 1988, Sazima 1992, Jared et al. 1998, Oliveira and Santori 1999, Almedia-Santos et al. 2000) and experimental (Kilmon 1976, Werner and Vick 1977, Werner and Faith 1978, Mousattche et al. 1978, 1979, Perez et al. 1979, Menchaca and Perez 1981, Soto et al. 1988, Moussatche and Perales 1989, Catanese and Kress 1993, Perales et al. 1994, Lovo-Farah et al. 1996) evidence suggest that species of *Didelphis*, *Philander*, and *Lutreolina* can survive envenomation by pitvipers (Jansa and Voss 2011). Whether *Chironectes* - the fourth genus in this clade - is venom resistant remains unknown. *Metachirus nudicaudatus*, the sister taxon to Didelphini, has been shown to have little if any resistance to whole venom injections (Perales et al. 1994). Consequently, this species and

many other small-bodied opossums (the remainder of family Didelphidae) known to be eaten by pitvipers (Voss 2013) are assumed to be susceptible to venom. Thus, it appears that snake-venom resistance has arisen at least once among South American opossums (in clade Didelphini), possibly as a dietary adaptation allowing them to exploit a dangerous prey item otherwise unavailable to non-resistant predators.

Venom resistance is a complex physiological trait which requires the neutralization of a diversity of biologically active venom peptides. Typically, venoms comprise proteins recruited from upwards of 14 protein families, resulting in venom cocktails containing as many as 40 different toxins (Calvete 2010, Voss and Jansa 2012, Fry 2012). Nearly all viper venoms include a combination of both enzymatic tissue damaging proteins (e.g. metalloproteinases, phospholipases), as well as integrin-like proteins that target and disrupt specific physiological functions (Calvete 2010). Because of the diverse function of venom components, venom resistance is also likely a complex suite of traits involving multiple mechanisms of toxin neutralization and target alteration. Venom resistance for opossums within Didelphini has been convincingly demonstrated by numerous *in vivo* and *in vitro* assays and is derived at least partially from specialized enzyme inhibitors present in the plasma (Kilmon 1976, Werner and Vick 1977, Perez et al. 1979, Moussatché and Perales 1989). While the biochemical basis of resistance to venom metalloproteinases and phospholipases has been well characterized, relatively little is known about how these opossums cope with other (non-enzymatic) venom protein classes.

Non-enzymatic venom proteins are known to cause significant destructive physiological effects in susceptible species, thus adaptations which counter these effects are likely vital to a successful venom-resistant phenotype (Read et al. 1993, Qi et al. 1994, Barchan et al. 1995). A particularly destructive class of these proteins known to be abundant in South American vipers are venom C-type lectins (CTL) (Arlinghaus and Eble 2012). A small subgroup of venom CTLs bind the mammalian blood protein von Willebrand Factor (vWF) to form a complex that binds, in turn, to the platelet-associated glycoprotein GP1bα, preventing blood clotting (Fukuda et al. 2002). This specific action

enhances the effects of other hemorrhagic venom factors and is thought to be responsible for the devastating systemic bleeding characteristic of snake bites from several species of *Bothrops* vipers. A wide diversity of mammalian taxa have been shown to be susceptible to vWF-targeting CTLs, including primates, carnivores, rodents, lagomorphs, bovids, and perissodactyls, in both *in vitro* and *in vivo* assays. Similarly, in the presence of an altered vWF (von Willebrand's disease; vWD) in pig and dog, the coagulopathic function of vWF-binding CTLs is abolished (Nichols et al. 2010, Sanders et al. 1995). Known venom CTLs with this function include botrocetin (from *Bothrops jararaca*), aspercetin (from *Bothrops asper*), and biticetin (from *Bitis arietans*). Although surveys of vWF binding activity have identified several additional species of *Bothrops* with vWF-binding activity, no other venom CTLs have been isolated and described (Arlinghaus and Eble 2012, Rucavado et al. 2001, Read et al. 1978).

While it is currently unknown how venom-resistant opossums cope with CTLs like botrocetin and aspercetin, recent research on the molecular evolution of vWF in marsupials has revealed that members of Didelphini show accelerated evolution in the vWF A1 region at sites that bind botrocetin (Jansa and Voss 2011). Although signatures of positive selection suggest adaptive function and possible coevolution, we still require explicit evidence that species with rapidly evolving vWF are physiologically resistant to vWF-targeting venom CTLs. Physiological resistance, in this case, is the failure of platelets to aggregate – measured by the quantity of platelets that remain suspended in solution – in the presence of venom CTLs.

In this work, we test the prediction that members of Didelphini – especially those that are known to prey on pitvipers – exhibit physiological resistance to venom CTLs (Jansa and Voss 2011). We expect opossums that eat pitvipers to show physiological resistance to CTLs from the species they prey upon (*Bothrops jararaca*: botrocetin, *Bothrops asper*: aspercetin), but not to CTLs from viper species with which they do not share a current or historical range (e.g. the African viper *Bitis arietans*: bitiscetin). Moreover, to assess whether vWF-mediated resistance to venom CTLs id restricted to Tribe Didelphini, we also assay a smaller-bodied species (*Monodelphis domestica*) likely

preyed upon by pitvipers and assumed to be susceptible to venom. We utilize a well-established *ex vivo* measure of vWF-mediated platelet aggregation to measure the aggregation response of opossum platelets to vWF-targeting venom CTLs both in the presence and absence of plasma to exclude the influence of any unknown CTL-inhibiting plasma proteins. These assays are well correlated with *in vivo* aggregation response, represent a strong predictor of organismal coagulopathy (Read et al. 1983, Nichols et al. 2010), and provide the first evidence of physiological resistance to venom CTLs in opossums.

Materials and Methods

Study Design

Species were selected for our assays based on their membership to clade Didelphini (Figure 3.1), as well as their availability for whole-blood draws. We used two opossum species from the clade Didelphini hypothesized to be venom resistant (*Didelphis virginiana* N=3, and *Didelphis aurita* N=4), as well as one presumably non-resistant species outside Didelphini (*Monodelphis domestica* N=2). Both *Didelphis virginiana* and *Didelphis aurita* were shown to have vWF that is evolving at an accelerated rate with respect to the remainder of Didelphidae (Jansa and Voss 2011). These species are also well known to exhibit organismal resistance to venom (Voss and Jansa 2012). *Monodelphis domestica* belongs to a distantly related genus of opossum that is not part of the clade shown to have accelerated vWF evolution, though this exact species was not included in previous work examining rates of vWF evolution (Figure 3.1). However, *M. domestica* belongs to a clade not expected to exhibit venom resistance and is a readily available laboratory animal.

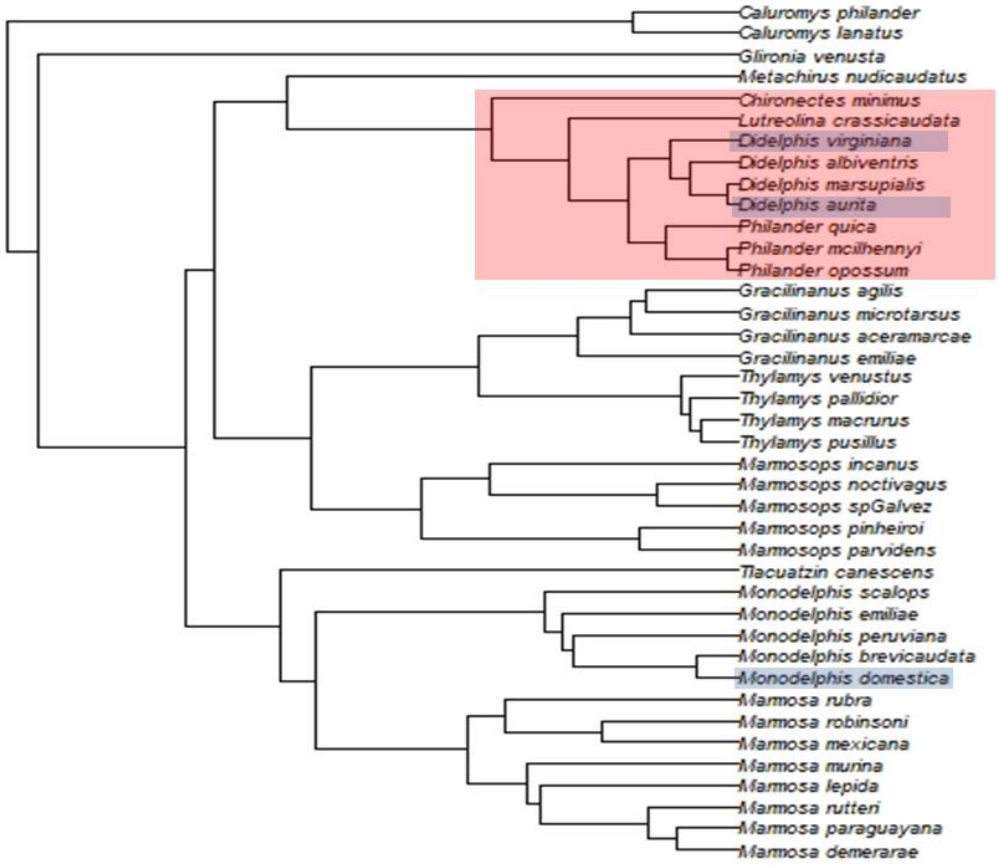


Figure 3.1 A phylogeny of didelphid opossums used for examination of rates of vWF evolution in Jansa and Voss (2011) with the addition of *Monodelphis domestica*. The clade Didelphini, known for both organismal venom resistance as well accelerated evolution of vWF, is shown in red (light grey in black and white prints). Species used for this work are highlighted in blue (dark grey in black and white prints).

For our assays using opossum blood, we adapted a standard method of vWF-mediated platelet aggregation used extensively in biomedical studies of vWF function in humans (Rucavado et al. 2001, Hamako et al. 1996, Coller et al. 1975, Read et al. 1983). Briefly, this assay uses platelet-rich plasma (PRP) that is isolated by centrifuging whole citrated blood at 250 x g, followed by a second centrifugation step at 13,000 x g to isolate platelet-poor plasma (PPP) (Saad et al. 1993). Platelet aggregation is measured by adding an agonist to PRP and measuring the amount of light that passes through the solution over time as compared to a PPP standard. If the agnoist induces platelet aggregation, PRP should become clear and transmit more light as the platelets precipitate; if the agonist is ineffective at inducing aggregation, the solution will remain

cloudy (Figure 3.2). This test is performed in an aggregometer, a specialized spectrophotometer designed specifically for this measurement (Chrono-log Co.; model 560ca). Aggregometers were calibrated with ddH20 according to standard guidelines, accepting a baseline error of 5% (Chrono-log Co.).

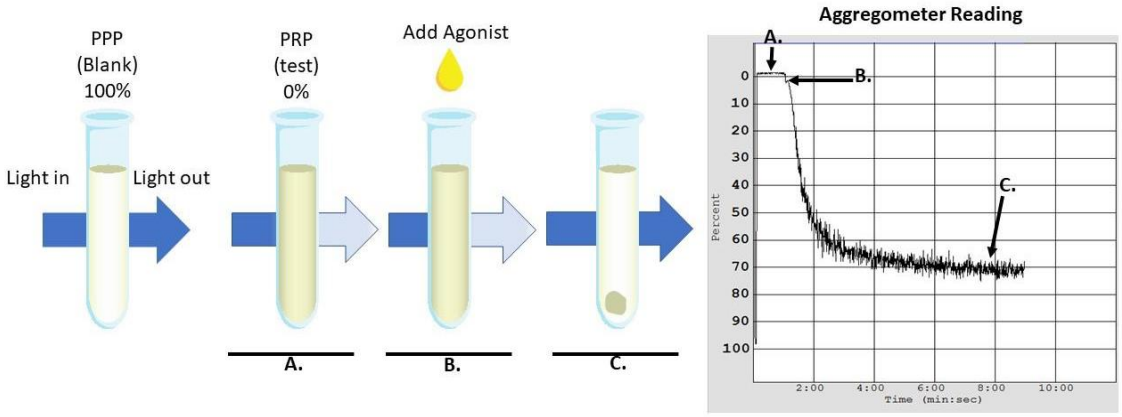


Figure 3.2- Principal design of a platelet aggregation assay. Platelet Poor Plasma (PPP) is used as a reference. Test sample with platelets, Platelet Rich Plasma (PRP), is measured against PRP for light transmittance. A. the test sample at baseline (0% aggregation). B. an agonist (a venom protein or other aggregating agent) is added to the PRP. C. Platelets aggregate in response to the agonist, allowing light to pass through the cuvette. On the right, an aggregometer sensogram reading of light passing through the cuvette for each stage (A, B, C). Aggregation percent is recorded as the transmittance reading (70% aggregation, in the above example).

Animal Handling and Blood Collection

Three individuals of *Didelphis virginiana* were live-trapped in urban and suburban areas of Minneapolis by licensed pest removal companies and transported in Tomahawk traps to the University of Minnesota Research Animal Resources facility (Minnesota Department of Natural Resources Permit #16312). Animal health was assessed upon arrival, and all animals were given a period of adjustment of 7-10 days before being used for blood draws. Blood draws were performed without anesthetization on the ventral tail vein, into sodium citrate coated syringes and stored in sodium citrate vacutainers (Moore 1984). Multiple blood draws were conducted on each individual, with intervals of a minimum of two weeks between blood draws. After use in this study, animals were released near the site of capture.

Four individuals of *Didelphis aurita* were wild caught in baited Tomahawk traps in urban forest at the Instituto Butantan (Brazil), anesthetized with 5% isoflurane by inhalation, and used for blood collection via tail vein puncture as previously described (CEUAIBU# 8346081018, SISBIO permit #64934-1, Moore 1984). Blood draws for *D. aurita* were not repeated, as all animals recovered within 30 minutes and were immediately released near the collection site. Two individuals of *Monodelphis domestica* were kindly gifted from the Oberlin College research colony by Dr. Yolanda Cruz and housed at the University of Minnesota according to protocols described in Rousmaniere et al. (2010). After a period of adjustment (3 weeks) individuals were anesthetized with 5% isoflurane and exsanguinated via cardiac puncture using a 3.2% sodium citrate coated syringe. All animals were handled in accordance with the American Society of Mammologists' standards, with supervision by veterinary staff at the University of Minnesota, and Instituto Butantan, Brazil (Sikes et al. 2011, IACUC Protocol #1303-3046A). Human samples used for controls and venom fraction isolation were obtained from healthy donors who had given informed consent. Volunteers did not consume alcohol, ibuprofen, aspirin, or any other drug which is known to disrupt coagulation within 24 hours of donation. Human blood draws were performed by University of Minnesota nurses either in the Special Coagulation Laboratory or at a clinic, using standard 3.2% sodium citrate vacutainers for PRP assays or Acid Citrate Dextrose (solution A) vacutainers for vWF purification (BD Pharmaceuticals).

vWF purification

Opossum and human vWF was purified from fresh blood collected in acid citrate dextrose (ACD) Solution A (BD Pharmaceuticals) vacutainers and centrifuged at 5000 x g for 10 minutes to remove blood cells, lipids, and large debris. Supernatant was removed and centrifuged for an additional 30 minutes at 28300 x g at 4° C to remove any remaining large cell or lipid remnants. Clear plasma supernatant was then placed directly onto a size exclusion liquid chromatography column (CL4B Sepharose XK26/100 column 3L volume, GE Life Sciences), and recovered as the first and largest protein in

the void volume as a single peak. vWF in circulation exists as very large multimers of 500-20,000 kDa, representing the largest multimeric protein complex in blood (Furlan 1996). This method of isolation was chosen as it was adaptable to small volumes of blood and excludes the vast majority of plasma proteins other than vWF by size. The only other plasma protein close to this size range is fibrinogen (340-350 kDa); other plasma proteins, including those known to inhibit venom proteins are much smaller (68 kDa) and are easily excluded with this method (Menchaca and Perez 1981). This void fraction (containing vWF) facilitated the aggregation of purified human platelets in the presence of ristocetin in physiological buffer, demonstrating this fraction contained concentrated and active vWF as previously described (De Marco et al. 1981).

DNA Sequencing

The vWF A1 region was sequenced for all opossum individuals used in aggregation assays to assess variation among individuals within each species. DNA was extracted from whole blood using a QIAGEN DNeasy Blood and tissue kit. The vWF A1 region was amplified with PHIRE polymerase at 90° C for 30 seconds, followed by 35 cycles of 98° C for 5 seconds, 65° C for 5 seconds, and 72° C for 15 seconds using primers DvWF F1 5'-TCACTGTGATGGTGTGAACTT-3'and DvWF R6 5'-GTCTGAGCCTTCTAGCACAAA-3' designed from a *Monodelphis domestica* genome scaffold. Samples were sequenced either at the University of Minnesota Genomics Center (*Didelphis virginiana* and *Monodelphis domestica*) or at the University of Butantan Laboratorio Especial de Toxinologia Aplicada (*Didelphis aurita*). Chromatograms were assembled and verified in GENEIOUS version 7.1.8; MUSCLE (Edgar 2004) was used to create alignments. Sequences have been deposited in GenBank (MN384757-59).

Platelet Agonists

Aspercetin was kindly gifted by Dr Alexandra Rucavado, Universidad de Costa Rica, having been purified as previously described (Rucavado et al 2001). Crude *Bitis arietans* venom was gifted to us by Kristen Wiley at the Kentucky Reptile Zoo. Biticetin

was purified first with salt precipitation described previously (Hamako et al. 1996), then eluted off both strong (SP-FF), and weak (DEAE-Sepharose-FF) FPLC cation exchange columns (GE Life Sciences). Fraction isolation was performed as previously described (Hamako et al. 1996).

To obtain the volume needed to test multiple replicates in several species, we used two sources of botrocetin. The first source (here designated "botrocetin A") was kindly provided by Dr Miguel Cruz (Baylor College of Medicine) and Dr Robert Andrews (Monash University). This sample was among several previously used to assess botrocetin activity and function (e.g. Dong et al 2001). Botrocetin was also purified from pooled *Bothrops jararaca* venom following the protocol described by Sekiya et al. (1999), with some modifications. Lyophilized venom (1.4 g) was reconstituted in 30 mL of 84 mM imidizole HCl, 2mM benzamidine pH 7.4, and dialyzed in the same buffer in a 10 kDa molecular weight cut off dialysis cassette (Thermo Scientific) at 4° C for 2 hours. Dialyzed venom was then subjected to ion exchange chromatography via a 15 mL DEAE sepharose column equilibrated in the same buffer. Elution buffer (84 mM imidizole HCL, 2 mM benzamidine, 1 M NaCl, pH 7.4) was applied first in a step gradient of 10%, and then in a linear gradient from 10-30%, with subsequent step gradients of 50%, 80% and 100%. Resulting fractions were tested for platelet aggregation activity using human PRP. Platelet aggregation activity was observed only in a single broad peak eluted at 10-30%. To further purify this sample, this peak was concentrated from 65 mL to 4 mL via ultrafiltration in 10kDa molecular weight cut off Vivispin columns (Sartorius, Goettingen, Germany), and dialyzed into 2 L TBS pH 7.4 at 4°C overnight. The dialyzed sample was then applied to a 16/600 200pg Superdex size exclusion column (GE Life Sciences). Five separate FPLC peaks were recovered, run out on an 8-20% Tris-Glycine protein gel with BlueStain protein ladder (GoldBio), and stained with Coomassie Brilliant Blue R-250. Three of these peaks contained bands indicative of botrocetin (subunits at 15.5 and 16 kDa).

To purify these three samples, each was dialyzed separately into 1 L 20mM Tris-HCl, pH 8.0 at 4° C overnight. Each was applied separately to a strong ion exchange

column (MonoQ, GE), and eluted using a linear gradient of NaCl (0-1M). Each recovered a fraction eluted at 0.5 M NaCl as the last peak (as in Sekiya et al. 1999). Two of these resulting peaks showed double bands at 15-16kDa as the only visible bands in a Tris-Glycine gel using the same gel conditions as above (indicative of botrocetin). Both samples also showed platelet-aggregation activity in human citrated platelet-rich plasma. Both fractions also aggregated purified lyophilized platelets reconstituted in physiological buffer (Chronolog Co.) only upon addition of a source of vWF (PPP), and were thus designated pure isoforms of botrocetin. Pure isoforms were dialyzed into TBS and concentrations were determined by absorbance at 280nm measured with a Nanodrop model 2000 (Thermo-Scientific), as well as gel-band imaging quantitation using Image J 1.x (Schneider et al. 2012). Both pure isoforms of botrocetin (named botrocetin B and botrocetin C) showed 70% aggregation at a 4 μg/mL test concentration after a 4-minute incubation with human platelet rich plasma. This activity matched that of botrocetin A provided by the Cruz and Andrews lab (data not shown). Due to limited protein availability, botrocetin A was used for all assays on *Didelphis virginiana*, while botrocetin B was used for all assays on *Didelphis aurita* and *Monodelphis domestica*. To minimize sources of variation we did not use Botrocetin C in any assay.

Ristocetin (Chronolog Co.), an antibiotic derived from bacteria, is known to induce platelet aggregation via vWF in humans and is often used as a control for vWF and platelet activity. We attempted to use ristocetin as a positive control for both human and opossum assays, however opossum platelets exposed to ristocetin became cloudy and formed a chalky precipitate without displaying an aggregation response. Though previously unknown in opossums, a similar response to ristocetin is known for dogs and pigs (Nichols et al. 2010, Read et al. 1983). Consequently, ristocetin could not be used as a positive control for vWF-mediated aggregation in opossums. Instead adenosine diphosphate (ADP) was used as an alternative control. Though ADP aggregates platelets, it does not do so via vWF, and so serves as a control of platelet activity only. Ristocetin and ADP were purchased (Chronolog Co.) and reconstituted as recommended by the manufacturer.

Platelet-Rich Plasma Assays

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were isolated by centrifugation for all species as described above. For each individual, a single aliquot of 500µl PPP and multiple aliquots of 450µl of PRP were placed into glass cuvettes (Chrono-log Co.). As PPP is not disturbed during data collection, a single PPP sample is used for each individual, though multiple PRP samples may be derived from that individual. Each individual assay (450µl PRP) was used either for a control (ADP, Ristocetin) or a treatment (botrocetin, aspercetin, bitiscetin). PRP samples which did not aggregate when exposed to a treatment agonist were either exposed to increasing concentrations of that same agonist by simply adding more agonist to the same sample or tested against a control agonist (ADP). A complete list of PRP tests performed on each individual organism can be found in Appendix Table 3.7.

Samples were warmed to 37°C and placed into appropriate PPP and PRP cuvette holders for a minimum of two minutes. A small disposable stir bar was added to each PRP cuvette and the sample was stirred at 1200rpm for the duration of the test. After setting a baseline at 0 on the aggregometer, the sample was allowed to sit undisturbed for 1 minute, after which 50ul of an agonist (botrocetin, aspercetin, bitiscetin, ristocetin, or ADP) was added to the PRP cuvette. Aggregation was observed via optical interference for 8 minutes. The slope of the aggregation curve (Figure 3.2) is the standard measure of aggregation response used for biomedical testing, however variation in platelet and vWF concentration can affect this slope and it should be adjusted for platelet counts in each test. Because we were working with species in field conditions and with limited blood volumes, we were unable to quantify and standardize these concentrations; therefore, we used total percent aggregation as our measure of aggregation response (Figure 3.2, Rucavado et al. 2001, Read et al. 1983, Nichols et al. 2010).

Platelet counts for PRP were estimated initially in both human and *D. virginiana* individuals, determined to be within normal range, and were not further adjusted or tested. No platelet counts were conducted for *Didelphis aurita* or *Monodelphis domestica*

because of sampling and facility limitations. Agonist concentrations were chosen to replicate previously established concentration ranges which produced aggregation in human samples, and to accommodate limited availability of venom protein (Hamako et al. 1996, Rucavado et al. 2001, Sekiya 1993). The range of final concentrations for each agonist are as follows: ristocetin: 1.5-3.75 mg/ml, botrocetin: 2-70 μg/ml, aspercetin: 20-200 μg/ml, ADP: 850 μg/ml, and bitiscetin: 10-12 μg/ml. PRP assays were carried out as previously described (Coller et al. 1975). Assays were prioritized by limited amounts of both blood and venom proteins. To accommodate limited plasma, samples which yielded no response were subjected to additional doses of agonist (increasing test concentration), and a subsequent dilution correction was applied to the final transmittance.

Fresh PRP from *Monodelphis domestica* was extremely limited, and thus used to test aggregation with botrocetin only. Two individual *Mondelphis domestica* were sampled, however, only one individual produced PRP which aggregated in the presence of ADP. The second individual's PRP did not aggregate in the presence of ADP, and thus could not be used for botrocetin testing, as it was uncler whether platelets were active. However, because vWF can induce platelet response (agglutination) even in formalin-fixed platelets, we utilized this sample to assess aspercetin agglutination response (Allain et al. 1975).

Washed Platelet Assays

To isolate the effect that plasma proteins might have on aggregation in PRP assays (Allain et al. 1975), an additional assay was conducted with washed platelets suspended in physiological buffer. This assay uses the same design as in Figure 3.2, using buffer without platelets as a reference. As this procedure removes vWF along with the plasma, we purified vWF from PPP and added it along with the washed platelets to each assay. This assay was performed only on *Didelphis virginiana* and human controls, as the platelet purification process - as well as the vWF purification process - (Allain et al. 1975, De Marco et al. 1981) both require repeated blood draws of large volumes, which

were only feasible for the larger bodied *D. virginiana* housed in a research facility. A complete list of washed platelet tests performed can be found in Appendix Table 3.8.

Platelets were washed via centrifugation as previously described (Allain et al. 1975) and re-suspended in phosphate buffer (0.15 M disodium phosphate diluted in 0.15 M monosodium phosphate until pH reached 7.0). Platelet preparations for human and *Didelphis virginiana* were prepared in tandem.

ADP controls were performed on purified platelets from *D. virginiana* to demonstrate that the purification processes did not inactivate platelets. To assess whether vWF was inactivated by the purification process, human vWF and pure platelets were assayed separately with ristocetin and bitiscetin. Because *D. virginiana* showed little to no aggregation to any vWF-targeting reagent (botrocetin, ristocetin, aspercetin, or bitiscetin), a similar control for opossum vWF purification inactivation could not be performed. To compensate for the lack of a positive vWF control in *Didelphis virginiana*, two sources of vWF were used to assure vWF activity. Assays were performed using either: 1) purified and concentrated vWF, or 2) 10% v/v platelet poor plasma (PPP), which contains vWF (Sekiya 1993, Kumar et al. 2006).

All vWF-binding agonists were added first to pure platelets and allowed to equilibrate before adding a vWF source. While the washing process removed nearly all plasma proteins, vWF occasionally remained in quantities sufficient to facilitate some vWF-mediated aggregation in human washed platelets in the presence of an agonist before the additional of purified vWF. Therefore, all estimates of aggregation in purified assays represent a conservative value.

Analyses

To test for differences in PRP aggregation response by concentration (dose-dependence), assays were grouped into low (botrocetin 2 µg/ml, aspercetin <50 µg/ml), and high concentrations (botrocetin >2 µg/ml, aspercetin >51 µg/ml). Differences in PRP aggregation response between high and low concentrations for each venom protein were tested using Wilcoxon tests. No significant differences in percent PRP aggregation were

observed between low and high concentrations for any venom protein, consequently, subsequent analyses did not distinguish between tests run at different concentrations (e.g. all *D. virginiana* botrocetin PRP tests were grouped and compared to all human botrocetin PRP test regardless of concentration; Appendix Table 3.2).

To compare opossum PRP aggregation with aggregation for the same agonist in human PRP, a one-way non-parametric analysis was performed. We used a Dunn joint-ranking method appropriate for testing each species-agonist treatment against the same treatment in human PRP, incorporating a Bonferroni correction for each group by agonist, as well as globally (Table 3.1).

Virtually no variation in PRP response to any agonist by individual was observed, except for *Didelphis virginiana* response to aspercetin. Because individuals of *Didelphis virginiana* were observed to vary in their PRP response to aspercetin, an additional test was done to determine if this variation in PRP aggregation response among individuals was statistically significant, using a standard Kruskal-Wallis rank sums test.

Monodelphis domestica PRP agglutination response to aspercetin from a sample which failed ADP control was compared against Human PRP and *Didelphis virginiana* PRP exposed to the same venom protein (aspercetin). A Tukey-Kramer HSD test for pairwise comparisons was used to evaluate pairwise differences between human, *Monodelphis domestica*, and *Didelphis virginiana* PRP aggregation response.

Differences in aggregation between vWF source (PPP vs purified vWF) in washed platelet (WP) assays were assessed using a one-way Wilcoxon test, and were grouped where no significant differences were found. The same test for dose-dependence used in PRP assays was also used to test for dose-dependent response in WP assays. No significant differences in percent aggregation were observed between low and high concentrations for any venom protein in WP assays; consequently, subsequent analyses did not distinguish between tests run at different concentrations (e.g. all *D. virginiana* botrocetin WP tests were grouped and compared to all *D. virginiana* PRP tests regardless of concentration) (Appendix Table 3.5).

Average values of percent aggregation for all WP assays were compared to the same treatment (ADP, or venom protein) in PRP, using one-way Wilcoxon tests, to assess whether the removal of plasma significantly altered aggregation results.

Results

Platelet Rich Plasma Assays

PRP assays were conducted to compare opossum vWF aggregation response to human aggregation response for the same agonist (venom protein). Averages and variation for human aggregation are consistent with previous work (Figure 3.3A) (Rucavado 2001, Hamako 1996, Sekiya 1993) for all treatments. By contrast, all three opossum species showed a significant reduction or loss in aggregation response to all venom proteins tested (Table 3.1, Figure 3.3A), but aggregated normally in the presence of ADP. *Didelphis virginiana* PRP did not aggregate in response to botrocetin or biticetin. Aspercetin caused *Didelphis virginiana* PRP to aggregate partially, although significantly less than observed in human samples (Figure 3.3A, Table 3.1). Variation in response to aspercetin among individual opossums (*Didelphis virginiana*) was observed, but this variation among individuals was not significant (Appendix Table 3.1). Changes in agonist concentration did not produce significant differences in aggregation. However, variation in response to aspercetin was reduced as all individuals showed at least partial aggregation at the highest concentration of aspercetin (100ug/ml) (Appendix Table 3.2). *Didelphis aurita* showed no aggregation response to either botrocetin or aspercetin but was not tested for bitiscetin due to limited blood volume available (Figure 3.3A). Two individuals of *Monodelphis domestica* were sampled; however, one of the two samples failed to aggregate in the presence of ADP and was thus excluded from analysis. The remaining *Monodelphis domestica* sample showed no aggregation response to botrocetin. *Monodelphis domestica* platelets from the sample that failed to aggregate with ADP did ultimately aggregate at an aspercetin concentration of 100 μg/ml (Appendix Figure 3.1). Both *D. virginiana* and *M. domestica* showed some aggregation in high concentrations of aspercetin (Appendix Figure 3.1, Appendix Table 3.3). The magnitude of the aggregation

response to aspercetin was similar between the two species, and both showed significantly less aggregation than human (Appendix Figure 3.1, Appendix Table 3.3).

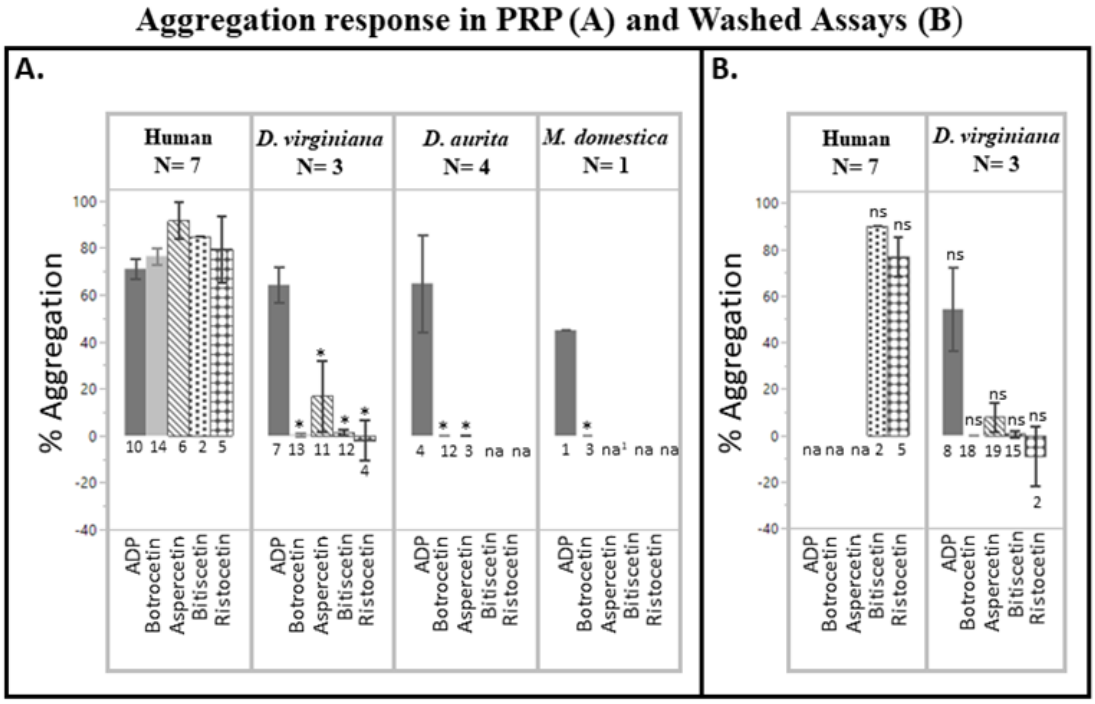


Figure 3.3- Mean percent platelet aggregation calculated as percent light transmittance (error bars 95% confidence intervals). N below species names in top panel indicates number of individual animals, numbers below each bar graph indicate total number of individual tests done for each agonist. NA indicates this test was not performed. 1 this test was performed with a sample which failed ADP control and is reported in supplementary material (Appendix Figure 3.1) Panel A are tests performed in platelet rich plasma (PRP); asterisks indicate values that are significantly different from the same PRP test in human samples. Panel B shows results from tests using washed platelets and purified vWF or 10% v/v PPP as a vWF source; ns indicates no significant difference from the same test performed with PRP (see in Panel A for comparison). ADP serves as a platelet activity control for all tests. Bitiscetin and ristocetin serve as vWF activity controls for washed assays.

Aggregation in Platelet Rich Plasma

Species	Control	Score Mean Difference	Std Err Dif	Z-score	P-Value
ADP					
Didelphis virginiana n=7		6.8769	3.453393	1.99135	0.1393
Didelphis aurita n=4	Human n=10	3.425	4.857221	0.70514	1
Monodelphis domestica n=1		-16.3	8.610932	-1.89294	0.1751
botrocetin					

Didelphis virginiana n=13		19.772	4.126265	4.79174	<.0001*
Didelphis aurita n=12	Human n=14	21.9226	4.21447	5.20175	<.0001*
Monodelphis domestica n=3		-21.7976	6.815705	-3.19815	0.0041*
aspercetin					
Didelphis virginiana n=11	Human n=11	15.39583	4.32599	3.558915	0.0007*
Didelphis aurita n=3		24.25	6.701795	3.618433	0.0006*
bitiscetin					
Didelphis virginiana n=12	Human n=2	12.35119	3.553028	3.476244	0.0005*
ristocetin					
Didelphis virginiana n=4	Human n=5	7.866667	2.453116	3.206806	0.0013*

Table 3.1 – One-way analysis of percent aggregation (% transmittance) in PRP. Analysis is a non-parametric comparison of PRP mean percent aggregation using Dunn method for joint ranking with Human as the control for each group. Dunn methods uses a Bonferroni adjustment within each test group. Between test groups (10 tests) Asterisk indicate significance at the Bonferroni adjusted α-value 0.006.

Washed Platelet Assays.

Washed platelet assays were done to test the effect of plasma proteins on venom CTL induced aggregation. These assays gave similar results to those using PRP: *Didelphis virginiana* showed a significant reduction in aggregation response compared to human for to all venom proteins tested, but platelets aggregated normally in the presence of ADP (Fig. 3.3B). There were no significant differences between aggregation values from the washed platelet assay and those from the PRP assay for either opossum or human tests (Figure 3.3B, Appendix Table 3.4). Bitiscetin and ristocetin treatments in human assays demonstrated that platelets and vWF remained active after being purified; similarly, ADP controls showed no reduction in the ability of opossum washed platelets to aggregate (Figure 3.3B, Appendix Table 3.4). Opossum samples treated with ristocetin showed increases in opaqueness due to formation of a chalky precipitate but did not show platelet aggregation. As with the PRP assays, no significant differences were seen among tests performed at increasing agonist concentration (Appendix Table 3.5). The source of vWF (PPP vs pure vWF) yielded no difference in response for botrocetin or bitiscetin but did for aspercetin ($p = 0.021$) (Appendix Table 3.6). When platelet poor plasma (as opposed to purified vWF) was used as a source of vWF, aggregation increased by an

average of 7.5%, suggesting some degradation of vWF during purification, or that a low concentration impurity such as a venom serine proteinase is enhancing aggregation via a target in the plasma (e.g. fibrinogen). However, an increase of 7.5% is small (only 2.5% above machine calibration error of 5%), and thus may not reflect a biologically relevant increase.

DNA Sequencing.

All DNA sequences were invariant within species at the vWF A1 region known to be responsible for venom CTL binding (residues 475-710 NCBI accession AAB39987.1). *Didelphis virginiana* sequences from this region were identical to Genbank accession JN415020, and *Monodelphis domestica* sequences were identical to Genbank sample accession NM_001246274. *Didelphis aurita* vWF was not previously available on Genbank.

Discussion

We provide the first physiological evidence that the coagulopathic effects of snake venom CTLs are disrupted in opossums, which may contribute to whole-organism resistance to pitviper envenomation. We challenged platelets from three species of opossums using vWF-binding venom CTLs from snakes known to currently or historically co-occur with those species (botrocetin and aspercetin from species of the South American pitvpers *Bothrops jararaca* and *Bothrops asper*, respectively), and one which is found on a different continent (bitiscetin from the African viper *Bitis arietans*). We confirm that human platelets aggregate as expected in the presence of all three venom CTLs and show that platelets from all examined opossum species (*D. virginiana*, *D. aurita*, and *M. domestica*) fail to show the same response (Figure 3.3A). Thus, contrary to the expectation that only opossums in Didelphini (with rapidly evolving vWF) will exhibit resistance to venom CTLs, all opossums tested to date appear to enjoy protection from CTL-mediated platelet aggregation. Furthermore, this protection appears to hold regardless of the geographic origin of the toxins tested.

Physiological Function within Didelphini

Didelphis aurita inhabits a geographic range which overlaps with that of *Bothrops jararaca*, as well as several other species of *Bothrops* which have been shown to have vWF-mediated platelet aggregating activity (Read et al. 1978, McManus 1974, Caceres and Monteiro-Filho 2010). Though *Didelphis virginiana* does not overlap geographically with *Bothrops jararaca*, it does share the southern portion of its range with *Bothrops asper* and likely shared a historical range with ancestral *Bothrops* species before its migration to North America ~3 mya (McManus 1974, Voss and Jansa 2012). These observations, along with behavioral and experimental data which show venom resistance, predation on venomous snakes, and venom targets (vWF) evolving under positive selection, lends credence to the assertion that these species are coevolving with venomous snakes (Wood 1954, Fitch 1960, Menchaca and Perez 1981, Moussatche and Perales 1989, Jared et al. 1998, Jansa and Voss 2011). Specifically, Jansa and Voss (2011) suggested that the detection of positive selection in didelphine vWF may be evidence of molecular changes that protect these opossums from the effects of vWF-targeting CTLs such as botrocetin. At first glance, the reduced aggregation response in these species to botrocetin and aspercetin shown in this study add support to this assertion.

While lack of aggregation response to botrocetin and aspercetin was expected, the same result for bitiscetin was not. This is likely due to a large degree of overlap in botrocetin and bitiscetin binding sites. Of 15 bitiscetin binding sites, 13 are within the botrocetin binding pocket, 5 directly overlap with botrocetin binding sites, and another 5 are directly adjacent to a botrocetin binding site (Maita et al. 2003). Given this overlap, it is not surprising that we observed similar responses to these two agonists.

Didelphis virginiana showed partial though greatly reduced aggregation for aspercetin, while all other tests involving opossums showed a nearly complete lack of platelet aggregation with aspercetin. Partial resistance to aspercetin in *D. virginiana* suggests that this protective function may not be effective against all venom CTLs in all

didelphines. Given that apparent resistance to botrocetin confers resistance to bitiscetin (a distantly related African CTL) it is thus surprising that a more closely related CTL (aspercetin) would not also fail to function in the same species. This result also suggests that aspercetin may be quite divergent from botrocetin particularly at vWF binding sites—and may be part of an adaptive radiation of venom proteins evolving to target vWF in mammals. Complete resolution of the aspercetin sequence and structure is not available at this time, but further work elucidating this would help decipher its divergence in form and function from botrocetin.

The observation of inter-individual variation of *D. virginiana* aggregation for aspercetin (though not statistically significant), suggests some variability in ability to resist this venom protein. Because no polymorphism in the vWF A1 region was present between these individuals, this result suggests that any variation in resistance to aspercetin between individuals of *Didelphis virginiana* is not due to changes in vWF. As vWF mediated aggregation requires that a venom protein first binds to vWF and subsequently binds to a platelet binding site (GP1bα), the variation in resistance observed may be due to aspercetin's ability to bind to GP1bα (variation in opossum GP1bα). Additional work examining the tertiary complex of vWF-aspercetin-and GP1bα, as well as variation in GP1bα among individual opossums are needed to assess the source of reduced aggregation response in this species.

Washed platelet assays excluded the possibility that opossum plasma inhibitors are responsible for the reduction in aggregation observed (Figure 3.3B). For aspercetin, a small increase in aggregation (7.5%) was seen when adding plasma to washed platelets - a pattern opposite to that expected if plasma proteins inhibited vWF CTLs. This result indicates either a small amount of a contaminating venom protein is causing aggregation via a plasma protein target, or some functional degradation of vWF caused by the purification process. Overall, these data are consistent with the assertion that amino acid changes in opossum vWF confer physiological resistance to vWF-binding venom CTLs, but do not rule out the role of the platelet site GB1Bα in contributing to this resistance.

Physiological Function Outside Didelphini

Surprisingly, *Monodelphis domestica*, an opossum not included in the clade known to be venom resistant (and distantly related to Didelphini), also showed a loss of aggregation response to botrocetin. This result is inconsistent with the hypothesis that vWF resistance to venom CTLs is restricted to Didelphini. Though several species of *Monodelphis* were included in the original work identifying rapid evolution in Didelphine vWF, *Monodelphis domestica* was not (Jansa and Voss 2011).

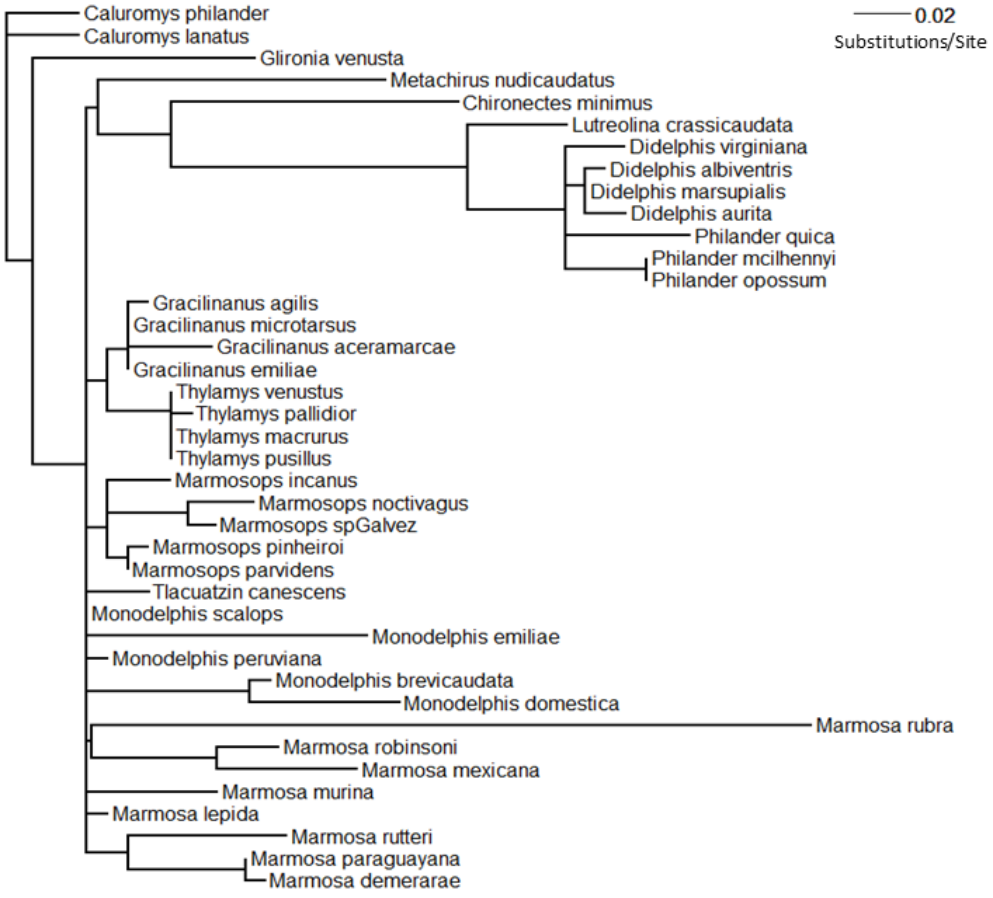


Figure 3.4- Topology of Didelphidae from Jansa and Voss (2011) with the addition of *Monodelphis domestica* according to Pavan et al (2014). Branch lengths reprisent the estimated number of amino acid substitions in vWF reconstructed with the JTT model of amino acid substitution available in PAML (Yang 2007).

Jansa and Voss (2011) utilized the branch-site test of positive selection which requires that a branch or branches thought to be evolving under selection be selected *a priori* to test a specific hypothesis (Yang 2007). Though this method is powerful tool for

hypothesis testing, the *a priori* selection of branches make it susceptible to missing instances of positive selection that were previously unexpected. Figure 3.4 shows that although Didelphini has a long branch (reflecting several amino acid changes), additional branches in Didelphidae outside this clade also exhibit several independent instances of long branches. *Monodelphis emiliae*, *Marmosa rubra*, *Monodelphis brevicaudata*, *Monodelphis domestica*, and *Metachirus nudicaudatus* all have branch lengths which are conspicuously long relative to other species (Figure 3.4). *Monodelphis domestica* specifically exhibits a long branch and shares several changes at botrocetin binding sites seen in Didelphini. Of 13 botrocetin binding sites, *M. domestica* has changes at six of these sites, three of which are identical to amino acid changes found in Didelphini. Additionally, *Monodelphis domestica* shares a geographic range with *Bothrops jararaca* and is likely to be a prey item of several South American vipers (Macrini 2004, Voss 2013). It has also been rarely described to eat snakes, though species were not specified (Streilein 1982). It is therefore possible that *Monodelphis domestica* represents an independent instance of rapid evolution of vWF among opossum species. Examining the physiology of CTL resistance among additional didelphid speices (Figure 3.4) could be a fruitful avenue for future research.

Though changes on vWF in this clade indicate that rapid adaptation may be shaping this gene, it is possible that these are artifacts of another unknown selection pressure unrelated to venom. Therefore, an alternative interpretation of this data may be that CTL-resistant vWF is the ancestral state for all opossums and may have facilitated a dietary adaptation (snake eating) in Didelphini. This initial disabling of venom CTL function may have disrupted coagulopathy enough to allow differential survival, which subsequently may have allowed these species to evolve additional mechanisms of venom resistance such as metalloproteinase and phospholipase inhibitors that are well known for this group (Voss and Jansa 2012). Whether this type of pre-existing resistance to CTLs would be effective for all opossums regardless of body size is unknown. Given that most members of Didelphini are large bodied compared to the remainder of Didelphidae, the combination of a large body size which is able to dilute venom dosage *in addition* to a

pre-adapted vWF may have combined to facilitate the evolution of whole venom resistance via metalloproteinase and phospholipase inhibitors in this group.

Though vWF resistance likely does not confer organismal venom resistance, it may be indicative of unexamined partial or complete organismal venom resistance. The results reported here suggest that the long-held assumption that *M. domestica* and potentially other related smaller bodied opossums are venom sensitive may need re-evaluation. Though analyses of positive selection which motivated this work are robust in detecting signals of persistent diversifying selection, they are poor at detecting selection in which a few advantageous nonsynonymous mutations are either quickly reaching fixation or toggling back and forth between states- as might be expected in the case of rapid directional selection or trench warfare coevolution (Yang and Reis 2011, Nuismer et al. 2007). Because of this, while tests of positive selection are a useful tool in directing attention to potential adaptive function, they should be followed up through examination of the functional effects of observed genotypes (Yokoyama et al. 2008). Although our results isolate the vWF-platelet-CTL interaction as the likely source of disruption of botrocetin/aspercetin coagulopathic response in opossums, further investigation is required to understand the underlying mechanism of CTL resistance conferred specifically by amino acid changes in vWF both in Didelphini and their more distant relatives. Identification of the molecular mechanism(s) of resistance and the ability to survey this function across more species in Didelphidae would help to better discern whether loss of aggregation response in these opossums is ancestral to all Didelphidae or is convergently derived as an antipredatory adaptation (in small bodied opossums) and as a dietary adaptation (in large bodied opossum).

CHAPTER 4. EVIDENCE FOR COEVOLUTION AND CRYPTIC CONVERGENCE BETWEEN OPOSSUMS AND PIT-VIPERS

Introduction

Coevolution is a process that has been central to evolutionary biology since its beginnings, when biologists and naturalists began to recognize that patterns of adaptation and counter adaptation produce reciprocal changes in phenotypes (Priestley 1764, Darwin 1859, 1862, and 1895, Müller 1873, Smith 1887, Andrews 1891, Kellogg 1896, von Ihering 1902). The best studied examples of these interactions include obligate mutualisms, host-pathogen models, and some predator-prey systems (Ehrlich and Raven 1964, Janzen 1966, Anderson and May 1979, Anderson and May 1982, Hanfin et al. 2008, Scanlan et al. 2011). However, Janzen (1980) argued that though we observe coevolution as matched phenotypes in the present day, the inability to observe the history of interactions among ancestral species renders us unable to distinguish the role that coevolutionary processes played in shaping modern species interactions. For example, predators may be able to eat novel toxic prey because they already carry detoxifying abilities attained through some other process. Such pre-adaptations may make a new predator-prey interaction possible, but the interaction did not result from trophic coevolution.

The study of coevolution in natural systems via large scale phenotypic and phylogenetic associations (using these as proxies for co-adaptive change) has been successful at demonstrating reciprocity through co-speciation and counter-adaptations (Langamore et al. 2003, Currie et al. 2003, Janzen 1980, Lawton and Hassel 1981, Klassen 1992). However, these approaches are limited to very recent and fast changing interactions that can be observed in contemporary species. However, distinguishing pre-adaptation from coevolution may be possible at deeper time scales if we can examine the pattern of evolution of ancestral phenotypes in interacting species. For example, coevolving species observed in present-day interactions may appear to have phenotypes which are tightly matched; however, examination of their ancestral phenotypes may show that they each already possessed their phenotypes before coming into contact with one

another, with no subsequent change. As coevolution is characterized by reciprocal evolutionary change in interacting partners, a pattern of unchanged phenotypes across ancestral forms in each species would exclude coevolution as the underlying process producing matched phenotypes, and instead would suggest that pre-adaptation is responsible for this observation.

Different models of the coevolutionary process make predictions about how ancestral phenotypes should change as a result of long-term reciprocal interactions between species. For example, arms-race coevolution — where antagonists evolve increasing defense/attack phenotypes perpetually escalating their trait values — has been described as the dominant model of antagonistic coevolution (Abrams 1986, Dawkins and Krebs 1979). A simple example of this model is predator speed versus prey speed. Arms-race coevolution would dictate escalating unidirectional selection for faster phenotypes in both interacting species. Thus, ancestral phenotypes in arms-race coevolution are expected to show unidirectional evolution of trait values (in this example, slow to fast) across the history of this interaction.

An alternative model of coevolution, where phenotypes change by alternating their ability to match one another, has been described as 'trench warfare', or 'phenotype matching' coevolution (Gomulkiewicz et al. 2000, Ridenhour and Nuismer 2007), by virtue of its characteristic reciprocal retaliation (Axelrod and Hamilton 1981). This model invokes a distinct process of cyclic fluctuating selection which generates traits that flip back and forth between trait values (Thompson 1994, Jokela et al. 2000). The best examples of this kind of coevolution include protein-matching models where a predator or disease must 'match' or identify a host in order to exploit it. Ancestral phenotypes in this type of coevolution would instead be expected to be changing in a non-unidirectional fashion, toggling between trait values over time.

These two models of coevolution are difficult to assess empirically without access to observations on ancestral interactions. These observations can come from analysis of the fossil record (e.g. Kelley 1992, Grossnickle and Polly 2013), from studies

of experimental evolution (Brockhurst and Kokella 2013), or from engineering ancestral proteins involved in a potential coevolutionary interaction (Golding and Dean 1998).

This latter approach — sometimes termed the 'functional synthesis'— places emphasis on testing the function of ancestral proteins to examine the plausibility of hypothesized evolutionary scenarios (Golding and Dean 1998). This approach uses models of sequence evolution to detect selection and reconstruct ancestral sequences, as well as *in-vitro* expression and functional assays to empirically test hypotheses of adaptive function in extant and ancestral proteins. Studies applying this approach have provided important new insights into the relative roles that additional complexities such as constraint, epistasis, and permissive mutations play in molecular evolution (Dean and Thornton 2007, Bridgham et al. 2009, Harms and Thornton 2013). However, the functional synthesis has primarily been applied to understanding adaptive evolution to abiotic selection pressure and has yet to be used to elucidate the history of protein evolution for coevolving molecules, including whether this history is consistent with an arms-race model or a trench warfare model of coevolution.

Ideally, the functional synthesis approach would be applied to both proteins involved in a coevolutionary interaction, but this goal has remained elusive due to the difficulty of reconstructing the evolutionary history of both participants within a coevolutionary interaction. Nevertheless, empirical ancestral phenotype data for just one of the interacting partners can still address predictions of different models of coevolution (Figure 4.1). For example, if interacting proteins are not coevolving, but rather are pre-adapted for their current interaction, then we might expect to see patterns of ancestral protein evolution consistent with pre-adaptation (Figure 4.1A) or a single adaptive event early in the clade's history (Figure 4.1B). Alternatively, if the protein shows evidence of rapid evolution in the clade of interest — consistent with expectations of ongoing coevolution — then different patterns of ancestral protein evolution would imply either arms-race coevolution (Figure 4.1C) or trench warfare coevolution (Figure 4.1D).

We apply this approach to examine the evolution of a blood protein (vWF or vonWillebrand Factor) that has apparently evolved to confer resistance to the

coagulopathic effects of snake venoms in some species of South American opossums. Prior research (Jansa and Voss 2011) discovered that vWF is evolving rapidly in a group of opossums known to be resistant to snake venoms. This finding suggests that vWF and its snake-venom agonist – the C-type lectin (CTL), botrocetin – might be involved in a co-evolutionary arms-race. However, to test this idea, we require evidence that snake venom proteins can no longer bind vWF to induce coagulopathy in resistant opossum species. Further, we require an understanding of how ancestral vWF proteins interact with these venom agonists. To elucidate these interactions, we use a functional synthetic approach to engineer modern and ancestral vWF proteins and assay their sensitivity to venom agonists in order to assess both the contemporary and ancestral interaction of vWF and venom CTLs. These data address the contemporary function of vWF, whether phenotype evolution of this protein in opossums is consistent with a coevolutionary process, and if so, which dynamic— arms-race dynamic or trench warfare coevolution— best describes this interaction.

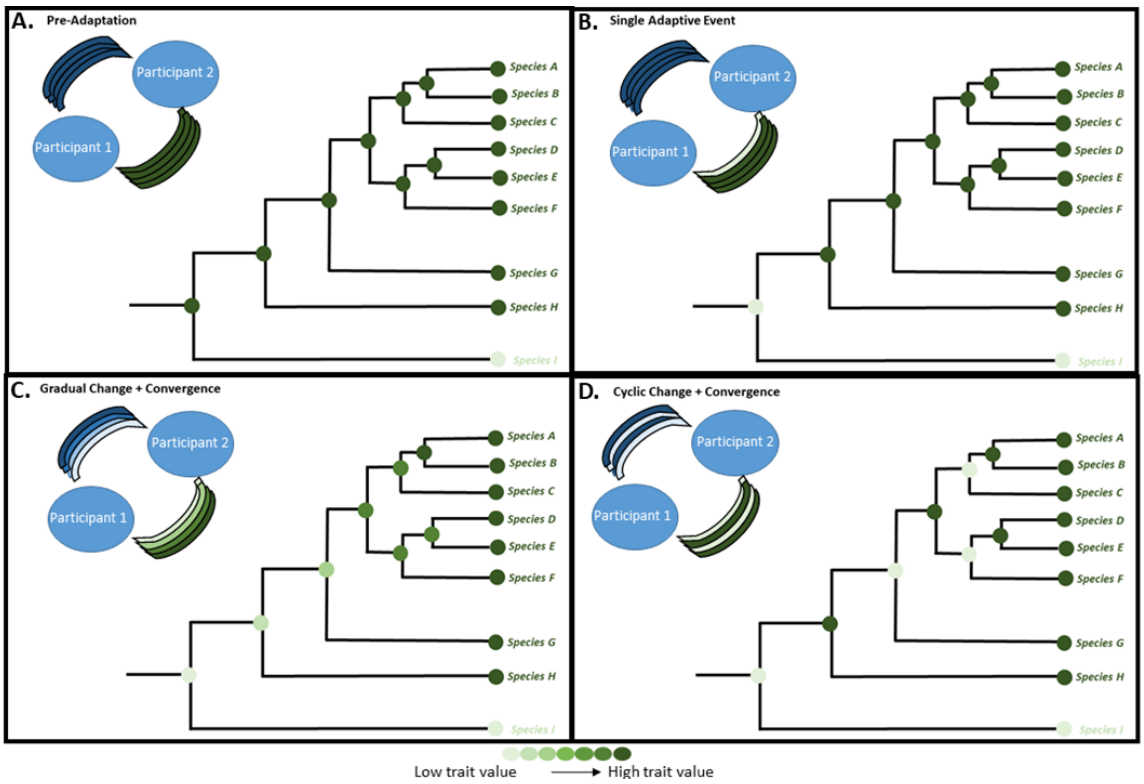


Figure 3.1- Figure showing four evolutionary histories of traits hypothesized to be coevolving because of a matched phenotype with an interacting partner. In each panel the evolutionary history is indicated by colored arrows with inner arrows representing ancestral phenotypes. In this case only the phenotype in green is known for ancestral forms. The evolutionary history for this single green trait is mapped onto a phylogenetic tree to show what the expected pattern of ancestral phenotype evolution would be given each of these histories. Panel A and B show expected pattern of phenotype evolution for histories which are not consistent with coevolution. Panel C shows an expected pattern of phenotype evolution given either arms race coevolution also consistent with gradual adaptive evolution towards an unchanging selection pressure. Panel D shows the expected pattern of phenotype evolution given trench warfare coevolution also consistent with adaptive evolution towards and changing selection pressure.

The Study System: Opossums and Pitvipers

Snake venoms are a complex cocktail of proteins which include both enzymatic tissue-destroying proteins (e.g. metalloproteinases, phospholipases), as well as integrin-like proteins (e.g. α-neurotoxins, C-type lectins), which target receptors involved in maintaining physiological processes (Markland 1998). Some venom C-type lectins (CTLs) are specialized to target vWF, causing disruption of normal blood coagulation. vWF is a multidomain protein (15 domains), whose main function is to initiate coagulation by binding to platelets in response to endothelial damage (Cruz et al. 2000). Botrocetin, a vWF-targeting CTL, functions by first binding tightly to the A1 domain of vWF, then inducing vWF to bind the platelet-associated glycoprotein GP1Bα, and finally binding to GP1Bα itself (Fukuda et al. 2005). This tri-molecular complex (botrocetin-vWF- GP1Bα) is the ultimate source of coagulopathy, preventing vWF and platelets from functioning normally (Maita et al. 2003). A second venom CTL, aspercetin, has been shown to induce coagulopathy via vWF, though the specific vWF domain(s) and platelet binding site it targets is unknown (Rucavado et al. 2001). Both botrocetin and aspercetin are derived from South American vipers (*Bothrops jararaca* and *Bothrops asper*, respectively), species that are known to be both prey and predators of various opossums (Oliveira and Santori 1999, Voss and Jansa 2012). A third vWF binding CTL, bitiscetin, is derived from an African viper (*Bitis arietans*) and is structurally and functionally similar to botrocetin (Hamako et al. 1996, Maita et al. 2003). Though many more vWF targeting CTLs likely exist within the genus *Bothrops* these are the only three that have been formally described to date (Read et al. 1979)

Some members of the South American opossum Tribe Didelphini (Voss et al. 2009) are resistant to snake venom and are known to prey upon venomous snakes in the genus *Bothrops* including *Bothrops jararaca* (Vellard 1945, Wood 1954, Fitch 1960, Perales et al. 1986, Melo and Suarez-Kurtz 1988, Sazima 1992, Jared et al. 1998, Oliveira and Santori 1999, Almedia-Santos et al. 2000). Several species in this group are known to possess multiple mechanisms which render them resistant to venom, including specific inhibitors which neutralize enzymatic venom components such as metalloproteinases and phospholipases (Kilmon 1976, Werner and Vick 1977, Werner and Faith 1978, Mousattche et al. 1978, 1979, Perez et al. 1979, Menchaca and Perez 1981, Soto et al. 1988, Moussatche and Perales 1989, Catanese and Kress 1993, Perales et al. 1994, Lovo-Farah et al. 1996, Voss and Jansa 2012). In addition, the A1 subunit of vWF has been shown to be evolving under positive selection at sites associated with botrocetin binding in species within Didelphini (Jansa and Voss 2011). Physiological assays have further shown that opossum platelets from two species of Didelphini have lost almost all aggregation response to botrocetin, aspercetin, and bitiscetin (a third CTL from an African viper) (Chapter 3). However, this loss of platelet aggregation response was also recovered in a species of opossum outside Didelphini (*Monodelphis domestica*), suggesting either that resistance to vWF-binding CTLs might be a shared ancestral trait for all Didelphids, or that it has arisen multiple times during opossum evolutionary history (Chapter 3).

Though tests of selection indicate that the vWF protein is rapidly evolving in Didelphini, these tests do not explicitly show that the causal selection pressure is indeed botrocetin, or that vWF has lost binding affinity for botrocetin. Furthermore, amino acids at the botrocetin-binding sites under positive selection in vWF differ among species in this clade, making it impossible to identify clear loss-of-function mutations by examining sequences alone, and indicating that there is likely functional variation among species. Therefore, testing the affinity of opossum vWF for venom CTLs is an important next step towards understanding the functional consequences of vWF evolution (Jansa and Voss 2011, Chapter 3). Moreover, synthesizing ancestral vWF proteins and assaying their

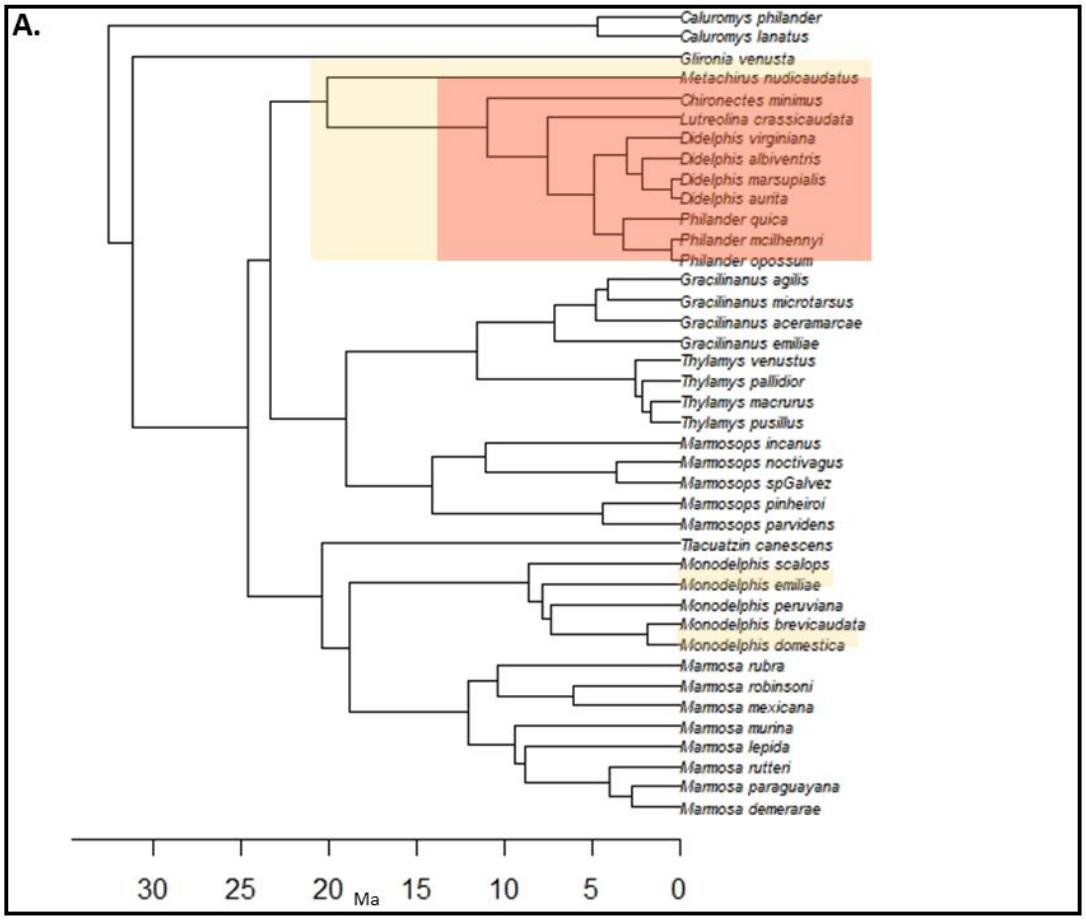
affinity for C-type lectins will extend our understanding of how coevolution may be shaping the interaction between these proteins.

Materials and Methods

Study Design.

We estimated the binding affinity between vWF A1 and venom CTLs (botrocetin, bitiscetin, and aspercetin) for several extant species from the tribe Didelphini, as well as species outside this clade, to assess the binding function of vWF across Didelphidae (Figure 4.2 Panel B). Species of Didelphini, and its sister taxon *Metachirus nudicaudatus* were chosen based on Jansa and Voss (2011). Though subsequent work has resulted in some species splits (Voss et al. 2018). Species within Didelphini are expected to be venom resistant based on several behavioral and experimental studies (Voss and Jansa 2012, Chapter 1), with the exception of *Chironectes minimus*, for which venom resistance status is unknown. *Metachirus nudicaudatus* was shown to be susceptible to venom injections from *Bothrops jararaca*, as two individuals tested died at 2x LD_{50} for mice, and has thus been assumed to be venom sensitive (Perales et al.1994). Other species outside Didelphini such as *Monodelphis spp* are assumed to be venom sensitive as they are small-bodied and common prey of *Bothrops spp* (Voss 2013). *Monodelphis domestica* was used, because it does not exhibit platelet aggregation in the presence of venom CTLs (Drabeck et al., in prep.), even though it is unknown it if is susceptible to pit-viper venom. We also included another species from this genus - *Monodelphis emiliae* – because we have observational evidence that pit-vipers attack and eat this species in the wild (Voss 2013). The genus *Monodelphis* has several unique changes in vWF botrocetin-binding sites which may disrupt the interaction between the two molecules, but this has never been tested. Though we attempted to express species from other genera of Didelphidae (e.g. *Gracilinanus*) these did not successfully express in *E. coli*. Human vWF was also chosen for expression as its affinity for botrocetin and bitiscetin (a third venom CTL from an African viper *Bitis arietans*) has previously been shown, and thus

served as a gauge for venom and binding assay function (Hamako et al. 1998, Fukuda et al. 2005).


A.
Caluromys philander
Caluromys lanatus
Glironia venusta
Metachirus nudicaudatus
Chironectes minimus
Lutreolina crassicaudata
Didelphis virginiana
Didelphis albiventris
Didelphis marsupialis
Didelphis aurita
Philander quica
Philander mcilhennyi
Philander opossum
Gracilinanus agilis
Gracilinanus microtarsus
Gracilinanus aceramarcae
Gracilinanus emiliae
Thylamys venustus
Thylamys pallidior
Thylamys macrurus
Thylamys pusillus
Marmosops incanus
Marmosops noctivagus
Marmosops spGalvez
Marmosops pinheiroi
Marmosops parvidens
Tlacuatzin canescens
Monodelphis scalops
Monodelphis emiliae
Monodelphis peruviana
Monodelphis brevicaudata
Monodelphis domestica
Marmosa rubra
Marmosa robinsoni
Marmosa mexicana
Marmosa murina
Marmosa lepida
Marmosa rutteri
Marmosa paraguayana
Marmosa demerarae
30
25
20
Ma
15
10
5
0

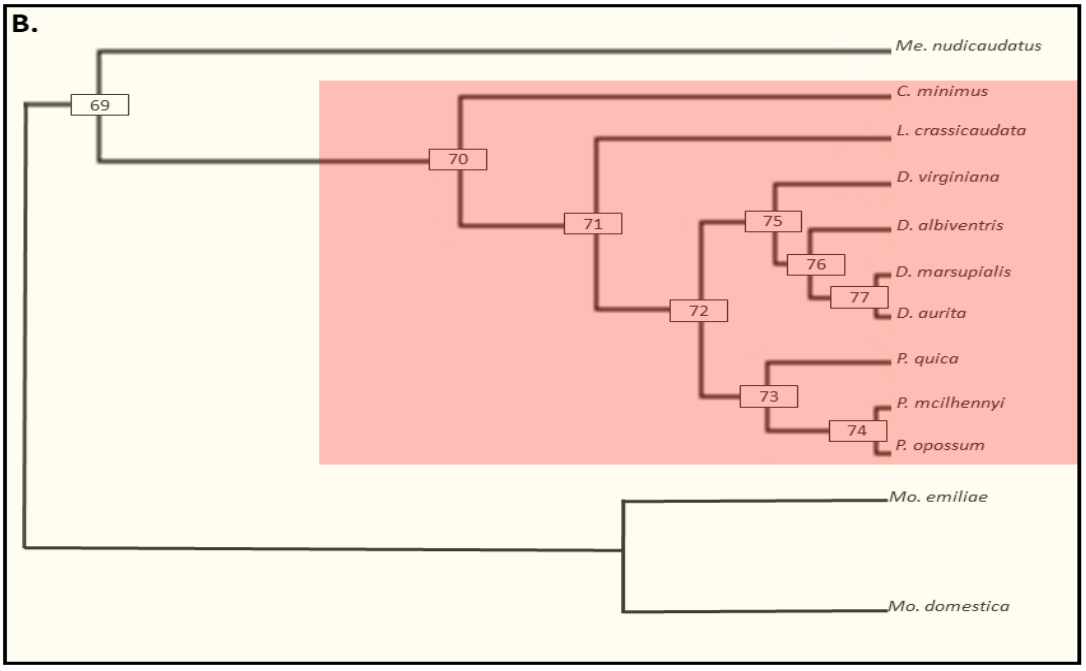

B.
Me. nudicaudatus
69
C. minimus
70
L. crassicaudata
D. virginiana
71
75
D. albiventris
76
D. marsupialis
77
72
D. aurita
P. quica
73
P. mcilhennyi
74
P. opossum
Mo. emiliae
Mo. domestica

Figure 4.2- Panel A: Pruned time-calibrated tree from Jansa et al. (In Prep) used for reconstructing ancestral vWF sequences. Tribe Didelphini is highlighted in salmon. We refer to Didelphini as defined by Jansa and Voss (2011) for this work, with the updated taxon designation for a specimen previously identified as Philander frenatus, which is now Philander quica (Voss et al. 2018). In yellow are species whose vWF was selected for overexpression outside Didelphini (human not pictured). Panel B: Topology of Didelphini and other opossums whose vWF was selected for overexpression. Nodes are numbered with reference to the larger phylogeny shown in Panel A; only node numbers for nodes selected for overexpression are shown.

Finally, to investigate the how the venom CTL-vWF binding interaction may have changed during opossum evolution, we inferred several ancestral opossum vWF sequences, expressed them, and quantified their binding affinity for various venom CTLs (Figure 4.2).

vWF Sequencing.

To express opossum vWF A1 protein, the entire sequence of the region used for previous expression studies on human vWF (residues 475-709) was required (Cruz et al. 2000, numbering scheme Maita et al. 2003). vWF sequences from species within and outside Didelphini were used from a previous study (Jansa and Voss 2011); however, the N-terminal region (~207bp) of the vWF-A1 region (residues 475-543) was missing from these individuals. We therefore sequenced this upstream region and assembled it with existing sequences using a ~50 bp overlapping region. The vWF-A1 region was newly sequenced for three species, either because ambiguities existed in the original sequence, or because the sequence was not available (Appendix Table 4.1). Sequences for human and *Monodelphis domestica* were downloaded from whole genome sequences available from NCBI (Appendix Table 4.1).

Polymerase Chain Reactions (PCRs) were carried out in a 25 μl reaction using 0.5 μl of 10 mM dNTPs, 15 μl ddH_20, 5 μl 5X Phire reaction buffer (Thermo-Scientific), 1 μl each of 10 mM primer solution of forward and reverse primers, 0.5 μl of Phire polymerase (Thermo-Scientific), and 2 μl of template DNA. Primers DvWF_F1 and DvWF_R1 were used for the partial upstream sequence, DvWF_F1 and DvWF_R6 was used for sequencing the complete vWF region (covering residues 475-543) (Appendix Table 4.2). Reactions included a 30 second 98° C activation step, after which 35 cycles

were programmed as follows; 98° C for 5 seconds, 65° C for 5 seconds, and 72° C for 15 seconds. Amplified PCR product was sequenced at the University of Minnesota Genomics Center for all species except *Didelphis aurita*, which was sequenced at the Instituto Butantan Laboratorio Especial de Toxinologia Aplicada (LETA). Permission for the capture and sampling of *D. aurita* was granted by SISBIO (permit #64934-1). Resulting sequences were edited and assembled in Geneious version 7.1.8. Upstream sequences were accessioned as amendments to the original sequence accession, and new sequences were accessioned separately (Appendix Table 4.1).

Ancestral Sequence Reconstruction.

We used a codon model for maximum likelihood reconstruction of ancestral sequences. To infer ancestral sequences and compare alternative models of selection in a likelihood framework, we used the codon model (CODEML) implemented in PAMLx version 1.3.1, PAML version 4.9 (Xu and Yang 2013, Yang 2007, Randall et al. 2016). These analyses require two components; (1) vWF sequences from extant taxa, and (2) a tree with branch lengths for all species included (Xu and Yang 2013, Yang 2007). Complete vWF sequences described above (Appendix Table 4.1, excluding human and *Didelphis aurita*) as well as 28 additional opossum vWF DNA sequences (covering residues 543-709) from a previous study (Jansa and Voss 2011) were used to make a 39 taxon vWF alignment in Geneious version 7.1.8 using MUSCLE (Edgar 2004) (Appendix Material 4.1- DidvWFAlign1.phy). An unpublished tree (Jansa et al., in prep.) resulting from Bayesian analysis of 6 genes (CYTB, BRCA, IRBP, and 3 nuclear introns) and 121 taxa with the same calibrations points as Jansa and Voss (2014) was pruned down to this same set of 39 species using R-package *ape* (Appendix Material 4.2 DidvWFtree1.tre) (Paradis and Schliep 2018).

We used a likelihood ratio test to compare maximum likelihood models of selection in PAMLx version 1.3.1, PAML version 4.9 (Xu and Yang 2013, Yang 2007). Specifically, we compared the β distribution models M8 and M7 to test whether a model (M8) that allows for positive selection (dN/dS >1) was a significantly better fit than a

model that only allows for neutral or purifying selection (M7). We the M7/M8 comparison as it is unconstrained by *a priori* designation of branches under positive selection, though Jansa and Voss (2012) used such a model (the branch-site test) to show positive selection in Didelphini. Subsequent data (Chapter 3) indicates that rapid evolution may be present throughout Didelphidae, and that species outside Didelphini may also be venom resistant. The relative fit of the two models was measured by the natural log of the likelihood ratio (Λ), where $-2[\Lambda]$ under the null hypothesis was assumed to follow a χ^2 distribution with two degree of freedom (Yang 2006).

Nine ancestral sequences generated under the best fit model (M8) were selected for expression in *E.coli*. These included all nodes within Didelphini as well as the most recent common ancestor of Didelphini with its sister taxa *Metachirus nuducadatus* (Figure 4.2). We used parsimony to reconstruct a deletion at the N-terminal end of ancestral sequences as well as a single deletion event mid-sequence on the branch leading to *Metachirus nudicaudatus* (Appendix Figure 4.1). Maximum likelihood sequence inference was originally conducted using an alignment of 39 species based on sequence availability of vWF (Appendix Material 4.1 DidvWFAlign1.phy). Sequences of *Didelphis aurita* were subsequently generated for expression and added to the alignment (Appendix Material 4.3 DidvWFAlign2.phy). Re-estimation of ancestral sequences with the addition *Didelphis aurita* resulted in one amino change in one ancestor (node 70). This alternative ancestral sequence was not subsequently cloned and used for *in-vitro* expression.

Gene Synthesis.

Extant and ancestral vWF sequences were edited in Geneious version 7.1.8 to contain N-terminal leader BamHI (5'- CACGGTAGC-3') and C-terminal HinDIII (5'-TAACAAGCTTAA-3') cut sites, optimized for *E.coli* codon usage, and submitted for gene synthesis by GenScript (Piscataway, NJ). Several sequences were identical at the protein level, and therefore synthesized only once: (Node 74, *Philander mcilhennyi* and *Philander opossum*), (Nodes 72 and 75), and (Node 76, 77 and *D. marsupialis*). A complete list of unique extant and ancestral constructs can be found in Appendix

(Material 4.5 Constructs.phy). Constructs were received inserted in a pUC57 backbone plasmid (Tai et al. 2013).

Vector Construction.

A pQE9 plasmid vector containing an N-terminal 6-Histidine tagged Human vWFA1 fragment (residues 475-710) was kindly provided by Dr Miguel Cruz (Baylor College of Medicine). This plasmid was used for expression of human vWF and subsequently modified for expression of extant and ancestral opossum vWF (Cruz et at 2000, Appendix Figure 4.1). Extant and ancestral opossum sequences were excised from pUC57 via BamHI/HinDIII restriction digest and ligated into purified pQE9 from which human vWF had been excised by the same digestion (Appendix Figure 4.1). pQE9 at this site has been engineered such that a ligated product will be placed directly downstream from a start codon and a 6-Histidine tag (Appendix Figure 4.1). For efficiency, a pQE9 plasmid for *Didelphis aurita* was constructed by site-directed mutagenesis PCR on the *Didelphis marsupialis* pQE9 construct. Two protein coding differences at the binding site exist between these two species and were altered accordingly via Golden Gate Assembly (Engler et al. 2008). A single protein coding difference 4 residues from the N-terminus and outside the botrocetin binding site was not altered.

To isolate mutant plasmids, ligation product was transformed into chemically competent M15 cells and allowed to recover in LB media at 37° C for 1 hour before being plated on LB-ampicillin plates (50 μg/mL) and grown at 37° C overnight. Single colonies were picked, grown overnight in liquid LB with 100 μg/ml ampicillin, and plasmids were isolated via a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). Isolated plasmids were checked for correct insert sequence by PCR amplification using 1 μl each of a 10mM primer solution of PQE9 Insert F1 and PQE9 insert R3 0.5 μl 10mM dNTPs, 15 μl ddH_20, 0.5 μl Phire polymerase, and 5 μl 5X Phire buffer (Thermo-Scientific) (Appendix Table 4.2). Reactions were run on a thermocycler using a 30 second 98° C activation step, and 35 cycles as follows: 98° C for 5 seconds, 65° C for 5 seconds, and 72° C for 15 seconds. PCR products were Sanger sequenced at the

University of Minnesota Genomics Center with the same primers. Sequences were edited and assembled in Geneious version 7.1.8; multi-species sequence alignments were created using MUSCLE (Edgar 2004). Vectors containing the correct inserted sequences were stored at -20° C for use in overexpression. A complete list of vWF constructs for extant and ancestral species can be found in Appendix (Materials 4.5 Constructs.phy).

vWF Expression and Purification.

To produce active vWF protein capable of binding to venom CTLs *in vitro*, plasmid constructs were overexpressed and purified. Overexpression and purification was carried out as described previously (Cruz et al. 2000), with the following modifications. After addition of DNase, cell lysate was subjected to 3 minutes of sonication with a 6mm microtip (Q500, Qsonica), (5 seconds on, 5 seconds off) with a 50% duty cycle. Purification was carried out as described previously (Cruz et al. 2000). Briefly, sonicated lysate was centrifuged, and supernatant was discarded. Remaining inclusion body proteins were unfolded in 6M Guanidium-HCL, filtered, and refolded by dilution. Diluent containing refolded protein was applied to a 15 mL nickel column (GE Life Sciences) overnight and fractions containing vWF were eluted at 350 mM imidazole. While Cruz et al. (2000) use a heparin column for further purification, the heparin binding site in human vWF A1 is not conserved in opossums, thus we used an altered protocol to ensure a folded monomeric product. Once eluted off nickel column, fractions were dialyzed into 100 mM Tris-HCl, 400 mM NaCl, 1 mM EDTA, pH 7.4 and applied to a 10 mL Thiopropyl Sepharose 6B column (GE Life Sciences). Folded protein was collected in the flow-through (Miura et al. 2000). Flow-through was then dialyzed in 10 mM Tris-HCL, 100 mM NaCl pH 7.5 at 4° C overnight, and concentrated to 5 mL or less via ultrafiltration in a Vivispin 15 10,000 molecular weight cut off spin column (Sartorius, Goettingen, Germany). This concentrated sample was applied to a size exclusion column (16/600 200 pg Superdex, GE Life Sciences) and the final peak was collected as monomeric folded vWF A1. Product was concentrated via ultrafiltration, checked for size and purity on a Tris-glycine gel, quantified by absorbance at 280 nm (NanoDrop model

2000, Thermo-Scientific), aliquoted, and frozen at -80 until use. Purification was done for each protein separately on an ATKA FPLC (GE Healthcare). Purified vWF protein for each species/node were stored in separate freezer boxes and labeled individually throughout to avoid cross-contamination.

Sequences inferred from ancestral-state reconstruction were transformed and overexpressed months apart from each other (with the exception of nodes 72 and 73 which were expressed on the same day) to reduce the possibility of samples being accidentally switched. As a control, vWF from *Monodelphis domestica* and *Philander quica* were expressed twice, months apart from one another. Resulting affinity measurements were comparable (Appendix Table 4.1).

Venom Purification.

Aspercetin was purified as previously described (Rucavado et al. 2001), reconstituted and dialyzed into TBS (25mM Tris-HCL, 150nM NaCl pH7.4). Two isoforms of botrocetin were utilized. One ('botrocetin A') was kindly gifted by Dr Robert Andrews, Monash University, and was among the batches used for the first studies of botrocetin structure and function (e.g. Dong et al. 2001). A second (botrocetin B) was purified from *Bothrops jararaca* venom as previously described (Chapter 3). Both samples botrocetin A and botrocetin B were the same isoforms used in Chapter 3.

Bitiscetin, a third vWF targeting CTL, is derived from the venom of a distantly related African viper (*Bitis arietans*) and has been shown to bind the vWF A1 domain and platelet receptor GB1Bα in a perpendicular conformation to botrocetin with several overlapping binding sites (Maita et al. 2003, Miura et al. 2000). Because bitiscetin is not proposed to be co-evolving with Didelphini, and is known to bind vWF A1 in humans, it was used as a positive control for assay function. Bitiscetin was purified as previously described and used to confirm that *E. coli*-expressed and refolded vWF A1 was capable of binding a venom CTL in the assay conditions described below (Chapter 3, Hamako et al. 1996).

Binding Assays.

Binding affinities for vWF and venom proteins were measured using a white light interferometry system, BLItz (ForteBio, Pall Corporation). Experimental design was optimized according to manufacturer recommendations (Sultana et al. 2015). The BLItz design was chosen as it allows for data collection of each binding interaction with extremely small volumes of venom protein (4 µl/curve), and therefore extended the ability to collect data given very limited sample volumes. Each binding curve is obtained by first immobilizing vWF (the ligand) to a surface, and then exposing it to a venom CTL (the analyte). Association and dissociation of analyte is observed in nearly continuous time, resulting in a directly observed binding curve (association and dissociation). Because curves from multiple concentrations of an analyte are required for accurate equilibrium estimation of binding affinity, each assay used 4-7 concentrations of analyte (CTL), resulting in 4-7 binding curves for each vWF-CTL pair (Shah and Duncan 2016). Where possible, replicate assays were run. However, because of limited sample volume for venom proteins, replicate assays for all species of vWF were not possible. Where replicate assays were possible, variation of resulting binding affinity was low in both tight and loose binders (Figure 4.3). Additional measures of assay accuracy are described below. Samples of venom and vWF were stored in separate boxes before and after data collection, and Human vWF A1 was used to spot-check venom protein activity periodically throughout data collection. A complete list of data collected for each vWF and venom protein pair by date can be found in the Appendix (Appendix Table 4.3).

Venom proteins (botrocetin A, botrocetin B, aspercetin, and bitiscetin) and vWF A1 aliquots were thawed from -80° C storage on ice and all samples were stored on ice until immediately prior to use. Both vWF and venom proteins were diluted into a buffer containing 25 mM Tris, 150 mM NaCl 1% BSA, 0.05% Tween 20, pH 7.4. vWF was diluted into test buffer to an approximate concentration of 200 nM for loading on to the chip. Venom proteins were serially diluted into concentration series which were appropriate to each $K_{D,}$ as low concentrations series are required for tight binders, and higher concentrations series are required for loose binders. Binding affinity was measured

on a BLItz instrument (ForteBio, Pall Corporation). Both HS1K (anti-histidine antibody) and NiNTA (Nickel) chip tips were used to immobilize vWF A1 onto the chip surface. This was done because HS1K chips loaded with vWF recovered tight binding to botrocetin and aspercetin, however, they loaded some forms of vWF poorly. NiNTA chips loaded all vWF forms well, and recovered tight binding with bitiscetin, but a reduced binding to botrocetin and aspercetin when compared to the same assays done on HS1K chips. As bitiscetin binds vWF at the same sites as botrocetin but in a perpendicular conformation, this difference is likely due to conformational differences of vWF when it is immobilized on each chip (Maita et al. 2003). To recover binding for vWFs which bound better to NiNTA than HS1K or vice versa, both chips were used. To compensate for K_D differences between chips, K_D for human samples (tight binders) were compared between chips and the derived ratio was used to scale binding data between data gathered on different chips. Botrocetin B NiNTA results (K_D, k_a, and k_d) were divided by 7.59, bitiscetin HS1K results (K_D, k_a, and k_d) were divided by 7.78.

Per manufacturer recommendations, biosensors were incubated in buffer for ten minutes prior to each test. Kinetic data collection was performed with a 30 second buffer baseline, followed by a 120 second loading step in which 4 µl of vWF A1 was exposed to the biosensor chip surface. This was followed by a second baseline for 30 seconds, an association phase of 60-120 seconds in which 4 µl of various concentrations of venom protein were allowed to associate to the chip, and a disassociation phase of the same length (either 60 or 120 seconds) where the biosensor was returned to buffer. A sample containing no venom protein was used to calibrate machine noise and subtracted from all curves, as is standard (Sultana et al. 2015). Association and disassociation times were chosen as sufficient time for the CTL to reach equilibrium association to vWF, as well as disassociation of more than 50% (Sultana et al. 2015). Nonspecific binding assays were performed by using a blank buffer for loading, and a sample containing the venom protein at up to the second highest concentration used for each curve series on each chip. Nonspecific binding was visually observed as a flat line for each CTL on each chip type, confirming no nonspecific binding, and not further analyzed (Sultana et al. 2015)

Data Analysis.

Raw kinetics data were globally fit to a 1:1 binding model using the BLItz Pro software v1.2.1.3. This software optimizes the observed association constant (k_{on}), dissociation constant (k_{off}), and R_{max} (the maximum binding of the venom protein, given the amount of vWF on the biosensor surface) over multiple curves (serial dilutions). BLItz Pro software then uses these optimized values to calculate an equilibrium constant K_D, where K_D for a given Analyte (A) and Ligand (L) is $\frac{[A][L]}{[AL]} = \frac{\text{koff}}{\text{kon}} = K_D$, given a 1:1 binding model. A 1:1 binding model was used as both botrocetin and bitiscetin have been shown to bind in a 1:1 conformation with vWF A1 (Maita et al. 2003, Fukuda et al. 2005). For a full description of curve fitting and affinity calculations see Appendix Materials 4.6. Curve sets were optimized by local fitting first, and curves which both had extremely low R^2 values and significantly changed the globally calculated K_D and R^2 were excluded per recommendation by the manufacturer (ForteBio, Pall Corporation). All resulting kinetics estimates were extrapolated from a minimum of 3 curves (concentrations of CTLs). All data estimated from the global fit for each curve set (vWF-venom protein pair) were exported into a CSV file for further analysis.

Summary statistics and subsequent analyses of binding curve data extracted from BLItz software were calculated in JMP Pro version 14.0.0 (SAS Institute). Variance in K_D is right skewed as larger numbers (higher K_D) will have associated larger error. A standard least squares regression was used to assess the assumption that error (variance) will increase with weaker binding, and identify data points that exhibited variance which was higher than expected (outside a 95% CI), above the regression curve. These data points are further addressed in the results. Because of the skewness of this data, K_D was $\log_{10}$ transformed for subsequent analyses. Log transformed K_D was also regressed against variance to determine resolution of skewness. As each exported assay has an associated K_D and R^2 value, R^2 was regressed with K_D to determine if goodness of fit (R^2) reduced with increasing K_D, which would indicate systemic poor fit for high K_D values.

To test how differences in on-rates (K_{on}) and off-rates (K_{off}) influence binding loss, data were grouped into binders (1 to $\frac{1}{3}$ human binding), and non-binders ($< \frac{1}{3}$ human binding). We chose this value because a 3 fold error is the reported expectation for error between BLItz measurements, though our standard error between K_D measures were much lower that this. A 3 fold change in binding therefore represents the smallest binding change which could not feasibly be due to measurement error, and is therefore likely to be biologically relevant. Non-parametric Wilcoxon tests were used on log-transformed K_{on} and K_{off} to assess differences between these groups (binders and non-binders).

Parsimony Reconstruction of Binding Phenotypes.

Estimates of ancestral phenotypes can be quite misleading when processes of molecular evolution are not adequately accounted for. To assess whether inferred ancestral binding affinity differed from actual measured affinity of expressed proteins, we estimated phenotypes (binding capacity) for the same nodes used for *in-vitro* expression for botrocetin A, botrocetin B, and aspercetin using phylogenetic inference of whole trait reconstruction. Binding capacity of extant vWF from biophysical assays was divided by human binding capacity for the same protein to generate a relative binding capacity and was used to generate predictions of ancestral node relative binding. Because relative binding spans values from 0.17-10000X, binding loss was coded by binning relative binding loss by increments of 5x binding loss: (1) 0-5x, (2) 6-10x, (3) 11-15x, (4) 16-20x, (5)>20x, and (6) no detectable binding (NDB) and treated as a discrete ordered trait. The same 121 taxon tree with branch lengths was pruned down to include only species within Didelphini and sister taxon *Metachirus nudicaudata* with the function drop.tip in Phytools (version 0.6-60). As *Philander mchilhennyi* had an identical vWF A1 protein sequence (and therefore phenotype) as *Philander opossum*, it was also pruned collapsing these two species into one, and collapsing their identical ancestral nodes (73 and 74) similarly into one node. We used several methods to reconstruct node states (parsimony and maximum likelihood) which all yielded similar results. Here we present results for squared change parsimony trait reconstruction done in the R package *castor* (function

asr_squared_change_parsimony, version 1.3.6) with unweighted transition costs (*weighted= FALSE*) (Maddison 1991, Louca and Doebeli 2017).

Results

Reconstruction of Ancestral vWF Sequences.

Consistent with previous work, an M8 model that allows a proportion of positively selected sites (with a dN/dS >1) was a significantly better fit than an M7 model that does not (Jansa and Voss 2011). This was true for both analyses with *Didelphis aurita* ($-2\cdot\ln[\Lambda] = 114.5$, d.f. = 2), and without *Didelphis aurita* ($-2\cdot\ln[\Lambda] = 112.8$, d.f. = 2) (Yang 2000, Jansa and Voss 2011). Inferred ancestral sequences from this model for nodes 69-77 were selected for expression (Figure 4.2). A total of 5 ancestral sequences were reconstructed representing 9 nodes, all of which had high marginal likelihood (<90%) for sites within and surrounding botrocetin binding regions (Appendix Material 4.5 Constructs.phy). Referring to node labeling in Figure 1, nodes 72 and 75 were identical to each other, node 76 and 77 were identical to *Didelphis marsupialis*, and node 74 was identical to both *P. mcilhennyi* and *P.opossum*. The addition of *Didelphis aurita* to the maximum likelihood reconstruction resulted in one amino acid difference in node 70 at a single botrocetin binding site (Site 636 L→ R): this alternative ancestor was not subsequently engineered or expressed.

Assessment of Kinetic Data Reliability

Extant Data Assessment

As synthetic refolded protein is not guaranteed to be recovered in an active conformation, Bitiscetin was used as a control to assess the ability of synthetic and refolded vWF (from inclusion bodies) to bind normally,. Normal binding curves were observed for vWF of all species with bitiscetin except *Philander opossum*, and thus is the only species for which loss of binding may be due to the expression/purification process (Figure 4.3). Separately expressed batches of *M. domestica* and *P. quica* were used for bitiscetin (batch 1) and botrocetin B (batch 2) assays and produced comparable binding

affinities (Figure 4.3). As is expected for kinetics data, measurement error increases with the magnitude of K_D. When average K_D is regressed with standard error a linear relationship is recovered (R^2= .90, P<0.0001). The only standard error value which falls above the 95% CI of this linear relationship is *Monodelphis emiliae* (for botrocetin B), though the range of this variation does not change the broad interpretation of binding loss relative to human (Figure 4.3). Mean Log-transformed K_D regressed with variance showed no relationship (R^2=0.03, p=0.48). Individual Log_{10} (K_D) regressed with its associated R^2 generated by BLItz model fitting also showed no significant correlation (R^2=0.03, p=0.12), indicating that log transformed data for both tight and loose binders are robust, including data which surpass the 1 μM limit of detection for BLItz.

The level of binding loss with respect to human vWF for botrocetin B across Didelphini is much less than that of botrocetin A. Between these samples we know that human platelets aggregate with botrocetin A, and its K_D for human vWF is 766 nM, whereas *Didelphis virginiana* platelets do not aggregate when exposed to botrocetin A and its vWF has a K_D for it of 25800 nM. For botrocetin B we know that human platelets aggregate in response to it, and it has a K_D of 50nM with its vWF. Whereas botrocetin B does not aggregate *Didelphis aurita* platelets and it binds to its vWF with a K_D of 428nM. This suggests that botrocetin A can induce aggregation even when binding fairly loosely to vWF (with a 766 nM K_D), while botrocetin B cannot as it fails to aggregate at 428nM, and requires a much tighter interaction to induce binding (50nM K_D).

Previously reported K_D derived from radiolabeling assays report a K_D for botrocetin and human vWF at 23 nM (Miura et al. 2000), though this assay was performed with an immobilized C-type Lectin, whereas our design immobilized vWF A1, which may account for differences in binding results. We recovered equilibrium constants for botrocetin B as 55.15 nM (SE±3.74 nM), quite similar to previously reported K_D, and botrocetin A as 766 nM (SE± 5.84 nM), a much higher K_D than previously reported. The low binding observed for botrocetin A could be considered suspect because the sample provided from the Andrews lab was at least several years old (Dr Robert Andrews Pers Comm) and could have experienced some degree of

degradation. However, if this was the case, the botrocetin A sample should have exhibited at least some observable reduction in ability to aggregate human platelets, which was not observed (Chapter 3).

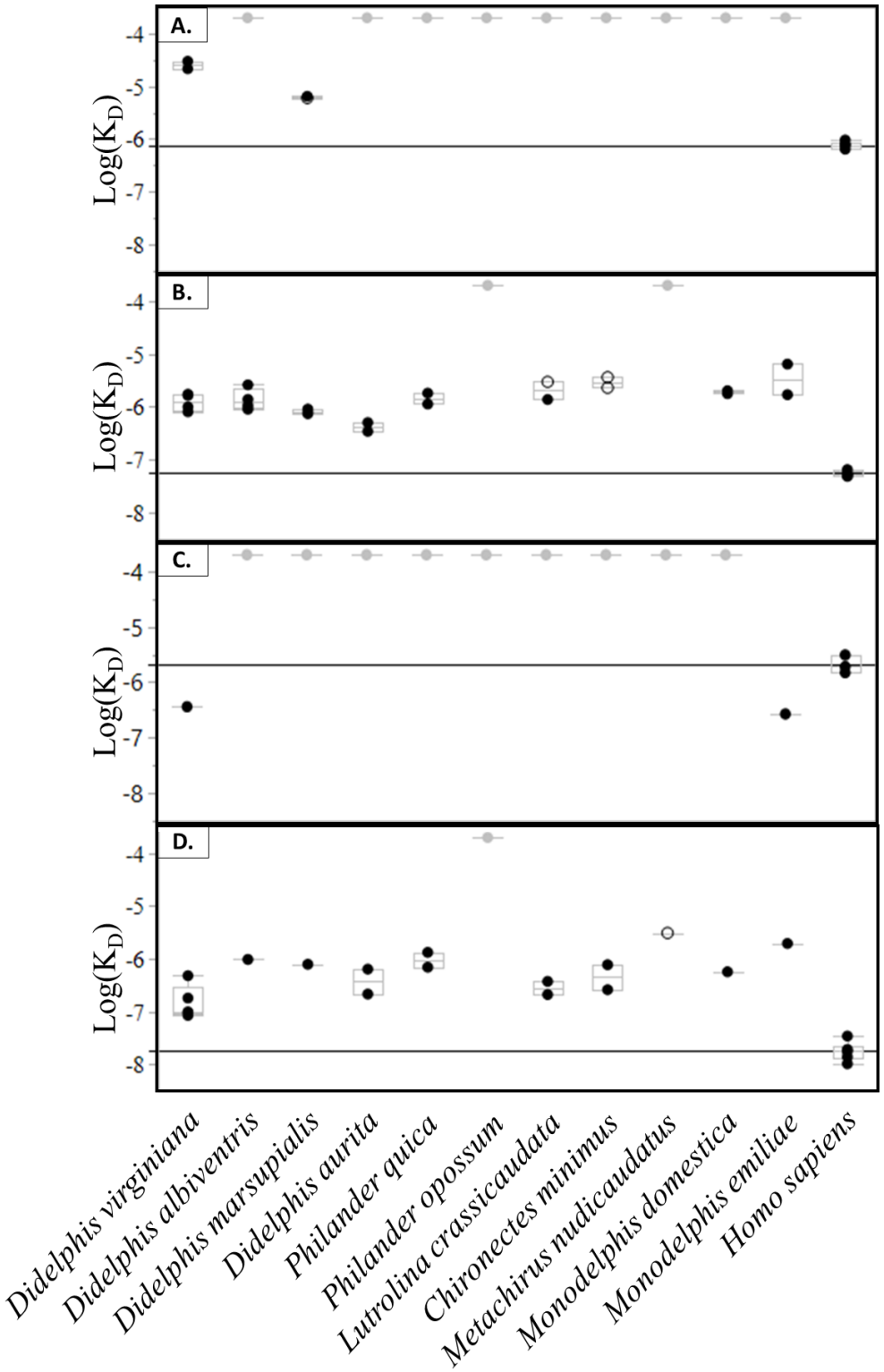

A.
B.
C.
D.
Log(K_D)
-4
-5
-6
-7
-8
Didelphis virginiana
Didelphis albiventris
Didelphis marsupialis
Didelphis aurita
Philander quica
Philander opossum
Lutrolina crassicaudata
Chironectes minimus
Metachirus nudicaudatus
Monodelphis domestica
Monodelphis emiliae
Homo sapiens

Figure 4.3- Log_{10} K_D [M] for vWF A1 against four venom proteins **A.** botrocetin A, **B.** botrocetin B **C.** aspercetin **D.** bitiscetin. Binding loss above -4, indicated by grey circles are data with no detectable binding curves (flat lines) and represent an arbitrarily low binding affinity. All other data points are derived from binding curve data. Data points with low R^2 values (0.91-0.95) are indicated by empty circles. As an R^2 value of >0.96 is generally used as a rule of thumb for acceptable data, K_D measurements with R^2 values of 0.96 or greater are indicated by black circles. Standard quantile box plots show a line at the median and enclose the 25th and 50th quantiles of the distribution. The line extending across each graph represents mean Human Log_{10} K_D. All graphs scales are identical.

Ancestral Data Assessment

Because all synthesized ancestral proteins produced measurable binding (some quite substantial) for at least one venom protein, all were presumed to be functionally folded. The sole function of bitiscetin in these assays was to confirm the ability to bind to the chip and produce measurable binding data (qualities of being functionally folded), therefore no bitiscetin data were not gathered. Variation among replicates was similar to data from extant proteins (Figure 4.4), with the exception of node 72 for aspercetin which had no detectable binding in one replicate and very little binding in a second replicate, with a notably low R^2 (0.94). This likely indicates that this protein is a weaker binder than is indicated by parameters estimated from the poor curve fit, and is on the border of detectable binding (50-200μM). However, the range of this variation does not change interpretation of a large binding loss relative to human. Similar to results from proteins representing extant species, several data points exceeded the detection limit of 1μM, but exhibited low variance and high R^2 in for globally fit binding curves.

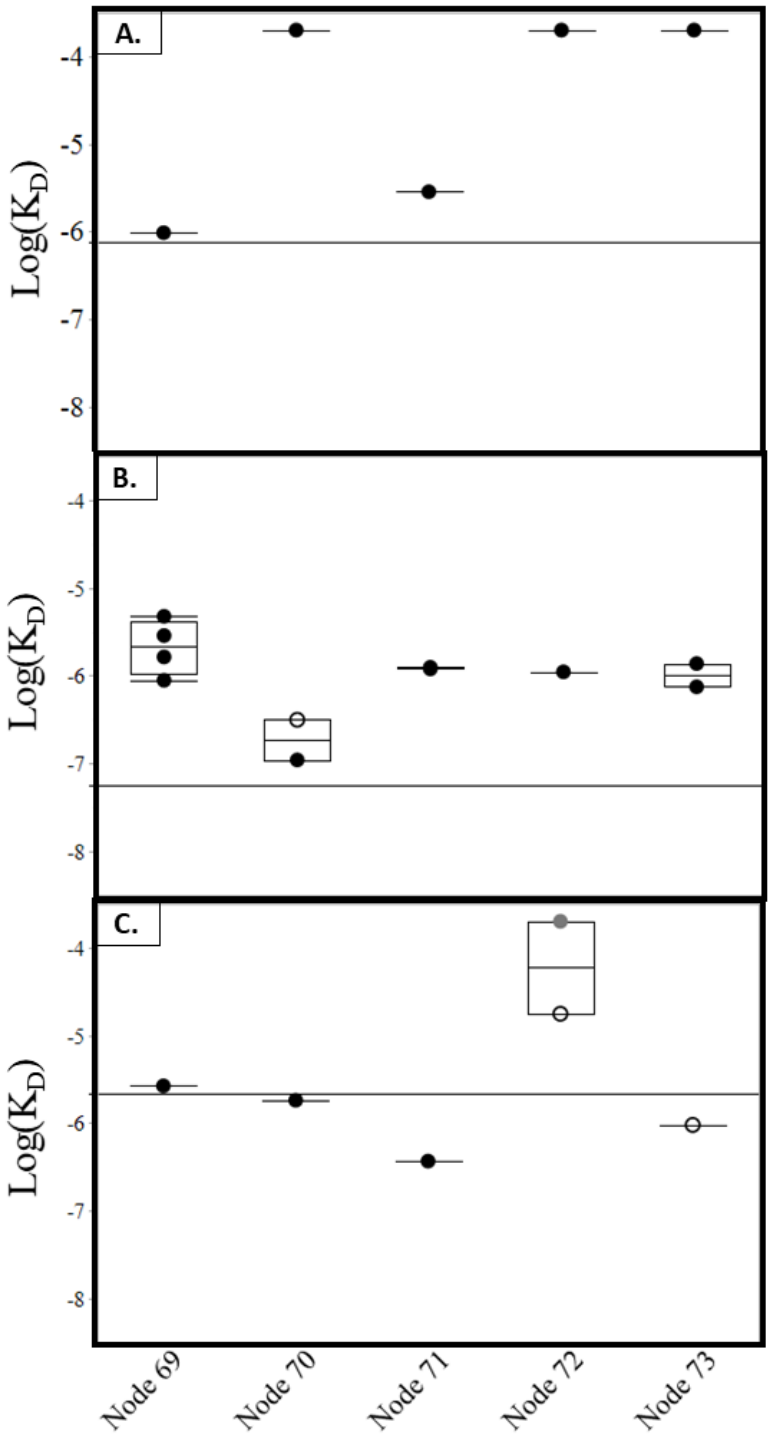


Figure 4.4- Log_{10} K_D for ancestral vWF A1 nodes against venom proteins. Panel A- botrocetin A, Panel B- botrocetin B, Panel C- aspercetin. Binding loss above -4, indicated by grey circles are data with no

detectable binding curves (flat lines) and represent an arbitrarily low binding affinity. All other data points are derived from binding curve data. Data points with low R^2 values (0.91-0.95) are indicated by empty circles. Data with R^2 values of 0.96 or greater are indicated by black circles). Standard quantile box plots show a line at the median and enclose the 25th and 50th quantile of the distribution. The line extending across each graph represents mean extant Human $Log_{10}(K_D)$ for reference.

Kinetics Results for Extant Taxa.

We report average K_D values for human vWF A1 for botrocetin B as 55.15 nM (SE±3.74 nM), botrocetin A as 766 nM (SE± 5.84 nM), bitiscetin as 19.2 nM (± 0.337 nM), and aspercetin as 2180 nM (SE± 485 nM). These results, as well as estimated K_D values for opossum species, are summarized in Figure 4.3.

Several species of vWF exhibited no binding curves i.e. flat lines, when challenged with a CTL. This lack of response indicates either a complete lack of binding, or a binding constant which is higher than our capacity to measure it given CTL concentrations and/or system detection limits. Flat lines or binding curves which were too small to be meaningfully fit to a binding model were therefore assigned a K_D value of 200 μM, in order to be included in analyses. This value is 4 times the largest K_D we were able to measure and one order of magnitude larger than expected machine error (3X K_D). Thus, this number represents the tightest K_D that could have been present but undetected as a result of the machine detection limitations of BLItz.

Binding loss for venom CTLs relative to human was observed both for species within and outside of clade Didelphini (Figure 4.5). All opossums showed binding loss when compared with human for both isoforms of botrocetin and bitiscetin (Figure 4.5). All but two species showed binding loss for aspercetin (Figure 4.5). All species known to exhibit total physiological resistance via loss of aggregation response for a botrocetin (A or B) and bitiscetin showed vWF-CTL binding loss between 8-36 fold (Figure 4.5). *Didelphis aurita* vWF A1 bound to botrocetin B with an 8 fold lower affinity than human vWF, but is known to have no aggregation response when its blood is exposed to this venom protein (Chapter 3). Because we know this species has a complete loss of physiological response given an 8-fold loss of binding we can surmise that an 8-fold binding loss is sufficient to abolish aggregation response. Using this as a benchmark, we

can interpret that the remaining species which have greater than 8-fold binding loss would not exhibit aggregation in response to botrocetin, bitiscetin, or aspercetin (though two species show tight binding to aspercetin).Two species' vWF A1 that bound to aspercetin showed binding tighter than that of human vWF A1 (Figure 4.5).

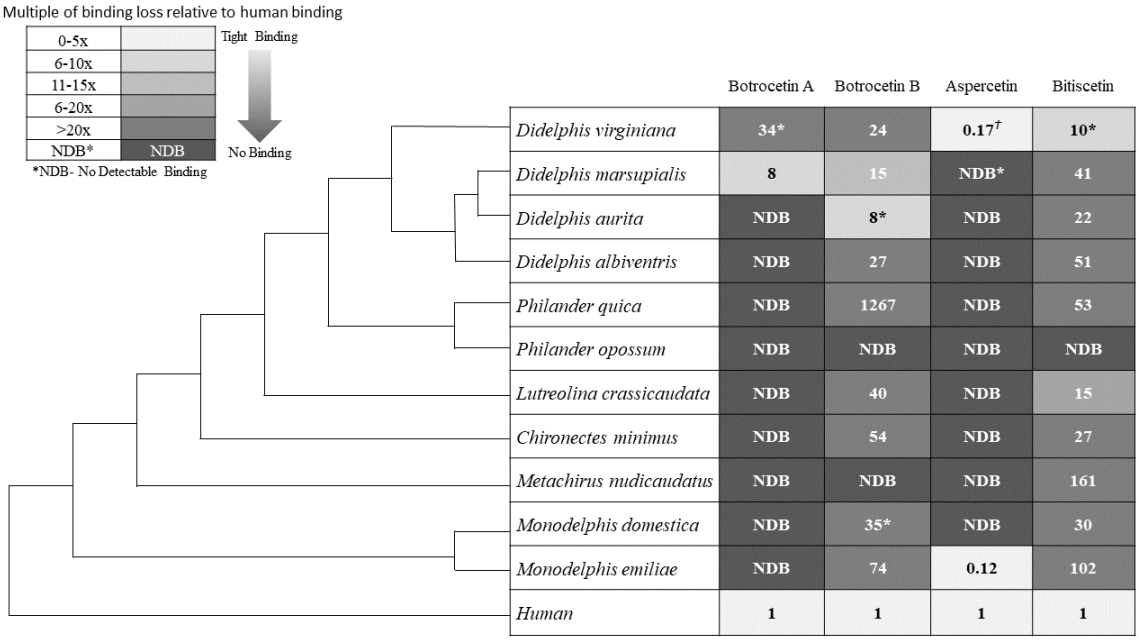


Figure 4.5 - Relative binding loss across the clade Didelphini and outgroup species. Binding loss is expressed as a multiple of loss from human binding (divided by human K_D). Heat map reflects the range of binding loss in increments of 5x up to 20x. This same 6 category classification is used for ancestral state reconstruction in Figure 4.6. NDB indicates no detectable binding. (*) species whose platelets have not aggregated in the presence of the venom protein indicated, and (†) have shown partial aggregation when compared to human samples, which aggregate completely (Chapter 2).

Kinetics Results for Ancestral vWF.

Kinetic data for proteins representing ancestral nodes was collected for botrocetin A, botrocetin B, and aspercetin. Relative binding loss of ancestors mapped to their location on a topology of Didelphini shows a remarkable pattern of repeated loss and gain of binding for botrocetin A and botrocetin B (Figure 4.6). This contrasts with the parsimony reconstruction of phenotype states (binding vs. non-binding) predicted from extant species (Figure 4.6). Large magnitude transitions in binding for these two proteins occur 4 times across internal nodes, and 4 times convergently in branches leading to extant taxa. Notably, this pattern is nearly in perfect opposition between the

two isoforms of botrocetin; as a reconstructed protein loses affinity for one isoform, it gains it for another. Switches from gain to loss are seen for aspercetin a total of 2 times across nodes, with convergent loss of binding in 5 branches leading to terminal taxa (Figure 4.6). Nearly all ancestral opossums bind strongly to aspercetin, while all extant taxa except *Didelphis virginiana* don't bind it at all. Only reconstructed ancestors of the genus *Didelphis* have lost binding in a way that matches extant taxa in a similar pattern to the parsimony reconstruction for this genus, and show that *D. virginiana* has regained binding for aspercetin (Figure 4.6).

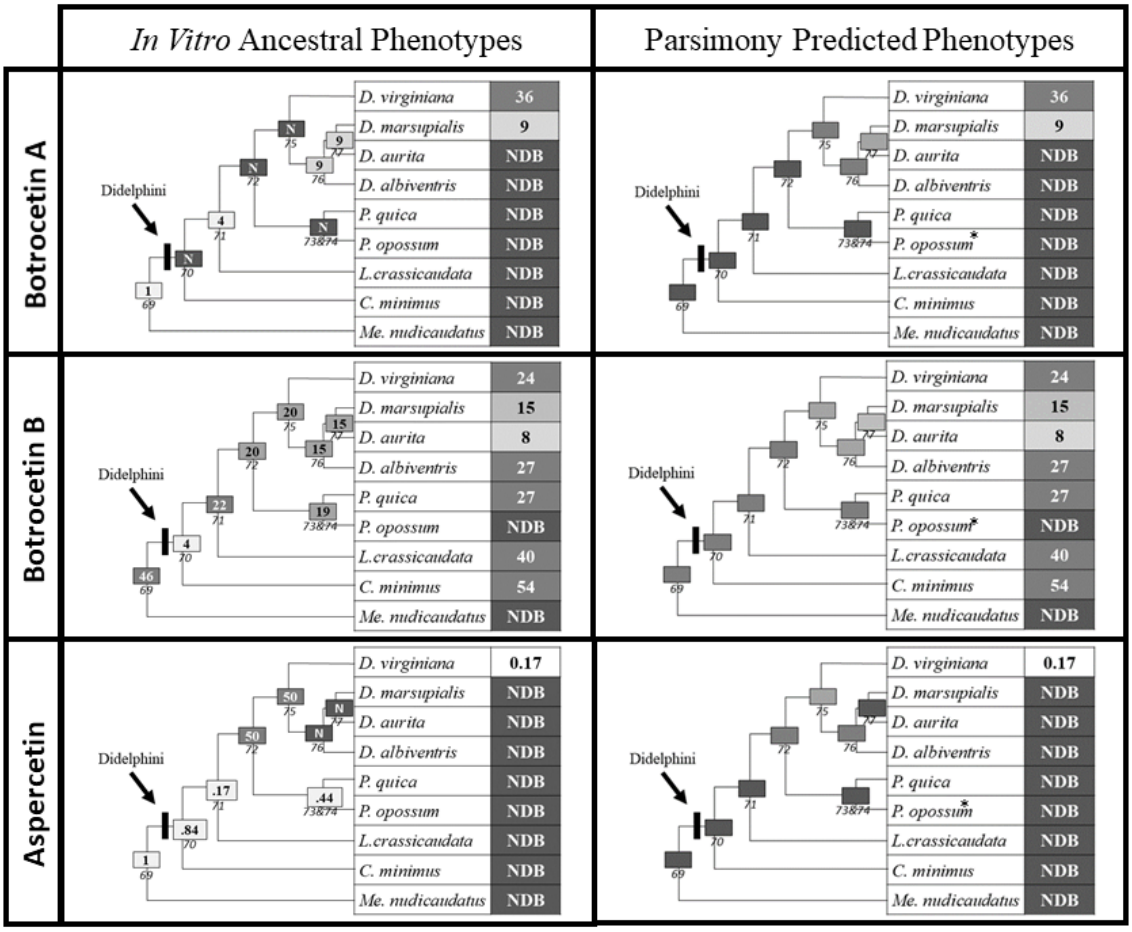


Figure 4.6- Relative binding of ancestral nodes of vWF A1 mapped onto a species topology. On left is the actual binding capacity of each node measured via biophysical assays of heterologously expressed ancestral protein. On right is the predicted phenotypes of the nodes based on a squared change parsimony

reconstruction of phenotypes given the binding data from tips. Asterisks indicated identical protein sequences *Philander opossum* and *Philander mcilhennyi* collapsed as one tip taxa, subsequent nodes (node 73 and 74) from Figure 1 are also identical and collapsed into one node. Panel A- botrocetin A, panel B- botrocetin B, C- aspercetin. Heat map indicates loss of binding with darkening shades, with increments of 0-5x, 6-10x, 11-15x, 16-20x, >20x, and NDB, identical to Figure 4.4. Number at each node indicates actual multiple of binding loss relative to human (K_D divided by human K_D). NDB at tips and N at nodes indicates no detectable binding. Extant relative binding is shown at the tips for reference. Node numbers correspond to Figure 4.2 and are labeled under each node's relative binding.

Influence of K_{on} and K_{off}.

To assess the influence of on and off-rates in binding loss we grouped all botrocetin A, botrocetin B, and aspercetin extant and ancestral data for which complete binding curves could be estimated. Species and ancestors were grouped as binders if they bound at 0.1-3 fold human binding loss (n=17), and grouped as non-binders if they exhibited binding loss greater than 3 fold (n=68). This value was chosen as a cut-off as a 3 fold maximum expected error between replicates for BLItz measurements, though our error between replicates was never this high. A three-fold difference in binding likely is the smallest binding change which is not expected to be recovered from measurement error alone. Binders did not have significantly faster on-rates from non-binders ($p=0.07$, $\chi^2=5.2$, df= 2). However, binders did have significantly slower off-rates when compared to non-binders ($p=0.0007$, $\chi^2= 14.5$, df= 2).

Analysis of Sequence and Functional Data

Seven sites within the botrocetin binding pocket exhibit independent changes across Didelphini, while 18 sites exhibit convergent changes across this clade, making it difficult to discern which are vital to functional binding loss by examination of sequence alone (Jansa and Voss 2011). Though sites evolving under positive selection narrow this pool, they cannot identify specific amino acid changes responsible for binding loss (Jansa and Voss 2011). The addition of functional data from reconstructed ancestral proteins allowed us to identify branches that exhibited extreme loss of binding (>10 fold binding loss) either between nodes or from nodes to tips (Table 4.1).

Using this approach we identified six branches exhibiting such loss for botrocetin A, 3 for botrocetin B, and 5 for aspercetin. Among these branches, multiple instances of

site convergence were observed (Table 4.1, Figure 4.7). The sites repeatedly associated with >10 fold loss of binding are summarized in Table 4.1. One site exhibited an additional level of convergence, having the same amino acid change multiple times at the same site (Table 4.1). Eight sites in total were associated with >10 fold loss of binding of venom proteins, and overlapped closely but not entirely with either botrocetin binding sites, or sites identified to be evolving under positive selection (Figure 4.7) (Jansa and Voss 2011).

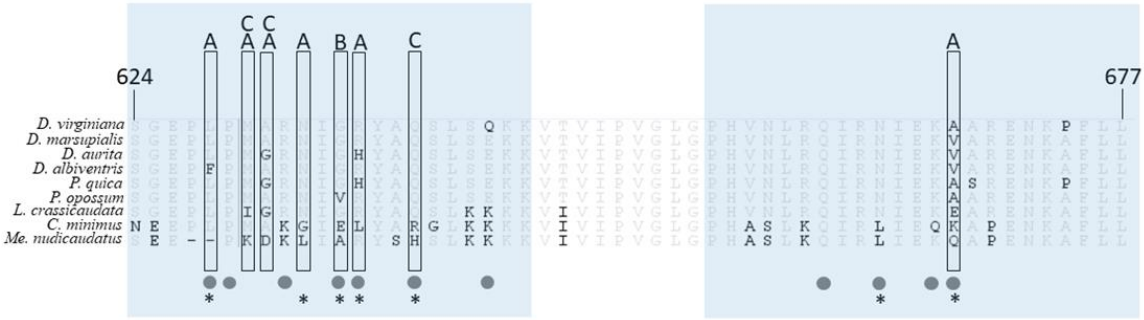


Figure 4.7- Protein alignment of Didelphini. vWF sites associated with multiple independent instances of >10 fold binding loss are enclosed in boxes, venom protein(s) for which binding loss is associated is denoted above the box. A- botrocetin A, B- botrocetin B, C- aspercetin. Known botrocetin binding sites are marked with a circle, and sites evolving under positive selection per Jansa and Voss et al. (2011), are denoted with an asterisk. Region 624-677 is shown as it encompasses the two botrocetin binding pockets (624-645), and (655-677) highlighted in blue. Majority amino acids are in grey, and variants are denoted in black.

Site	Change	Branch	Binding loss for
628*	deletion	node 69→*Me. nudicaudatus*	botrocetin A
	L→F	node 76→*D. albiventus*	botrocetin A
630	M→I	node 71→ *L. crassicaudata*	botrocetin A, aspercetin
	M→K	node 69→Me. Nudicaudatus	botrocetin A, aspercetin
631	A→G	node 71→ *L. crassicaudata*	botrocetin A, aspercetin
	A→G	node 73→*P. quica*	botrocetin A, aspercetin
	A→D	node 69→ *Me. nudicaudatus*	botrocetin A, aspercetin
633*	S→L	node 69→*Me. nudicaudatus*	botrocetin A
	S→G	node69→node 70	botrocetin A
635*	D→E	node 70→*C. minimus*	botrocetin B, aspercetin
	D→G	node 70→ Node 71	botrocetin B, aspercetin
636*	R→H	node 77→ *D. aurita*	botrocetin A
	R→L	node 69→node 70	botrocetin A

639*	Q→R	node 70→ *C. minimus*	aspercetin
	Q→H	node 69→ *Me. nudicaudatus*	aspercetin
668	Q→K	node 69→node 71	botrocetin A
	E→A	Node 71→ node 72	botrocetin A

Table 4.1- Sites which are associated with >10 fold binding loss at least twice (on two branches independently) within Didelphini + *Metachirus nudicaudata*. Sites in bold are known botrocetin binding sites, and sites with asterisks are evolving under positive selection per Jansa and Voss (2011). Changes with the exact same amino acid changes are colored in grey.

Discussion

Ancestral reconstruction and in-vitro testing of vWF A1 phenotypes show the capacity for large losses and gains of binding capacity in single steps, repeated loss and gains of function across ancestors, and convergent loss of function across species. Though vWF A1-CTL binding loss was expected to be found exclusively in the Tribe Didelphini, it was instead found to be more widespread across the family Didelphidae. These results suggest that both trench warfare coevolution and convergent evolution have played important roles in the evolution of resistance to venom CTLs in opossums.

How Ancestral Binding Results Inform Coevolution.

Kinetic testing of ancestral vWF A1 shows a pattern of repeated loss and gains of function across nodes. This pattern diverges from the expectation of phenotype evolution under a simple parsimony reconstruction of binding affinity (Figure 4.6). While surprising, results from Jansa and Voss (2011) showing strong positive selection suggest that coevolution may be working on vWF A1. Data from ancestral binding reveal that trench warfare rather than arms race coevolution is likely the dominant process at work in this system. Ancestral binding results also reveal several cases of unexpected—cryptic—convergent evolution.

The mismatch between observed *in vitro* reconstruction and trait reconstruction in this study likely stems partially from an overly simplified model of trait change which is not meant to handle traits with complex genotype-phenotype interactions like protein binding which can jump between states not represented in tip taxa in a saltatorial manner

(Webster and Purvis 2002, Wiens et al. 2006). Secondarily, assumptions of these models do not account for more complex evolutionary dynamics such as coevolution. Though ancestral sequences expressed *in vitro* also used models of evolution to predict ancestral states, they differ as they rely on models of codon evolution which do not make broad assumptions about the basis and mode of complex whole-trait evolution (Yang 2006, Dean and Thornton 2007, Randall et al. 2012, Harms and Thornton 2013). Thus, this method is more capable of recovering unusual tempo and mode which are not allowed in models of whole-trait evolution (Dean and Thornton 2007, Randall et al. 2012, Harms and Thornton 2013).

Data presented here show a departure from expectations commonly assumed in models of trait evolution (gradual evolution) and stress the importance of ancestral reconstruction and testing in uncovering otherwise hidden evolutionary histories. Models of trait evolution in this case would likely never recover the pattern seen in *in vitro* testing as vWF A1 binding capacity varies in a continuous manner, but is capable of large jumps in trait values in single steps. Though increased taxon sampling may result in increased resolution of change in deeper nodes (and may in fact be more gradual), at least one set of sister taxa (*Didelphis aurita* and *Didelphis marsupialis*) differ by two changes at the binding site which abolishes binding of botrocetin A. This demonstrates that at least to some degree few changes of large effect can abolish binding and suggests that saltatorial change is important in the evolution of this interaction.

These data also exhibit a trajectory uncharacteristic of simple adaptive evolution. While adaptive evolution for an unchanging selection pressure would predict states evolving unidirectionally (Figure 4.1 Panel B and C), actual ancestral trait data here show a distinct pattern of loss and gain across nodes, with repeated convergence between species (Figure 4.6). Such losses and gains (as well as repeated convergence) are expected in models of trench warfare coevolution, referred to as phenotype matching coevolution in Nuismer (2007) (Figure 4.1 Panel D). This pattern of functional flipping is strongest for the botrocetin isoforms, and less so for aspercetin, which may be a further

reflection of either its flexibility in target (A2, or A3) or the GP1Bα site being a more important player in the latter interaction.

Utilizing the framework of the functional synthesis, we have reconstructed the pattern of evolution in one interacting gene and uncovered a cryptic evolutionary mode (convergence and repeated loss and gain of function) which is consistent with that predicted by trench warfare coevolution (Thompson 1994, Jokela et al. 2000). Though these data add to previous evidence that opossums and pit-vipers may be coevolving, the pattern recovered here could also be explained by intermittent selection for binding loss, where very high cost drives reversals when selection is not present. This pattern may also be consistent with opossums expanding their diet to multiple species of *Bothrops* with differing CTLs, essentially mimicking reciprocal venom changes via prey-switches. Ultimately, further work reconstructing the history of venom vWF-binding CTLs and testing ancestral botrocetins *in vitro* would be necessary to confirm the assertions that venom CTLs are in fact evolving reciprocally with opossum vWF. With recent advances in snake venom genomics and venomics (Holding et al. 2018, Amazonas et al. 2018) as well as recent work in developing an *in vitro* expression system for botrocetin (Matsui et al. 2017), this system in uniquely poised to characterize reciprocal functional and molecular changes in ancestors for *both* interacting partners in a natural system; a goal that has remained elusive in coevolutionary work thus far (Lovell and Robertson 2010, Scanlan et al. 2011).

The Role of vWF in Venom CTL Resistance

Botrocetin*:*

Here we show that vWF A1 from the majority of opossums within and outside Didelphini have lost binding to two isoforms of botrocetin. All species that were previously shown to have near complete loss of platelet aggregation response for botrocetin showed a substantial loss of vWF A1 binding by botrocetin, suggesting that this protein interaction plays an important role in the observed physiological resistance to this venom protein (Chapter 3).

The threshold of binding needed for aggregation loss seems to be much higher for botrocetin A compared to botrocetin B, suggesting that these proteins may differ substantially both in underlying amino acid sequence and functional potency. Given that botrocetin A did not show reduced aggregation response, and that binding in ancestral vWF differed between these isoforms not just by magnitude but in pattern (botrocetin A bound ancestors that botrocetin B did not), we assume that differences between botrocetin A and B are due to variation in the amino acid sequence, as opposed to preparation quality or degradation. This kind of functional diversification is consistent with rapid evolution in response to a constantly changing, coevolving target. Future work using broader taxon sampling of Botrocetin-like CTLs and population level studies of CTL diversity may shed light on the sequence and functional variation of Botrocetin like CTLs in the genus *Bothrops*.

Though previous workers have observed variation in botrocetin activity by batch (R. Andrews, pers. comm.), our work is the first to report variation in K_D. Between these samples a 36-fold vWF binding loss appears to protect *Didelphis virginiana* from botrocetin A- induced aggregation, whereas an 8-fold loss of vWF binding appears to protect *Didelphis aurita* from botrocetin B. A possible explanation for this may be in the tertiary complex of vWF-botrocetin-GB1bα, as botrocetin must first bind to vWF and then bind efficiently and persistently with GP1Bα in order to initiate an aggregation response. Botrocetin A may retain its potency even with relatively low affinity for vWF, as long as it retains its ability to bind tightly with GP1bα and initiate aggregation. Similarly, botrocetin B may bind as tightly or even more tightly to vWF but may have a reduced ability to bind to GP1Bα, raising the threshold of vWF binding loss required for a loss of aggregation response in *in vitro* assays. These suggest that a secondary mode of resistance (GP1Bα binding) is likely playing a supporting role in botrocetin B resistance. However, confirmation of amino acid differences between these two isoforms in needed to confirm this result.

<u>Bitiscetin:</u>

Bitiscetin has been previously reported to have a K_D for human vWF A1 of 2 nM, a value obtained by surface plasmon resonance, with no associated replicate number or error estimates (Maita et al. 2003). While we report a K_D nearly ten times this value via our immobilized vWF assay, our assay showed high replicability and low error. Bitiscetin also bound nearly every species of vWF (except *P. opossum*), though all species showed significant binding loss relative to human vWF. This is likely a result of the overlap in binding sites shared between these two venom proteins. Of 15 known bitiscetin binding sites, 13 are within the botrocetin binding pocket, 5 directly overlap with botrocetin binding sites, and another 5 are directly adjacent to a botrocetin binding site (Maita et al. 2003). Given this, it is not surprising that we saw a greatly reduced affinity (though still larger than botrocetin A, and very similar to botrocetin B) for bitiscetin in nearly all species tested with respect to human (Figure 4.4). Furthermore, previous work has shown that *Didelphis virginiana* platelets do not aggregate in response to bitiscetin, consistent with these data (Chapter 3).

Aspercetin:

Aspercetin bound with the lowest affinity for human vWF A1 (2180 nM), though it has been shown to exhibit vWF specific aggregation (Rucavado et al. 2001). These data suggest that aspercetin may not primarily target vWF A1, but may target vWF flexibly via the A2 or A3 domains, similar to bitiscetin (Hirotsu et al. 2001). While binding loss for aspercetin was nearly ubiquitous in opossums, vWF from two species (*Didelphis virginiana*, and *Monodelphis emiliae*) bound aspercetin more tightly than human vWF. Puzzlingly, though *Didelphis virginiana* exhibit partial (though variable) loss of aggregation response to aspercetin, it bound nearly 6 times more tightly than human vWF A1 (Chapter 3, Figure 4.4). Another species, *Didelphis aurita*, showed complete binding loss for aspercetin as well as complete physiological resistance (no aggregation response; Chapter 3). These data further suggest either that aspercetin may not primarily target vWF A1, or that the tertiary interactor (GP1Bα) may be a more important factor in resistance to aspercetin, or both. Further work examining aspercetin's

capacity to bind vWF A1, A2, A3, and GB1Bα across taxa would serve to distinguish these hypotheses.

vWF Resistance Outside Didelphini.

Chapter 3 showed that *Monodelphis domestica* exhibits physiological resistance to botrocetin measured in lack of platelet aggregation response. Kinetics data reported here from botrocetin are consistent with the results of Chapter 3, and further suggest that *Metachirus nudicaudatus* and *Monodelphis emiliae* likely also exhibit physiological resistance to botrocetins. If we use the 8-fold loss of binding seen in *D. aurita* as a threshold for protection for all venom CTLs, we can predict that all species tested in this work are resistant to all CTLs except *D. virginiana* and *M. emiliae* for aspercetin. The general agreement of physiological data from previous work and kinetics data reported here strongly suggest that all opossums tested in this work either within or outside Didelphini enjoy physiological resistance to multiple isoforms of botrocetin.

Though this result was expected for species within Didelphini (particularly species for which organismal venom resistance has been demonstrated), it was not expected for species outside this group. Previous work has reported *Metachirus nudicaudatus* as venom sensitive, however, this assertion relies on a single study in which 2 individuals died after an injected dose of *Bothrops jararaca* venom (at 2X LD_{50} for mice) (Perales et al. 1994). Though these data certainly indicate that *M. nudicaudatus* whole venom resistance is less than that of species that survive higher doses of the same venom, it not necessarily inconsistent with the existence of venom CTL resistant vWF in this species. While resistant vWF alone would likely not confer organismal resistance to whole venom, it may suggest that partial venom resistance for *B. jararaca* venom, or the venom of a closely related viper, may be important in this species.

While the ecological selection pressure driving venom resistance within Didelphini appears to be a dietary adaptation that enabled large bodied opossums to exploit venomous snakes as a food source (Wood 1954, Fitch 1960, Jared et al. 1998, Sazima 1992, Voss and Jansa 2012, Voss 2013), opossums outside this clade are smaller

bodied and more likely prey of *Bothrops spp*. (Voss 2013). While *Monodelphis domestica* has been reported to eat snakes, specific species are not reported, and known instances of predation on *Monodelphis spp* by *Bothrops spp* are more common (Streilein 1982, Voss 2013). Other species known to have evolved venom resistance as a dietary adaptation include mammals that exhibit exceptionally strong and generalized antivenom competence (e.g. Hedgehogs, Mongoose, Grasshopper mice) (Barchan et al. 1995, Rowe et al. 2013). By contrast, species known to have evolved venom resistance as a predator defense generally show weaker venom resistance that is highly variable by species and geographic range (Holding et al. 2016a, Pomento et al. 2016). While CTL resistant vWF does not by itself appear to confer resistance to whole venom, it may be indicative of selection pressures for partial or local resistance for species outside Didelphini which experience heavy predation by *Bothrops spp*.

Given that at least 3 species outside Didelphini exhibit loss of aggregations response to botrocetins (Chapter 3), it is possible that venom resistance at vWF A1 is ancestral for all opossums and is rapidly evolving only in species (Didelphini) who have engaged in a coevolutionary relationship with vipers via a dietary adaptation. However, examination of ancestral nodes shows repeated convergent evolution of CTL binding loss, with several ancestral nodes that are sensitive to CTLs at levels comparable to human vWF A1. Similarly, if we examine rates of vWF A1 evolution from Jansa and Voss (2011), several additional branches leading to species of *Monodelphis* and *Marmosa* are as long if not longer than the branch leading to Didelphini. This suggests that these lineages also have accelerated evolutionary rates on vWF A1 and indicate that possible repeated convergent evolution for vWF CTL resistance in opossums was missed because branch-site rate tests require an *a priori* designation of branches under selection. Below, we examine how repeated loss of function recovered in this work may inform the extent of venom CTL resistance across the remaining species in the family Didelphidae.

Repeated Loss of Function.

Changes within the botrocetin binding pockets (624-645, 655-677; Figure 4.7) indicate that both convergent and independent changes may be important for vWF-CTL resistance in Didelphini (Jansa and Voss 2011). Previous work using alanine-scanning mutagenesis has shown that single alanine mutants at 629, 632, 636, and 667 each result in >60% loss of binding (Matsushita et al. 2000). Of these 629 and 632 also disrupt binding to GP1Bα (Matsushita and Sadler 1995, Matsushita et al. 2000). Though most mutations in vWF observed in Didelphini seem to be working via steric hindrance or charge disruption rather than elimination of amino acids with essential side-chains (as alanine scanning does) we similarly recover sites 632, and 636 as essential for binding loss. However, we find two additional sites adjacent to those previously identified, 628 (a known botrocetin binding site) and 630 a site not identified previously, which seem to be repeatedly associated with large degrees of binding loss (Table 4.1). Alanine scans have also shown that tandem mutations at the lysine stretch 642-645, disrupt botrocetin binding (Matsushita and Sadler 1995, Matsushita et al. 2000). While we do see a loss of two of these lysines from node 71 to 72 in Didelphini, this branch only shows significant loss of binding for botrocetin A and aspercetin, indicating that it may contribute to binding loss (as an important independent change), but may not be as vital to opossum vWF-botrocetin B binding loss. As these changes are only present once in this clade, the effect of these mutations would need to be tested via direct mutagenesis to decipher their specific effect on CTL binding. Previous work has also shown that a stretch outside the binding pocket 613-616 induces loss of binding for botrocetin only for tandem mutants (when more than one site is replaced with A) (Matsushita and Sadler 1995, Matsushita et al. 2000). However, this stretch is conserved between humans in almost all opossums; *M. emiliae* has one lysine in this region, and *M. robinsoni* has a phenylalanine in this stretch. Though these differ from alanine and could disrupt binding more efficiency, previous work suggests that single mutations in this region have little effect on binding (Matsushita et al. 2000).

Using the sites and changes in Table 4.1, we examined the protein sequence of the remaining species outside Didelphini used for this work (Figure 4.1, Appendix Material 4.1 DidvWFAlign1.phy). Several species exhibit many of the same amino acids found in Table 4.1 at these sites, as well as different amino acid changes at the same sites (Table 4.2). In particular, *Glironia venusta*, several species of *Monodelphis* and *Marmosa* had 3 or more changes which are associated with >10 fold binding loss for several venom CTLs (Table 4.2). *Marmosa rubra* showed the highest number of changes (8) repeatedly associated with >10 fold binding loss (Table 4.2).

	Parallel Changes	**Divergent Changes**	**Total**
Caluromys philander	635		2
Caluromys lanatus	628	635	2
Gilronia venusta	628	635, 639	3
Metachirus nudicaudatus		635	1
Gracilinanus agilis		635	1
Gracilinanus microtarsus		635	1
Gracilinanus aceramarcae		635	1
Gracilinanus emliliae		635	1
Thylamys venustus	635	628	2
Thylamys pallidior	635		1
Thylamys macrurus	635	628	2
Thylamys pusillus	635	628	2
Marmosops incanus		635	1
Marmosops noctivagus		635	1
Marmosops spGalves		635, 639	2
Marmosops pinheiroi		635	1
Marmosops parvidens		635	1
Tlacuatzin canescens		635	1
Monodelphis scalops		635	1
Monodelphis emiliae	635, 636, 668	631, 633	5
Monodelphis pervuviana	635		1
Monodelphis brevicaudata		628, 630, 631, 635	4
Monodelphis domestica		628, 630, 631, 635	4
Marmosa rubra	628, 630, 635, 636, 668	633, 639	8
Marmosa robinsoni		630, 631, 635	3
Marmosa mexicana		630, 631, 635	3
Marmosa murina		628, 631, 635	3
Marmosa lepida		635	1

Marmosa rutteri	628	630, 631, 635	4
Marmosa paraguayana		635	1
Marmosa demerarae		635	1

Table 4.2- Species outside of Didelphini that have parallel changes (the same amino acid replacement) associated with >10 fold binding loss for venom proteins seen in Table 4.1, and divergent changes at the same site. Sites associated with loss of binding by venom protein are: botrocetin A [628, 630, 631, 633, 636, 668], botrocetin B [635], aspercetin [630, 631, 639]. One site highlighted in grey (635) indicates that every species had a change from the known binding state (D-Aspartic Acid) at this site. Rows of species that have associated vWF A1 affinity data are highlighted in grey.

Sites associated with loss of function identified in this work represent a subset of known botrocetin binding and adjacent sites, and further narrow down our ability to identify additional taxa which may be venom resistant, and potentially coevolving with venomous snakes (Table 4.2). Future work should focus on examining the physiological resistance of species in Table 4.2 with exceptionally high numbers of changes at sites associated with binding loss (CTL resistance), as these may represent species which are convergently evolving venom resistance.

Additionally, further work examining the ability of vWF from species with very few changes (Table 4.2), as well as species with slower evolutionary rates of vWF shown in Jansa and Voss (2011) should be examined to assess if these species are in fact botrocetin binders. These data will be vital to deciphering if all species of Didelphid opossums exhibit some degree of venom resistance, or if this is observed only in species with accelerated rates of evolution on vWF (convergently). Though sites identified here are associated with repeated loss of function, site directed mutagenesis on extant opossums for single mutations (one by one) identified to promote binding in ancestral forms would ultimately quantify the exact extent to which each of the mutations seen in extant taxa is responsible for binding loss.

Future Directions

While coevolution with resistant prey has been cited as a major driver of venom evolution (Casewell et al. 2012b), this study is the first to report a potentially coevolving molecular mechanism of venom resistance across several species of both prey items and predators of venomous snakes. This work also highlights that the role of the invasion and radiation of a generalist predator (Viperids) may be an important driver of adaptive evolution for all South American opossums, not just those who have evolved as specialist snake predators. South American opossums are unique in exhibiting a high extinction fraction, and an unusual period of net-zero lineage accumulation mid-Miocene, suggesting a mass extinction event unique to Didelphidae (Jansa et al. 2014). It has been suggested that the invasion of Viperids to South America ~23 mya played a role this extinction event (Jansa et al. 2014). If surviving opossum lineages are in fact coevolving with vipers, this may be the driver of the high variation in botrocetin binding sites and sites associated with botrocetin binding loss across all Didelphids found in this study (Jansa and Voss 2011). Evidence presented here of loss of venom CTL binding associated with loss of aggregation function across several extant members of Didelphidae (not seen in several other placental mammals Read et al. 1983), as well as a pattern which suggests trench warfar coevolution add credence to this assertion and argue for the merit of further study of the diversity, physiology, and ecology of this evolutionary relationship.

THE INFLUENCE OF CONVERGENCE AND COEVOLUTION IN THE EVOLUTION OF VENOM RESISTANCE IN MAMMALS

This dissertation examines the role of convergent evolution and coevolution in the evolution of venom resistance in mammals. In Chapter 1 I outlined and reviewed how mammalian resistance to snake venom can be used to elucidate evolutionary tempo and mode of complex potentially coevolving adaptations. In Chapter 2 I examined convergent evolution of resistance to α-neurotoxins across mammals, and found strong evidence that resistance has evolved at least 4 times across mammals via two distinct biophysical

mechanisms. In Chapter 3 I demonstrated the function of a molecule previously hypothesized to confer resistance to a class of venom CTLs, and discover that this resistance is present more broadly across opossums than expected. In Chapter 4 I developed an experimental model to test the specific function of vWF, the protein hypothesized to confer CTL resistance. In this work I demonstrate lack of binding broadly across opossums and use ancestral reconstruction to identify mutations relevant to binding. By testing the function of vWF A1 ancestors with modern CTLs we find that the mode of vWF evolution is non-linear and departs from expectations of adaptive evolution towards an unchanging selection pressure, or arms race coevolution. Rather, we find that vWF A1 evolution is consistent with expectations of tit-for tat coevolution (though this pattern does not exclude other non-linear histories). These results revealed repeated convergence which is otherwise not predicted by models of trait evolution and show that vWF evolutionary tempo is saltatorial.

These results indicate that the evolution of venom resistance may be an important driver of adaptive evolution across species which are both prey and predators of venomous snakes. Chapter 4 demonstrates of the need to reconstruct and empirically test ancestral states to determine if traits hypothesized to be coevolving conform to a mode which is expected for a coevolving trait. Chapter 4 also demonstrates the untapped potential to apply a functional synthesis framework to other cases of hypothesized coevolution, in order to explicitly test for the presence of this dynamic in one or both interacting partners. This work presents a case study in examining convergence and coevolution in the molecular basis of ecologically-relevant traits in a natural system. Chapters 3 and 4 establish an experimental system in which it is possible to explicitly test the tempo and directionality of traits hypothesized to be coevolving. My goal was to lay the groundwork for establishing an experimental system which could eventually be developed (over the course of a career) to explicitly test the functional and molecular evolution of *both* interacting partners in a coevolutionary relationship. This elusive but important goal is necessary to test long held assumptions of how coevolution plays out in natural systems.

Further work utilizing site-directed mutagenesis to explicitly identify the functional effect of each site identified in chapter 4 is needed to further our understanding of the biophysical interaction between vWF and CTLs. Establishing the same robust time -calibrated species and genes trees for Bothrops and CTLs respectively are the next steps in facilitating the development of this system to include botrocetin ancestors and test whether snake venom is indeed evolving *reciprocally* in response to mammalian venom resistance.

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APPENDIX 1. SUPPLEMENTARY MATERIAL FOR CHAPTER 1.

Appendix Table 1.1. Potentially coevolving venom and venom resistant proteins within mammals which meet conditions outlined for evolutionary studies involving phylogenetic and functional reconstruction as described in the text.

Resistant Protein	**Venom component**	**Heterologous expression system developed previously**		**Resistant Species**	**V Citations for heterologous expression systems**	**R Citations for heterologous expression systems**	**Citations for Resistance**
		R	**V**				
muscular nAChR	alpha neurotoxins	Y	Y	*Herpestes ichneumon, Erinaceus concolor, Erinaceus europus, Mellivora capensis, Sus scrofa*	*Lyukmanova et al. 2007,* (Botes 1971, Utkin 2012, Ogawa et al. 2004, Tsetlin 1999, Kini and Doley 2010)	Barchan et al. 1992, Utkin et al. 2001	Barchan et al. 1992, Barchan et al. 1995, Takacs et al. 2004, Drabeck et al. 2015
vWF	botrocetin	Y	Y*	*Didelphis virginiana, Didelphis albiventris, Didelphis marsupialis, Philander mcilhennyi, Philander opossum, Philander frenatus, Lutreolina crassicaudata, Chironectes minimus*	Yamamoto-Susuki et al. 2012	Cruz et al. 1993, Schulte am Esch II et al. 2007, Emsley et al. 1998	Jansa and Voss 2011
SVMPIs (e.g.: oprin, DM43, DM40, Erinacin)	SVMPs	N**	Y***	*Didelphis virginiana, Didelphis marsupialis, Didelphis albiventris, Lutreolina crassicaudata, Philander frenatus, Erinaceus europus, Otospermophilus beecheyi, Ictidomys mexicanus, Neotoma micropus, Sigmodon hispidus, Herpestes edwardsii,*	None	***Shimokawa et al. 1993, Zhu et al. 2010, Ramos et al. 2003, Assakura et al. 2003, Wang and Huang 2002, Wu et al. 2001, Selistre-de-Araujo et al. 2000	For a review see: Sanchez and Rodriguez-Aucosta 2008
Nav1.8/ Nav1.7	Unnamed toxin/ CvIV4	Y	N	*Onychomys torridus, Onychomys arenicola*	None	Rowe et al. 2013, Dib-Hajj et al. 2010	Rowe and Rowe 2008, Rowe et al. 2013

* botrocetin-2, a similar but altered version of botrocetin has been expressed in mammalian cells (Yamamoto-Susuki et al. 2012). Native botrocetin is commercially available as E.coli expressed product, however, only as individual monomers. To our knowledge, the heterodimeric form is not commercially available, and has not been experimentally expressed in E.coli. Several other venom CTLs have been successfully expressed ex: Kassab et al. 2004

** Though not completed, partial expression of opossum SVMPIs (DM43) is being undertaken at San Jose State University by Dr Claire Komives as a part of a project aimed at improving antivenom technology (Komives et al. Poster 2016)

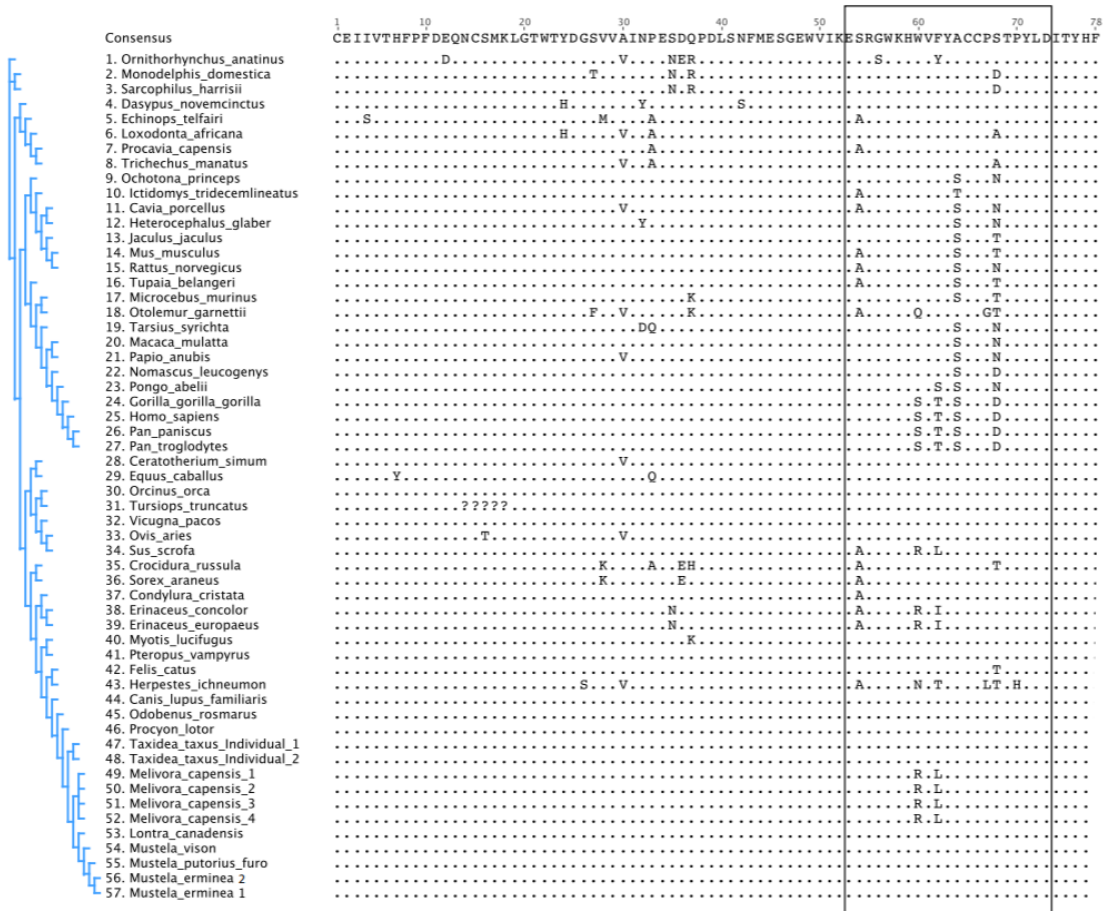
*** This is a diverse set of proteins, however, many researchers have developed expression systems to study this class of protein, far too many to list here.

APPENDIX 2. SUPPLEMENTARY MATERIAL FOR CHAPTER 2.

Appendix Table 2.1- Species and acccession numbers for CHRNA1 sequences that were obtained from public databases and used in this report.

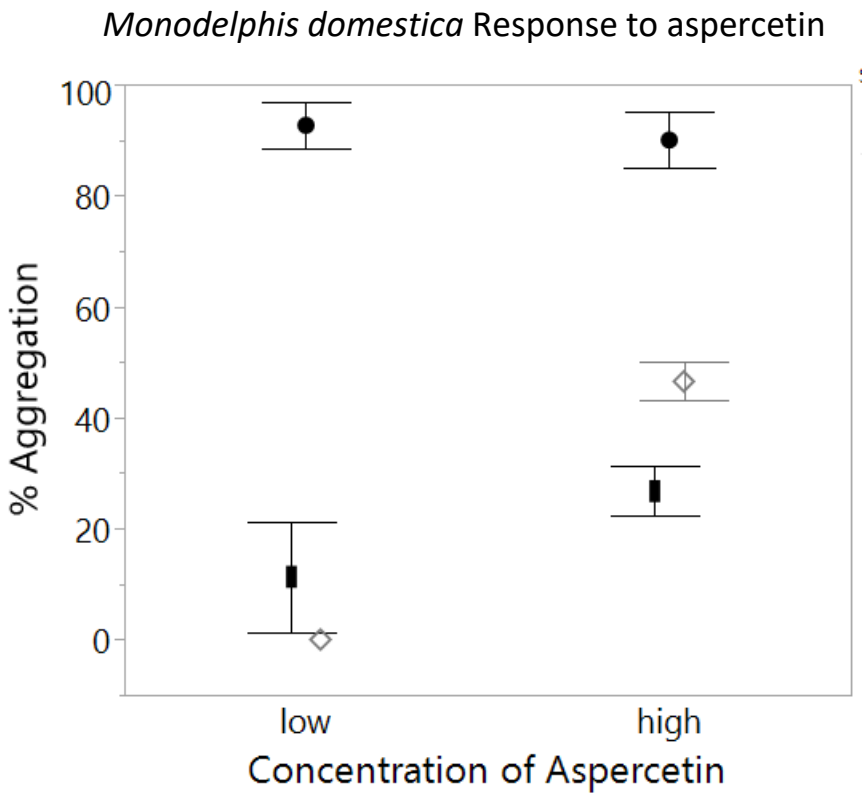
Species	**Genbank/EnsEMBL accession**
Canis lupus familiaris	chromosome:CanFam3.1:36:18726243:18742505:1
Cavia porcellus	scaffold:cavPor3:scaffold 3:15383510:15398211:1
Ceratotherium simum simum	Genbank Accession XM 004426644
Condylura cristata	Genbank Accession XM 004674514
Crocidura russula	Genbank Accession U17006
Dasypus novemcinctus	genescaffold:dasNov2:GeneScaffold 3470:18400:33825:1
Echinops telfairi	genescaffold:TENREC:GeneScaffold 4122:3564:109793:1
Equus caballus	chromosome:EquCab2:18:53455099:53470210:1
Erinaceus concolor	Genbank Accession U17016
Erinaceus europaeus	genescaffold:HEDGEHOG:GeneScaffold 4250:14792:92863:1
Felis catus	chromosome:Felis catus 6.2:C1:163017266:163029388:1
Gorilla gorilla gorilla	chromosome:gorGor3.1:2b:62651955:62669340:1
Herpestes ichneumon	Genbank Accession M93639
Heterocephalus glaber	Genbank Accession XM 004908522
Homo sapiens	chromosome:GRCh37:2:175612320:175629200:1
Ictidomys tridecemlineatus	scaffold:spetri2:JH393319.1:4453205:4465194:1
Jaculus jaculus	Genbank Accession XM 004660327
Loxodonta africana	supercontig:loxAfr3:scaffold 3:52617234:52634961:1
Macaca mulatta	chromosome:MMUL 1:12:38373466:38390438:1
Microcebus murinus	genescaffold:micMur1:GeneScaffold 2120:3839:19770:1
Monodelphis domestica	chromosome:BROADO5:4:185745550:185772251:1
Mus musculus	chromosome:GRCm38:2:73563215:73580338:1
Mustela putorius furo	scaffold:MusPutFur1.0:GL897095.1:951737:970798:1
Myotis lucifugus	scaffold:Myoluc2.0:GL429772:8136691:8152800:1
Nomascus leucogenys	supercontig:Nleu1.0:GL397263.1:18550090:18569431:1
Ochotona princeps	genescaffold:pika:GeneScaffold 2413:62892:79747:1
Odobenus rosmarus	Genbank Accession XM 004408997
Orcinus orca	Genbank Accession XM 004267331
Otolemur garnettii	scaffold:OtoGar3:GL873567.1:11514795:11524631:1

Ovis aries	Genbank Accession XM 004004575
Pan paniscus	Genbank Accession XM 003824235
Pan troglodytes	chromosome:CHIMP2.1.4:2B:179204218:179221621:1
Papio anubis	Genbank Accession XM 003907633
Pongo abelii	chromosome:PPYG2:2b:64841691:64858791:1
Procavia capensis	genescaffold:proCap1:GeneScaffold 3561:16616:35113:1
Pteropus vampyrus	genescaffold:pteVam1:GeneScaffold 1768:18325:32806:1
Rattus norvegicus	chromosome:Rnor 5.0:3:66925970:66940958:1
Sarcophilus harrisii	scaffold:DEVIL7.0:GL849635.1:349074:365803:1
Sorex araneus	Genbank Accession XM 004601170
Sus scrofa	chromosome:Sscrofa10.2:15:89889110:89903466:1
Tarsius syrichta	genescaffold:tarSyr1:GeneScaffold 3989:3074:23223:1
Trichechus manatus	Genbank Accession XM 004375490
Tupaia belangeri	genescaffold:TREESHREW:GeneScaffold 2670:43101:54292:1
Tursiops truncatus	genescaffold:turTru1:GeneScaffold 1529:17744:33028:1
Vicugna pacos	genescaffold:vicPac1:GeneScaffold 980:219012:234612:1



Appendix Figure 2.1- Part of the amino acid alignment for the region of the nicotinic acetylcholine receptor (CHRNA1) used for this study and others (Barchan et al. 1992, 1995). On the left is the evolutionary tree of mammals that have been sequenced for the α1 subunit of the nicotinic acetylcholine receptor gene (CHRNA1). The α-bungarotoxin-binding region of nAChR (Barchan et al. 1995) is outlined with a square. The consensus sequence is shown at the top, and dots indicate sequence identity with this reference sequence.

APPENDIX 3. SUPPLEMENTARY MATERIAL FOR CHAPTER 3.



Appendix Figure 3.1- Percent aggregation of *Monodelphis domestica* samples from failed ADP control for high and low concentrations of aspercetin. Grey diamonds are *Monodelphis domestica*, black rectangles are *Didelphis virginiana*, and black circles are Human. All tests are in PRP, and error bars are the standard error of the mean (%Transmittance as %Aggregation). All samples of *M. domestica* are from an individual who failed to aggregate in response to ADP. *M. domestica* samples contained botrocein which failed to aggregate platelets, after which aspercetin was added to the same samples and results above were recorded.

Intraspecific Variation in Aggregation Response

Species	Ind ID	N tests	ChiSquare	DF	P-Value
Didelphis virginiana	F1	9	4.0319	2	0.1332
Didelphis virginiana	M1	5			
Didelphis virginiana	M2	10			

Appendix Table 3.1- Kruskal-Wallis rank sums test of aggregation between individuals. N tests is number of tests including high and low concentrations and all test types (PRP and washed assay). DF- degrees of freedom. Ind ID- Individual identification.

High versus Low Concentration PRP Tests

Venom	Species	N high	N low	ChiSquare	DF	P-Value	Corrected P
aspercetin	*Didelphis aurita*	2	1	0	1	1	1
aspercetin	*Didelphis virginiana*	4	7	3.74	1	0.053*	0.265
aspercetin	Human	4	2	0.2143	1	0.6434	1
botrocetin	*Didelphis virginiana*	11	2	0.3939	1	0.5302	1
botrocetin	Human	12	2	0.3033	1	0.5818	1

Appendix Table 3.2- One-way Wilcoxin tests of differences in percent aggregation in PRP between high and low concentrations of venom proteins used for botrocetin and aspercetin. DF- degrees of freedom N high- number of tests using a high concentration, N low- number of tests using a low concentration. Bonferroni corrected P values are multiplied by the number of tests and reported as 1 if >1. Asterisk indicate significance at α=0.05.

Comparison of aspercetin Aggregation Response of *Monodelphis domestica*

Agonist [C]	Species	Species	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
	Human	*M. domestica*	92.65	24.74003	23.5756	161.7244	0.0115*
Low	Human	*D. virginiana*	81.36429	13.86956	42.6403	120.0882	0.0006*
	D. virginiana	*M. domestica*	11.28571	23.656	54.7621	77.3335	0.8836
	Human	*D. virginiana*	63.25	6.898369	40.8037	85.69635	0.0006*
High	Human	*M. domestica*	43.5	7.965551	17.5812	69.41881	0.0065*
	M. domestica	*D. virginiana*	19.75	6.898369	-2.6963	42.19635	0.0767

Appendix Table 3.3- Tukey-Kramer HSD comparison of percent aggregation of *Monodelphis domestica* PRP samples from failed ADP control in response to high (100ug/ml) and low (<51ug/ml) concentrations of aspercetin. Responses are compared to both *D. virginiana* and Human PRP aggregation to the same aspercetin concentrations. *M. domestica* samples contained both Botrocein which failed to aggregate platelets, after which aspercetin was added to the same samples and results were recorded. Asterisk indicate significance at α=0.05.

PRP versus Washed Platelet Tests

Agonist	Species	N WP	N PRP	ChiSquare	DF	P-Value	Corrected P
ADP	*Didelphis virginiana*	6	7	1.317	1	0.2511	1
aspercetin	*Didelphis virginiana*	13	11	0.2245	1	0.6356	1

bitiscetin	*Didelphis virginiana*	9	12	1.9106	1	0.1669	1
botrocetin	*Didelphis virginiana*	8	13	1.2923	1	0.2556	1
ristocetin	*Didelphis virginiana*	2	4	1.4205	1	0.2333	1
bitiscetin	Human	2	2	3	1	0.0833	0.5831
ristocetin	Human	5	5	0.1756	1	0.6752	1

Appendix Table 3.4- One-way Wilcoxin tests of differences in percent aggregation between tests run in PRP versus washed assays. DF- degrees of freedom, N WP- number of tests done in washed platelets, N PRP- number of tests done in PRP. Bonferroni corrected P values are multiplied by the number of tests and reported as 1 if >1.

High versus Low Concentration Tests in Washed Assays

Venom	Species	N high	N low	ChiSquare	DF	P-Value	Corrected P
aspercetin	*Didelphis virginiana*	10	3	0.1895	1	0.6633	1
botrocetin	*Didelphis virginiana*	2	6	0	1	1	1

Appendix Table 3.5- One-way Wilcoxin tests of differences in percent aggregation between low and high concentrations in washed assays. DF- degrees of freedom, N high- number of tests done in high concentrations of agonist, N low- number of tests done in low concentrations of agonist. Bonferroni corrected P values are multiplied by the number of tests and reported as 1 if >1.

Comparison of Source of vWF in Washed Assays

Agonist	Species	N PPP	N pvWF	ChiSquare	DF	P-Value	Corrected P
aspercetin	*Didelphis virginiana*	9	4	8.1866	1	0.0042*	0.021*
bitiscetin	*Didelphis virginiana*	8	1	0.125	1	0.7237	1
botrocetin	*Didelphis virginiana*	7	1	0	1	1	1
ristocetin	*Didelphis virginiana*	1	1	1	1	0.3173	1
ristocetin	Human	1	3	3.2	2	0.2019	1

Appendix Table 3.6- One-way Wilcoxin tests of differences in percent aggregation between washed assays which used 10% v/v PPP or cleaned up concentrated vWF as a source of vWF for washed assays. DF- degrees of freedom, N PPP- number of tests using 10% v/v PPP, N pvWF- number of tests using cleaned up vWF from size exclusion chromatography. Bonferroni corrected P values are multiplied by the number of tests and reported as 1 if >1. Asterisk indicate significance at α=0.05.

List of Platelet Rich Plasma Assays

TestID	Test Material	Agonist	[C]	units	%Trasmittance	Species	Ind ID
21	PRP	ADP	850	µg/ml	61	Human	CM
22	PRP	ADP	850	µg/ml	72	Human	CM
23	PRP	ADP	850	µg/ml	63	Human	CM
35	PRP	ADP	850	µg/ml	68	Human	DD

TestID	Test Material	Agonist	[C]	units	%Trasmittance	Species	Ind ID
36	PRP	ADP	850	µg/ml	76	Human	DD
89	PRP	ADP	850	µg/ml	79	Human	CM
90	PRP	ADP	850	µg/ml	79	Human	CM
19	PRP	ADP	850	µg/ml	70	*Didelphis virginiana*	M2
20	PRP	ADP	850	µg/ml	69	*Didelphis virginiana*	M2
24	PRP	ADP	850	µg/ml	72	*Didelphis virginiana*	F1
28	PRP	ADP	850	µg/ml	59	*Didelphis virginiana*	M1
31	PRP	ADP	850	µg/ml	50	*Didelphis virginiana*	M1
33	PRP	ADP	850	µg/ml	70	*Didelphis virginiana*	F1
102	PRP	ADP	850	µg/ml	60	*Didelphis virginiana*	F1
160c	PRP	ADP	10	µM	78	*Didelphis aurita*	DE001
161	PRP	ADP	10	µM	68.9	Human	EHZ
170	PRP	ADP	10	µM	52.5	*Didelphis aurita*	DE003
171	PRP	ADP	10	µM	72	Human	PC
172	PRP	ADP	10	µM	72	Human	PC
182	PRP	ADP	10	µM	55	*Didelphis aurita*	DE006
185	PRP	ADP	10	µM	74	*Didelphis aurita*	DE005
195	PRP	ADP	850	µg/ml	45	*Monodelphis domestica*	MdomA
196	PRP	ADP	850	µg/ml	45	*Monodelphis domestica*	MdomA
154	PRP	aspercetin	20	µg/ml	100	Human	NZ
3	PRP	aspercetin	20	µg/ml	99	Human	DD
93	PRP	aspercetin	100	µg/ml	85	Human	CM
94	PRP	aspercetin	100	µg/ml	95	Human	CM
85	PRP	aspercetin	100	µg/ml	30	*Didelphis virginiana*	M2
86	PRP	aspercetin	100	µg/ml	38	*Didelphis virginiana*	M2
106	PRP	aspercetin	100	µg/ml	20	*Didelphis virginiana*	F1
107	PRP	aspercetin	100	µg/ml	19	*Didelphis virginiana*	F1
156	PRP	aspercetin	20	µg/ml	0	*Didelphis virginiana*	F1
9a	PRP	aspercetin	20	µg/ml	5	*Didelphis virginiana*	F1
12a	PRP	aspercetin	20	µg/ml	0	*Didelphis virginiana*	M1
25	PRP	aspercetin	20	µg/ml	3	*Didelphis virginiana*	F1
12b	PRP	aspercetin	36.36	µg/ml	0	*Didelphis virginiana*	M1
9b	PRP	aspercetin	36.3	µg/ml	71	*Didelphis virginiana*	F1
12c	PRP	aspercetin	50	µg/ml	0	*Didelphis virginiana*	M1
162	PRP	aspercetin	20	µg/ml	81.6	Human	EHZ
168	PRP	aspercetin	20	µg/ml	0	*Didelphis aurita*	DE003
173	PRP	aspercetin	20	µg/ml	90	Human	PC
178	PRP	aspercetin	20	µg/ml	0	*Didelphis aurita*	DE006
186	PRP	aspercetin	20	µg/ml	0	*Didelphis aurita*	DE005
91	PRP	bitiscetin	12.75	µg/ml	85	Human	CM
92	PRP	bitiscetin	12.75	µg/ml	85	Human	CM

TestID	Test Material	Agonist	[C]	units	%Trasmittance	Species	Ind ID
76a	PRP	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M1
80	PRP	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M2
81	PRP	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M2
82	PRP	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M2
100	PRP	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M2
101	PRP	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	F1
108	PRP	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	F1
145	PRP	bitiscetin	12.75	µg/ml	5	*Didelphis virginiana*	F1
146a	PRP	bitiscetin	12.75	µg/ml	6	*Didelphis virginiana*	F1
146b	PRP	bitiscetin	11.59	µg/ml	1	*Didelphis virginiana*	F1
146c	PRP	bitiscetin	10.625	µg/ml	1	*Didelphis virginiana*	F1
147	PRP	bitiscetin	12.75	µg/ml	3	*Didelphis virginiana*	F1
153	PRP	botrocetin	2	µg/ml	75	Human	NZ
2	PRP	botrocetin	2	µg/ml	75	Human	DD
5	PRP	botrocetin	4	µg/ml	90	Human	DD
17	PRP	botrocetin	4	µg/ml	0	*Didelphis virginiana*	M2
18	PRP	botrocetin	4	µg/ml	2	*Didelphis virginiana*	M2
8c	PRP	botrocetin	10	µg/ml	0	*Didelphis virginiana*	F1
11c	PRP	botrocetin	10	µg/ml	0	*Didelphis virginiana*	M1
8d	PRP	botrocetin	12.3	µg/ml	0	*Didelphis virginiana*	F1
11d	PRP	botrocetin	12.3	µg/ml	0	*Didelphis virginiana*	M1
14	PRP	botrocetin	20	µg/ml	5	*Didelphis virginiana*	M1
155	PRP	botrocetin	2	µg/ml	0	*Didelphis virginiana*	F1
159	PRP	botrocetin	2	µg/ml	0	*Didelphis virginiana*	M1
8a	PRP	botrocetin	4	µg/ml	0	*Didelphis virginiana*	F1
11a	PRP	botrocetin	4	µg/ml	0	*Didelphis virginiana*	M1
8b	PRP	botrocetin	7.27	µg/ml	0	*Didelphis virginiana*	F1
11b	PRP	botrocetin	7.27	µg/ml	0	*Didelphis virginiana*	M1
160a	PRP	botrocetin	4	µg/ml	0	*Didelphis aurita*	DE001
160b	PRP	botrocetin	10	µg/ml	0	*Didelphis aurita*	DE001
163	PRP	botrocetin	10	µg/ml	66.8	Human	EHZ
164	PRP	botrocetin	10	µg/ml	69.5	Human	EHZ
165	PRP	botrocetin	4	µg/ml	71	Human	EHZ
166	PRP	botrocetin	4	µg/ml	70.6	Human	EHZ
167	PRP	botrocetin	4	µg/ml	0	*Didelphis aurita*	DE003
169	PRP	botrocetin	10	µg/ml	0	*Didelphis aurita*	DE003
174	PRP	botrocetin	10	µg/ml	75	Human	PC
175	PRP	botrocetin	4	µg/ml	79	Human	PC
176	PRP	botrocetin	10	µg/ml	78	Human	PC
177	PRP	botrocetin	4	µg/ml	82	Human	PC
179	PRP	botrocetin	10	µg/ml	0	*Didelphis aurita*	DE006

TestID	Test Material	Agonist	[C]	units	%Trasmittance	Species	Ind ID
180	PRP	botrocetin	4	µg/ml	0	*Didelphis aurita*	DE006
181	PRP	botrocetin	4	µg/ml	0	*Didelphis aurita*	DE006
181a	PRP	botrocetin	10	µg/ml	0	*Didelphis aurita*	DE006
183	PRP	botrocetin	4	µg/ml	0	*Didelphis aurita*	DE005
183a	PRP	botrocetin	10	µg/ml	0	*Didelphis aurita*	DE005
184	PRP	botrocetin	4	µg/ml	0	*Didelphis aurita*	DE005
184a	PRP	botrocetin	10	µg/ml	0	*Didelphis aurita*	DE005
187	PRP	botrocetin	10	µg/ml	80	Human	MW
188	PRP	botrocetin	10	µg/ml	78	Human	MW
189	PRP	botrocetin	10	µg/ml	81	Human	MW
194	PRP	botrocetin	10	µg/ml	0	*Monodelphis domestica*	MdomA
194b	PRP	botrocetin	17	µg/ml	0	*Monodelphis domestica*	MdomA
194c	PRP	botrocetin	71	µg/ml	0	*Monodelphis domestica*	MdomA
152	PRP	ristocetin	1.5	mg/ml	90	Human	NZ
1	PRP	ristocetin	1.5	mg/ml	84	Human	DD
4	PRP	ristocetin	1.5	mg/ml	89	Human	DD
48	PRP	ristocetin	1.5	mg/ml	68	Human	JD
49	PRP	ristocetin	1.5	mg/ml	67	Human	JD
155	PRP	ristocetin	1.5	mg/ml	0	*Didelphis virginiana*	F1
6a	PRP	ristocetin	1.5	mg/ml	0	*Didelphis virginiana*	F1
6b	PRP	ristocetin	2.72	mg/ml	2	*Didelphis virginiana*	F1
6c	PRP	ristocetin	3.75	mg/ml	-10	*Didelphis virginiana*	F1

Appendix Table 3.7- List of all PRP assays. Individual ID corresponds to a single unique organism. Test ID is each 450µl Test. When test ID is a subset a,b,c this indicates that this test was a continuation of a previous test (the same 450µl PRP sample). Sections highlighted in grey denote a different venom protein (for visual ease).

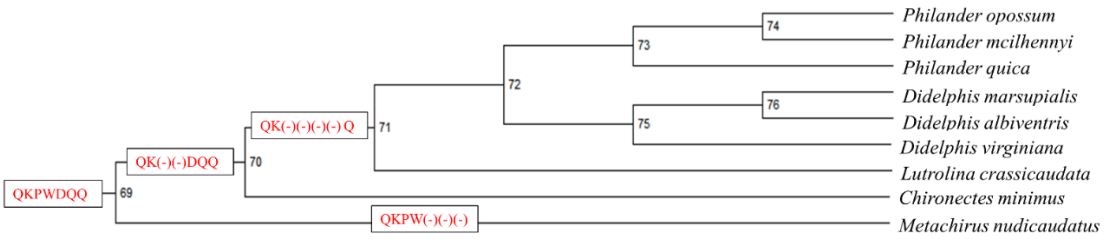
List of Washed Platelet Assays

TestID	Test Material	vWF source	reagent	[C]	units	%Transmittance	Species	Ind ID
66	Washed Platelets	PPP opossum	ADP	850	µg/ml	82	*Didelphis virginiana*	M2
77	Washed Platelets	PPP opossum	ADP	850	µg/ml	65	*Didelphis virginiana*	M1
110	Washed Platelets	PPP opossum	ADP	850	µg/ml	49	*Didelphis virginiana*	F1
110	Washed Platelets	PPP opossum	ADP	850	µg/ml	49	*Didelphis virginiana*	F1
116c	Washed Platelets	PPP opossum	ADP	850	µg/ml	50	*Didelphis virginiana*	F1
120	Washed Platelets	PPP opossum	ADP	850	µg/ml	65	*Didelphis virginiana*	M1
141	Washed Platelets	PPP opossum	ADP	850	µg/ml	20	*Didelphis virginiana*	F1
64	Washed Platelets	PPP opossum	aspercetin	20	µg/ml	10	*Didelphis virginiana*	M2
68	Washed Platelets	PPP opossum	aspercetin	20	µg/ml	10	*Didelphis virginiana*	M2
74	Washed Platelets	PPP opossum	aspercetin	100	µg/ml	5	*Didelphis virginiana*	M1
78	Washed Platelets	PPP opossum	aspercetin	100	µg/ml	3	*Didelphis virginiana*	M1

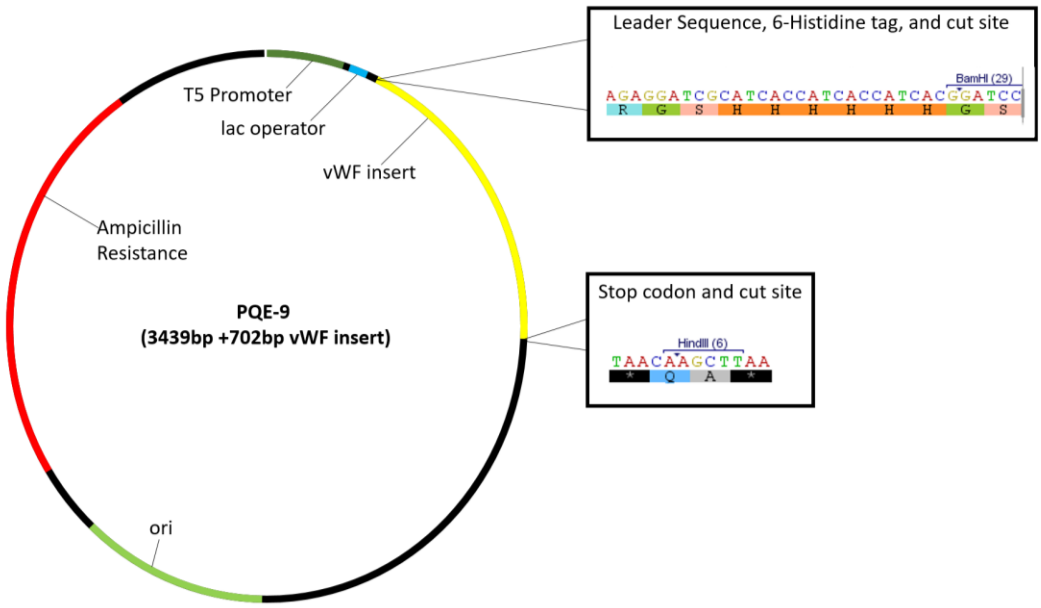
TestID	Test Material	vWF source	reagent	[C]	units	%Transmittance	Species	Ind ID
115a	Washed Platelets	PPP opossum	aspercetin	100	µg/ml	40	*Didelphis virginiana*	F1
150a	Washed Platelets	PPP opossum	aspercetin	100	µg/ml	5	*Didelphis virginiana*	F1
127	Washed Platelets	PPP opossum	aspercetin	100	µg/ml	10	*Didelphis virginiana*	M2
128	Washed Platelets	PPP opossum	aspercetin	100	µg/ml	10	*Didelphis virginiana*	M2
129	Washed Platelets	PPP opossum	aspercetin	100	µg/ml	9	*Didelphis virginiana*	M2
65	Washed Platelets	purified opossum	aspercetin	20	µg/ml	0	*Didelphis virginiana*	M2
96	Washed Platelets	purified opossum	aspercetin	100	µg/ml	0	*Didelphis virginiana*	M2
99	Washed Platelets	purified opossum	aspercetin	100	µg/ml	0	*Didelphis virginiana*	M2
111	Washed Platelets	purified opossum	aspercetin	100	µg/ml	0	*Didelphis virginiana*	F1
73	Washed Platelets	PPP human	bitiscetin	12.75	µg/ml	90	Human	SJ
73	Washed Platelets	PPP human	bitiscetin	12.75	µg/ml	90	Human	SJ
75	Washed Platelets	PPP opossum	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M1
79	Washed Platelets	PPP opossum	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M1
109	Washed Platelets	PPP opossum	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	F1
116a	Washed Platelets	PPP opossum	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	F1
121	Washed Platelets	PPP opossum	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M1
122	Washed Platelets	PPP opossum	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M1
123	Washed Platelets	PPP opossum	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M1
148	Washed Platelets	PPP opossum	bitiscetin	12.75	µg/ml	5	*Didelphis virginiana*	F1
98	Washed Platelets	purified opossum	bitiscetin	12.75	µg/ml	0	*Didelphis virginiana*	M2
61	Washed Platelets	PPP opossum	botrocetin	2	µg/ml	0	*Didelphis virginiana*	M1
124	Washed Platelets	PPP opossum	botrocetin	2	µg/ml	0	*Didelphis virginiana*	M2
125	Washed Platelets	PPP opossum	botrocetin	2	µg/ml	0	*Didelphis virginiana*	M2
126	Washed Platelets	PPP opossum	botrocetin	2	µg/ml	0	*Didelphis virginiana*	M2
140	Washed Platelets	PPP opossum	botrocetin	10	µg/ml	0	*Didelphis virginiana*	F1
143a	Washed Platelets	PPP opossum	botrocetin	2	µg/ml	0	*Didelphis virginiana*	F1
143b	Washed Platelets	PPP opossum	botrocetin	10	µg/ml	0	*Didelphis virginiana*	F1
62	Washed Platelets	purified opossum	botrocetin	2	µg/ml	0	*Didelphis virginiana*	M1
54	Washed Platelets	PPP Human	ristocetin	1.5	mg/ml	68	Human	CM
55	Washed Platelets	purified human	ristocetin	1.5	mg/ml	78	Human	CM
56	Washed Platelets	purified human	ristocetin	1.5	mg/ml	86	Human	CM
57	Washed Platelets	purified human	ristocetin	1.5	mg/ml	80	Human	CM
69	Washed Platelets	purified human	ristocetin	1.5	mg/ml	73	Human	SJ
58	Washed Platelets	PPP opossum	ristocetin	2	mg/ml	-10	*Didelphis virginiana*	M2
59	Washed Platelets	purified opossum	ristocetin	2	mg/ml	-8	*Didelphis virginiana*	M2

Appendix Table 3.8- List of all washed platelet assays. Individual ID corresponds to a single unique organism. Test ID is each 450µl Test. When test ID is a subset a,b,c this indicates that this test was a continuation of a previous test (the same 450µl WP sample). Sections highlighted in grey denote a different venom protein (for visual ease).

APPENDIX 4. SUPPLEMENTARY MATERIAL FOR CHAPTER 4.



Appendix Figure 4.1- Parsimony reconstruction of N-terminal sequence gaps for clade Didelphini. Didelphini is defined as the clade beginning at node 70. Node labels are arbitrarily designated by the program PAML and are used for reference in ancestral reconstruction. Ancestral reconstruction identifies nodes/ species identical at the proteins level as nodes (72,75), (76, *D. marsupialis*), (74, *P. mcilhennyi*, *P.opossum*). An additional node generated by the addition of *D. aurita* as sister to *D. marsupialis* (not shown) is identical to *D. marsupialis.*



Appendix Figure 4.2- Cartoon depiction of the pQE9 vector with the vWF insert pictured. Zoom shows the exact sequences of the leader and terminating sequence. Not all features shown.

Species	**NCBI Accession #**
Chironectes minimus	FJ159330.1*
Didelphis virginiana	FJ159335.1*
Lutreolina crassicaudata	FJ159342.1*
Philander quica	FJ159362.1*
Philander mcilhennyi	#########
Philander opossum	FJ159364.1*
Metachirus nudicaudata	FJ159353.1*
Didelphis marsupialis	#########
Homo sapiens	**AAB39987.1**
Monodelphis domestica	**NW_001582018.1**
Monodelphis emiliae	FJ159358.1*
Didelphis aurita	#########

Appendix Table 4.1- List of specimens used for vWF constructs. GenBank accession numbers with asterisks were amended with the newly sequenced upstream portion of vWF for this work. Accession numbers in bold were novel and accessioned uniquely, sequences with hash marks will be accessioned upon publication. Sequences not in bold were used directly from GenBank and not re-sequenced.

Primer name	Primer Sequence
DvWF_F1	5'- TCACTGTGATGGTGTGAACTT-3'
DvWF_R6	5'- GTCTGAGCCTTCTAGCACAAA-3'
DvWF_R1	5'-ACATTGAACTGAAAGATCGGAAGC-3'
PQE9 Insert F1	5'- CACAGAATTCATTAAAGAGGAGA-3'
PQE9 insert R3	5'-CTGAGGTCATTACTGGATCTATCAACAGGA-3'

Appendix Table 4.2- List of primers used.

date	molecule	chip	species	K_D [M]	k_a	k_d	Rmax	R equilibrium	R^2
9/14/2016	bitiscetin	NiNTA	Human	1.97E-08	2.07E+04	4.07E-04	5.32E-01	5.28E-01	0.9882
9/14/2016	bitiscetin	NiNTA	*Didelphis virginiana*	1.81E-07	2.95E+04	5.35E-03	3.69E-01	3.22E-01	0.9776
11/1/2016	bitiscetin	NiNTA	Human	1.03E-08	1.02E+05	1.05E-03	4.22E-01	4.16E-01	0.9841
11/1/2016	bitiscetin	NiNTA	Human	1.77E-08	9.83E+04	1.74E-03	2.69E-01	2.57E-01	0.9835
11/2/2016	bitiscetin	NiNTA	*Didelphis virginiana*	8.47E-08	7.06E+04	5.98E-03	5.44E-01	4.88E-01	0.9832
11/3/2016	bitiscetin	NiNTA	*Didelphis virginiana*	9.52E-08	4.63E+04	4.41E-03	4.30E-01	3.81E-01	0.9863
11/12/2016	bitiscetin	NiNTA	Human	1.39E-08	8.01E+04	1.12E-03	7.65E-01	7.51E-01	0.9941
11/15/2016	bitiscetin	NiNTA	Human	1.92E-08	8.74E+04	1.67E-03	1.15E+00	1.10E+00	0.9963
11/15/2016	bitiscetin	NiNTA	*Didelphis virginiana*	9.90E-08	1.19E+05	1.18E-02	7.71E-01	6.80E-01	0.9842
12/9/2016	botrocetin A	HS1K	*Didelphis virginiana*	3.00E-05	2.11E+03	6.33E-02	1.76E+00	1.73E-01	0.9905
12/13/2016	botrocetin A	HS1K	Human	6.50E-07	7.23E+03	4.70E-03	5.34E-01	4.45E-01	0.9794
1/2/2017	botrocetin A	HS1K	Human	6.47E-07	7.41E+03	4.80E-03	3.31E-01	2.37E-01	0.9838
1/2/2017	botrocetin A	HS1K	*Didelphis virginiana*	2.16E-05	3.61E+03	7.78E-02	1.72E+00	1.21E-01	0.9835
5/15/2017	bitiscetin	HS1K	*Chironectes minimus*	2.03E-06	6.79E+04	1.38E-01	4.30E-01	2.38E-01	0.9745
5/18/2017	botrocetin A	HS1K	Human	7.69E-07	9.48E+03	7.29E-03	4.50E-01	3.64E-01	0.9896
5/18/2017	botrocetin A	HS1K	*Monodelphis domestica*	2.00E-04	na	na	na	na	na
5/24/2017	aspercetin	HS1K	Human	1.92E-06	2.49E+04	4.78E-02	4.14E-01	3.47E-01	0.9944
5/24/2017	aspercetin	HS1K	*Metachirus nudicaudatus*	2.00E-04	na	na	na	na	na
5/25/2017	aspercetin	HS1K	*Lutreolina crassicaudata*	2.00E-04	na	na	na	na	na
5/25/2017	botrocetin A	HS1K	*Metachirus nudicaudatus*	2.00E-04	na	na	na	na	na
5/25/2017	botrocetin A	HS1K	*Lutreolina crassicaudata*	2.00E-04	na	na	na	na	na
5/25/2017	botrocetin A	HS1K	*Philander quica*	2.00E-04	na	na	na	na	na
5/25/2017	botrocetin A	HS1K	*Didelphis albiventris*	2.00E-04	na	na	na	na	na
5/30/2017	bitiscetin	HS1K	*Didelphis marsupialis*	9.36E-07	2.27E+04	2.12E-02	9.00E-01	6.55E-01	0.9591
5/30/2017	bitiscetin	HS1K	*Chironectes minimus*	6.03E-06	1.89E+04	1.14E-01	1.07E+00	3.13E-01	0.9846

date	molecule	chip	species	K_D [M]	k_a	k_d	Rmax	R equilibrium	R^2
5/30/2017	botrocetin A	HS1K	*Chironectes minimus*	2.00E-04	na	na	na	na	na
5/30/2017	botrocetin A	HS1K	*Didelphis marsupialis*	6.03E-06	3.03E+04	1.83E-01	3.71E-01	1.30E-01	0.9509
6/1/2017	aspercetin	HS1K	*Chironectes minimus*	2.00E-04	na	na	na	na	na
6/1/2017	aspercetin	HS1K	*Didelphis virginiana*	3.64E-07	4.42E+03	1.61E-03	3.59E-01	3.46E-01	0.9865
6/1/2017	bitiscetin	NiNTA	Human	3.44E-08	2.76E+04	9.51E-04	6.71E-01	6.62E-01	0.997
6/1/2017	bitiscetin	NiNTA	*Didelphis virginiana*	4.80E-07	1.90E+04	9.10E-03	1.46E+00	1.23E+00	0.9948
6/2/2017	aspercetin	HS1K	Human	1.50E-06	3.08E+04	4.63E-02	4.09E-01	3.56E-01	0.9941
6/2/2017	aspercetin	HS1K	Human	3.12E-06	2.01E+04	6.25E-02	3.73E-01	2.84E-01	0.9901
6/2/2017	aspercetin	HS1K	*Monodelphis domestica*	2.00E-04	na	na	na	na	na
6/2/2017	aspercetin	HS1K	*Didelphis marsupialis*	2.00E-04	na	na	na	na	na
6/2/2017	aspercetin	HS1K	*Didelphis albiventris*	2.00E-04	na	na	na	na	na
6/19/2017	aspercetin	HS1K	*Philander quica*	2.00E-04	na	na	na	na	na
6/19/2017	aspercetin	NiNTA	*Philander opossum*	2.00E-04	na	na	na	na	na
6/19/2017	botrocetin A	NiNTA	*Philander opossum*	2.00E-04	na	na	na	na	na
6/27/2017	bitiscetin	NiNTA	*Didelphis albiventris*	9.77E-07	9.09E+03	8.89E-03	3.17E+00	2.28E+00	0.9947
6/27/2017	bitiscetin	NiNTA	*Didelphis marsupialis*	7.91E-07	7.82E+03	6.18E-03	3.99E+00	3.03E+00	0.9973
6/27/2017	bitiscetin	NiNTA	*Monodelphis domestica*	5.70E-07	1.85E+04	1.06E-02	1.27E+00	1.03E+00	0.9928
6/27/2017	bitiscetin	NiNTA	*Metachirus nudicaudatus*	3.10E-06	3.80E+03	1.18E-02	3.56E-01	1.59E-01	0.9141
2/23/2018	botrocetin A	HS1K	Human	7.99E-07	8.51E+03	6.81E-03	4.04E-01	3.25E-01	0.9608
2/23/2018	botrocetin A	HS1K	Node 69	9.79E-07	1.13E+04	1.11E-02	5.53E-01	4.25E-01	0.9721
2/23/2018	botrocetin A	HS1K	*Monodelphis emiliae*	2.00E-04	na	na	na	na	na
2/24/2018	bitiscetin	NiNTA	*Monodelphis emiliae*	1.96E-06	9.50E+03	1.87E-02	2.31E+00	1.29E+00	0.9977
2/24/2018	bitiscetin	NiNTA	Node 69	3.03E-07	1.80E+04	5.45E-03	1.16E+00	1.04E+00	0.9934
2/25/2018	aspercetin	HS1K	Node 69	2.67E-06	1.97E+04	5.26E-02	3.21E-01	2.54E-01	0.9769
3/27/2018	bitiscetin	NiNTA	*Lutreolina crassicaudata*	2.11E-07	1.64E+04	3.47E-03	6.91E-01	6.37E-01	0.9921
3/27/2018	bitiscetin	NiNTA	*Lutreolina crassicaudata*	3.74E-07	1.31E+04	4.89E-03	8.68E-01	7.55E-01	0.9938
3/28/2018	bitiscetin	NiNTA	*Philander quica*	6.95E-07	1.49E+04	1.04E-02	5.88E-01	4.60E-01	0.9925
3/28/2018	bitiscetin	NiNTA	*Philander quica*	1.33E-06	1.05E+04	1.39E-02	5.05E-01	3.30E-01	0.9768
3/28/2018	bitiscetin	NiNTA	*Philander opossum*	2.00E-04	na	na	na	na	na
3/29/2018	botrocetin A	HS1K	*Philander quica*	2.00E-04	na	na	na	na	na
3/29/2018	botrocetin A	HS1K	*Didelphis marsupialis*	6.48E-06	7.31E+03	4.74E-02	7.37E-01	2.46E-01	0.9807
7/16/2018	botrocetin B	HS1K	Human	5.09E-08	5.20E+05	2.65E-02	2.39E-01	2.38E-01	0.9699
12/13/2018	botrocetin A	HS1K	Node 70	2.00E-04	na	na	na	na	na
12/13/2018	botrocetin A	HS1K	Node 71	2.87E-06	3.05E+04	8.75E-02	3.85E-01	2.05E-01	0.9788
12/13/2018	botrocetin A	HS1K	Node 72	2.00E-04	na	na	na	na	na
12/13/2018	botrocetin A	HS1K	Node 73	2.00E-04	na	na	na	na	na
12/16/2018	botrocetin B	HS1K	*Didelphis marsupialis*	7.49E-07	2.22E+05	1.66E-01	1.56E-01	1.13E-01	0.9569
12/16/2018	botrocetin B	HS1K	*Lutreolina crassicaudata*	1.39E-06	9.69E+04	1.35E-01	2.42E-01	2.23E-01	0.9645
12/16/2018	botrocetin B	HS1K	*Lutreolina crassicaudata*	3.00E-06	6.24E+04	1.87E-01	1.30E+02	3.26E+01	0.9394

date	molecule	chip	species	K_D [M]	k_a	k_d	Rmax	R equilibrium	R^2
12/17/2018	botrocetin B	NiNTA	*Chironectes minimus*	2.78E-05	3.14E+03	8.73E-02	5.38E-01	1.97E-01	0.9377
12/17/2018	botrocetin B	HS1K	*Didelphis virginiana*	1.66E-06	1.51E+05	2.51E-01	4.67E-01	4.23E-01	0.9841
12/18/2018	botrocetin B	HS1K	*Didelphis albiventris*	2.63E-06	1.39E+05	3.64E-01	4.61E-01	3.47E-01	0.9784
12/18/2018	botrocetin B	NiNTA	*Didelphis albiventris*	1.06E-05	4.24E+04	4.50E-01	9.04E-01	7.78E-02	0.9917
1/8/2019	botrocetin B	NiNTA	Human	4.99E-07	3.85E+04	1.92E-02	1.39E+00	1.31E+00	0.9812
1/8/2019	botrocetin B	NiNTA	Human	3.72E-07	7.28E+04	2.71E-02	6.10E-01	5.97E-01	0.9892
1/9/2019	botrocetin B	NiNTA	*Didelphis virginiana*	1.33E-05	2.19E+04	2.90E-01	4.71E-01	6.18E-02	0.9626
1/9/2019	botrocetin B	NiNTA	Human	4.18E-07	7.11E+04	2.97E-02	7.67E-01	7.29E-01	0.9895
1/10/2019	botrocetin B	NiNTA	*Didelphis albiventris*	6.91E-06	4.05E+04	2.79E-01	2.26E+00	1.58E+00	0.9928
1/10/2019	botrocetin B	NiNTA	*Didelphis albiventris*	8.16E-06	3.71E+04	3.03E-01	2.64E+00	1.75E+00	0.9916
1/10/2019	botrocetin B	NiNTA	*Didelphis marsupialis*	6.16E-06	2.73E+04	1.68E-01	3.11E+00	2.25E+00	0.9921
1/10/2019	botrocetin B	NiNTA	*Didelphis marsupialis*	6.96E-06	3.85E+04	2.68E-01	3.07E+00	2.14E+00	0.9797
1/10/2019	botrocetin B	NiNTA	*Chironectes minimus*	1.76E-05	1.89E+04	3.32E-01	2.12E-01	1.01E-01	0.9416
1/10/2019	botrocetin B	NiNTA	*Didelphis virginiana*	6.19E-06	3.28E+04	2.03E-01	1.31E+00	9.45E-01	0.9974
1/10/2019	botrocetin B	NiNTA	*Didelphis virginiana*	7.65E-06	3.52E+04	2.70E-01	1.58E+00	1.07E+00	0.9965
1/10/2019	botrocetin B	NiNTA	*Philander opossum*	2.00E-04	na	na	na	na	na
1/15/2019	botrocetin B	NiNTA	*Didelphis aurita*	3.87E-06	5.71E+04	2.21E-01	2.37E-01	1.91E-01	0.9829
1/15/2019	botrocetin B	NiNTA	*Didelphis aurita*	2.64E-06	5.10E+04	1.35E-01	2.63E-01	2.25E-01	0.9843
1/15/2019	botrocetin B	NiNTA	*Metachirus nudicaudatus*	2.00E-04	na	na	na	na	na
1/15/2019	botrocetin B	NiNTA	*Metachirus nudicaudatus*	2.00E-04	na	na	na	na	na
1/15/2019	botrocetin B	NiNTA	*Monodelphis emiliae*	4.92E-05	1.27E+04	6.23E-01	2.09E+00	5.13E-01	0.9954
1/15/2019	botrocetin B	NiNTA	*Monodelphis emiliae*	1.30E-05	3.47E+04	4.51E-01	8.88E-01	4.90E-01	0.9938
1/18/2019	aspercetin	HS1K	Node 72	2.00E-04	na	na	na	na	na
1/18/2019	botrocetin B	NiNTA	Node 69	6.77E-06	2.83E+04	1.91E-01	1.03E+00	7.21E-01	0.9924
1/18/2019	botrocetin B	NiNTA	Node 71	9.14E-06	2.29E+04	2.09E-01	2.79E+00	1.78E+00	0.9978
1/18/2019	botrocetin B	NiNTA	Node 71	9.52E-06	2.35E+04	2.24E-01	3.41E+00	1.56E+00	0.9937
1/18/2019	botrocetin B	NiNTA	Node 72	8.45E-06	2.14E+04	1.81E-01	4.06E+00	2.66E+00	0.9947
1/18/2019	botrocetin B	NiNTA	Node 69	1.25E-05	2.16E+04	2.69E-01	1.77E+00	9.92E-01	0.9965
1/21/2019	botrocetin B	NiNTA	Node 69	3.62E-05	7.76E+03	2.81E-01	2.24E+00	6.86E-01	0.9773
3/3/2019	botrocetin B	NiNTA	*Monodelphis domestica**	1.55E-05	2.32E+04	3.60E-01	1.07E+00	5.46E-01	0.9905
3/3/2019	botrocetin B	NiNTA	*Monodelphis domestica**	1.36E-05	2.73E+04	3.72E-01	7.30E-01	3.94E-01	0.9968
3/3/2019	botrocetin B	NiNTA	Node 69	2.19E-05	1.17E+04	2.57E-01	1.82E+00	7.66E-01	0.9955
3/3/2019	botrocetin B	NiNTA	Node 70	8.39E-07	5.59E+04	4.69E-02	1.14E-01	1.03E-01	0.9823
3/3/2019	botrocetin B	NiNTA	Node 70	2.41E-06	4.71E+04	1.14E-01	1.55E-01	1.35E-01	0.9418
3/3/2019	botrocetin B	NiNTA	*Philander quica**	1.39E-05	2.52E+04	3.51E-01	1.62E+00	5.91E-01	0.9974
3/3/2019	botrocetin B	NiNTA	*Philander quica**	8.65E-06	3.58E+04	3.10E-01	1.16E+00	5.57E-01	0.9966
3/4/2019	aspercetin	HS1K	*Monodelphis emiliae*	2.69E-07	1.03E+05	2.76E-02	2.64E-01	2.08E-01	0.9758
3/4/2019	aspercetin	HS1K	Node 71	3.71E-07	1.77E+04	6.58E-03	1.35E-01	1.30E-01	0.9616
3/4/2019	aspercetin	HS1K	Node 73	9.59E-07	1.40E+04	1.34E-02	1.68E-01	1.54E-01	0.9595

date	molecule	chip	species	K_D [M]	k_a	k_d	Rmax	R equilibrium	R^2
3/4/2019	aspercetin	HS1K	Node 70	1.83E-06	4.47E+04	8.17E-02	9.06E-02	7.66E-02	0.9719
3/4/2019	aspercetin	HS1K	Node 72	1.77E-05	1.73E+04	3.06E-01	3.83E-01	1.38E-01	0.9418
3/4/2019	aspercetin	HS1K	*Didelphis aurita*	2.00E-04	na	na	na	na	na
3/4/2019	botrocetin B	NiNTA	Node 73	5.71E-06	2.65E+04	1.52E-01	2.61E+00	1.92E+00	0.9949
3/4/2019	botrocetin B	NiNTA	Node 73	1.05E-05	1.85E+04	1.95E-01	5.33E+00	4.63E-01	0.9973
3/20/2019	bitiscetin	NiNTA	*Didelphis aurita*	2.15E-07	3.34E+04	7.20E-03	4.08E-01	3.48E-01	0.9777
3/20/2019	bitiscetin	NiNTA	*Didelphis aurita*	6.40E-07	1.77E+04	1.13E-02	5.62E-01	4.47E-01	0.9842
3/21/2019	botrocetin A	HS1K	Human	9.65E-07	7.10E+03	6.85E-03	4.61E-01	3.56E-01	0.98250
3/22/2019	botrocetin A	HS1K	*Didelphis aurita*	2.00E-04	na	na	na	na	na

Appendix Table 4.3- All data collection for curve sets by date from oldest to newest. Table is color coded by venom protein. All data is from a globally fit 1:1 binding model performed in BLItz Pro software. Date is the date the assay was performed. Chip types are Nickel coated (NiNTA) or pentahistidine tagged (HS1K). Molecular indicates the venom protein used. Species indicates the species (or node) of vWF used. K_D is given here in [M] units. Off rates (k_a) is 1/Ms, and off rates (k_d) are in 1/s (seconds). Rmax is the maximum response determined from the fit of the binding data (nm), R equilibrium is the calculated response (nm) at equilibrium resulting from globally fit binding data. R^2 is a measure of goodness of fit of globally fit binding data. K_D, k_a, and k_d between chips are scaled --for botrocetin B NiNTA results are divided by 7.59, bitiscetin HS1K results are divided by 7.79. Separately expressed bactches of *M. domestica* and *P. quica* are indicated with an asterisk.

Appendix Material 4.1.

DidvWFAlign1.phy

39 702
Caluromys_lanatus ----------

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TGACCTGGTACCTGATATTCCTGCTCCTACCATCCCCTCC
Caluromys_philander ----------

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TGACCTGGTGCCTGATATTCCTGCTCCTACCATCCCCTCC
Chironectes_minimus CAGAAG------
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C
Didelphis_albiventris CAGAAG----------
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Didelphis_marsupialis CAGAAG----------
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Didelphis_virginiana CAGAAG----------
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Glironia_venusta ----------

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Gracilinanus_aceramarcae ----------

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Gracilinanus_agilis ----------

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Gracilinanus_microtarsus ----------

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Marmosa_demerarae ----------

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Marmosa_murina ----------

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Marmosa_rutteri ----------

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Marmosops_incanus ----------

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Marmosops_noctivagus ----------

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Marmosops_parvidens ----------

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Marmosops_pinheiroi ----------

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Marmosops_spGalvez ----------

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Metachirus_nudicaudatus CAGAAGCCATGG---------
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Appendix Material 4.2.

DidvWFTree.tre

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Appendix Material 4.3.

DidvWFAlign2.phy

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Marmosa_demerarae ----------

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Marmosa_rubra ----------

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Marmosops_noctivagus ----------

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Marmosops_pinheiroi ----------

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Marmosops_spGalvez ----------

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Monodelphis_scalops ----------

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Thylamys_pallidior ----------

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Thylamys_venustus ----------

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TCCCACTCCTACATTGAACTGAAAGATCGGAAGCGGCCTTCAGAGCTTCGTCGGATT
GCCAGTTCTGTGAGATATCCTGGCAGCAAGATGGCATCCACCAGTGAGGCACTGAAG
TTTACCCTTTTCCATGTCTTTGGCAAGGCTGACCGCCCTGAAGCATCTCGGATCGCCT
TGCTTCTGACGGCCAGTGAGGAGCCCCCTCCCATGGCTAAGTCTATAGGTCGCTATG
CCCAAGGTCTGAAGAAGAAGAAGGTCATCGTGATCCCAGTGGGCTTAGGGCCCCAT
GCTAGCCTCAAGCAGATCCGTATCATTGAGAAGCAGGCACCAGAAAACAAGGCCTTT
TTGCTTAGTGGCGTGAATGAGCTAGAGCAAAGGCGGGATGACATCTTGGGCTACCTC
TGTGACCTGGTACCTGATATTCCTGCTCCTACCATCCCCTCC
Tlacuatzin_canescens ----------

CAGAGGCGCATCAGGGTGGCTGTGGTGGAATACCATGATGGATCCCACTCCTACATT
GAACTGAAGGATCGGAAGCGGCCCTCGGAGCTTCGTCGGATTGCCAGTTCTGTGAGA
TATCCTGGCAGCAAGATGGCATCCACCAGCGAGGCACTGAAGTTTACCCTTTTCCAT
GTCTTTGGCAAGGCTGACCGCCCTGAAGCATCTCGGATTGCTTTGCTTCTGACGGCCA
GTGAGGAGCCCCTTCCCATGGCTAAGTCTATAGCTCGCTTTACCCAAGGTCTGAAGA
AGAAGAAGGTCATTGTGATCCCAGTGGGCTTAGGGCCCCATGCTAGCCTCAAGCAGA
TTCGTCTCATTGAGAAGCAGGCACCAGAAAACAAGGCCTTTTTGCTTAGTGGTGTGA
ATGAGCTAGAGCAAAGGCAGGATGACATCTTGGGCTACCTCTGTGACCTGGTACCTG
ATGTTCCTGCTCCTACCATCCCCTCC

Appendix Material 4.4.

DidvWFTree2.tre
((((((((((Marmosa_demerarae:2.765345451,Marmosa_paraguayana:2.765345451):1.273905464,
Marmosa_rutteri:4.039250914):4.807878906,Marmosa_lepida:8.847129821):0.6181647349,Mar
mosa_murina:9.465294557):2.563326222,((Marmosa_mexicana:6.095467925,Marmosa_robinso
ni:6.095467925):4.271309193,Marmosa_rubra:10.36677712):1.661843661):6.811332203,((((Mo
nodelphis_domestica:1.910844392,Monodelphis_brevicaudata:1.910844392):5.417041016,Mono
delphis_peruviana:7.32788541):0.5183117649,Monodelphis_emiliae:7.846197173):0.754799745
2,Monodelphis_scalops:8.600996918):10.23895606):1.563429229,Tlacuatzin_canescens:20.4033
8221):4.157254367,((((Marmosops_parvidens:4.419065303,Marmosops_pinheiroi:4.419065303)
:9.729347487,((Marmosops_spGalvez:3.685183951,Marmosops_noctivagus:3.685183951):7.422
23026,Marmosops_incanus:11.10741421):3.040998578):4.907926781,((((Thylamys_pusillus:1.6
91742116,Thylamys_macrurus:1.691742116):0.5182666689,Thylamys_pallidior:2.210008785):0
.317471128,Thylamys_venustus:2.527479913):9.042200902,(Gracilinanus_emiliae:7.154086816
,(Gracilinanus_aceramarcae:4.776400484,(Gracilinanus_microtarsus:4.134707034,Gracilinanus_
agilis:4.134707034):0.6416934509):2.377686333):4.415593998):7.486658755):4.257517681,((((

((Philander_opossum:0.5543911959,Philander_mcilhennyi:0.5543911959):2.69796657,Philander_quica:3.252357766):1.647067035,(((Didelphis_aurita:0.4874729729,Didelphis_marsupialis:0.4874729729):1.661041458,Didelphis_albiventris:2.148514431):0.9146554021,Didelphis_virginiana:3.063169833):1.836254969):2.661929493,Lutreolina_crassicaudata:7.561354295):3.428728553,Chironectes_minimus:10.99008285):9.138166744,Metachirus_nudicaudatus:20.12824959):3.18560766):1.246779326):6.533314423,Glironia_venusta:31.093951):1.445331938,(Caluromys_lanatus:4.708844969,Caluromys_philander:4.708844959):27.83043798);

Appendix Material 4.5.

Constructs.phy

17 250
Human_Construct
MRGSHHHHHHGSQEPGGLVVPPTDAPVSPTTLYVEDISEPPLHDFYCSRLLDLVFLLDGSSRLSEAEFEVLKAFVVDMMERLRISQKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRIASQVKYAGSQVASTSEVLKYTLFQIFSKIDRPEASRIALLLMASQEPQRMSRNFVRYVQGLKKKKVIVIPVGIGPHANLKQIRLIEKQAPENKAFVLSSVDELEQQRDEIVSYLCDLAPEAPPPTLPPD*QA
Monodelphis_domestica_Construct
MRGSHHHHHHGSQKPGDLQLSPSFTTVAPTAEYLEETPEPPLHSFYCSKMLDLAFLLDGSSKLSEAEFEVLKAFVVGVMERLHISQRRIRVALVEYHEGSHSYIELKDRKRPSELRRIASSVRYPGSKMASTSEVLKFTLFHVFGKVDRPEASRIALLLTASEEPRPTSKSIARYSQDLMEKKVIVIPVGLGPHASLRQIRLIEKQAPENKAFLLSGVNELEQRRDDILGYLCDLVPDSPAPTISS*QA*
Monodelphis_emiliae_Construct
MRGSHHHHHHGSQKPGDLQLSPSFTTVAPTTEYLEETPEPPLHSFYCSKMLDLAFLLDGSNKLSESEFEVLKAFVVGVMERLHISQRRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASSVRYPGSKMASISEALKFTLFHVFGKADRPKASRIALLLTASEEPLRMTKFIGHYAQSLKKKKVIVIPVGLGPHASLKHIRLIENKAPENKAFLLSGVNELEQRRDDILGYLCDLVPDIPAPTIPS*QA*
Metachirus_nudicaudatus_Construct MRGSHHHHHHGSQKPW---
LSPSFTTATPTTAYLEETPEPPLDSFHCSKILDLAFLLDGSSKLSEAEFEVLKAFVVDMMERLHISQRRIRVAVVEYHEGSHSYIELKDRKRPSELRRIASSVRYPGSKIASTSEALKFALFHIFGKADRPEASRIALLLTASEEP--
KDKLIARYSHSLKKKKVIVIPVGLGPHASLKQIRLIEKQAPENKAFLLSGVNELQQRRDDILGYLCDLVPDIPAPTKPS*QA*
Chironectes_minimus_Construct MRGSHHHHHHGSQK--
DQKLSPSFTTATPTTAYLEETPEPPLHSFHCSKILDLAFLLDGSSKLSEAEFEVLKAFVVGVMERLHISQRRIRVAVVEYHDGCHSYIQLKDRKRPSELRRIASSVRYPGSNMASTSEVLKFALFHVFGKADRPEASRIALLLTANEEPLPMAKGIELYARGLKKKKVIVIPVGLGPHASLKQIRLIEQKAPENKAFLLSGVNELEQRRDDILDYFCDLVPDIPAPTIPS*QA*
node_69_M8_Construct
MRGSHHHHHHGSQKPWDQQLSPSFTTATPTTAYLEETPEPPLHSFHCSKILDLAFLLDGS

SKLSEAEFEVLKAFVVGVMERLHISQRRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASS
VRYPGSKMASTSEVLKFALFHVFGKADRPEASRIALLLTASEEPLPMAKSIARYAQSLKK
KKVIVIPVGLGPHASLKQIRLIEKQAPENKAFLLSGVNELEQRRDDILGYFCDLVPDIPAPT
IPS*QA*
node_70_M8_Construct MRGSHHHHHHGSQK--
DQQLSPSFTTATPTTAYLEETPEPPLHSFHCSKILDLAFLLDGSSKLSEAEFEVLKAFVVGV
MERLHISQRRIRVAVVEYHDGCHSYIQLKDRKRPSELRRIASSVRYPGSNMASTSEVLKF
ALFHVFGKADRPEASRIALLLTANEEPLPMAKGIDLYAQGLKKKKVIVIPVGLGPHASLK
QIRLIEKKAPENKAFLLSGVNELEQRRDDILGYFCDLVPDIPAPTIPS*QA*
node_71_M8_Construct MRGSHHHHHHGSQK----
QLSPSFTTATPTTAYVEETPEPPLHSFHCSKILDLAFLLDGSSKLSEAEFEVVKAFVVGVM
ERLHISQKRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASSVRYPGSKMASISEVLKFTLF
HVFGKADRPEASRIALLLTASGEPLPMARNIGRYAQSLKKKKVTVIPVGLGPHVNLRQIR
NIEKEARENKAFLLSGVNELEQRRDDILGYFCDLVPDIPAPTIPS*QA*
node_72_M8_Construct MRGSHHHHHHGSQK----
QLSPSFTTATPTTAYVEETPEPPLHNFHCSKILDLAFLLDGSSKLSEAEFEVVKAFVVGVM
ERLHISQKRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASSVRYPGSKMASISEVLKFTLF
HVFGKADRPEASRIALLLTASGEPLPMARNIGRYAQSLSEKKVTVIPVGLGPHVNLRQIR
NIEKAARENKAFLLSGVNELEQRRDDILGYFCDLVPDIPAPTIPS*QA*
node_73_M8_Construct MRGSHHHHHHGSQK----
QLSPSFTTATPTTAYVEETPEPPLHNFHCSKILDLVFLLDGSSKLSEAEFEVVKAFVVGVM
ERLHISQKRIRVAVVEYHEGSHSYIELKDRKRPSELRRIASSVRYPGSNMASISEVLKFTLF
HVFGKAKRPEASRIALLLTASGEPLPMARNIGRYAQSLSEKKVTVIPVGLGPHVNLRQIR
NIEKAARENKAFLLSGVNELEQRRDDILGYFCDLVPDIPAPTIPS*QA*
Didelphis_aurita_Construct MRGSHHHHHHGSQK----
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ERLHISQKRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASSVRYPGSKMASISEVLKFTLF
HVFGKADRPEASRIALLLTASGEPLPMGRNIGHYAQSLSEKKVTVIPVGLGPHVNLRQIR
NIEKVARENKAFLLSGVNELEQRRDDILGYFCDLVPDIPAPTIPS*QA*
Didelphis_virginiana_Construct MRGSHHHHHHGSQK----
QLSPSFTTATPTTAYVEETPEPPLHNFHCSKILDLAFLLDGSSKLSEAEFEVVKAFVVGVM
ERLHISQKRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASSVRYPGSKMASISEVLKFTLF
HVFGKADRPEASRIALLLTASGEPLPMARNIGRYAQSLSQKKVTVIPVGLGPHVNLRQIR
NIEKAARENKPFLLSGVNELEQRRDDILSYFCDLVPDIPAPTIPS*QA*
Didelphis_marsupialis_Construct MRGSHHHHHHGSQK----
QLSPSFTTATPTTAYVEETPEPPLHNFHCSKILDLAFLLDGSSKLSEAEFEVVKAFVVGVM
ERLHISQKRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASSVRYPGSKMASISEVLKFTLF
HVFGKADRPEASRIALLLTASGEPLPMARNIGRYAQSLSEKKVTVIPVGLGPHVNLRQIR
NIEKVARENKAFLLSGVNELEQRRDDILGYFCDLVPDIPAPTIPS*QA*
Didelphis_albiventrus_Construct MRGSHHHHHHGSQK----
QLSPSFTTATPTTAYVEETPEPPLHNFHCSKILDLAFLLDGSSKLSEAEFEVVKAFVVGVM
ERLHISQKRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASSVRYPGSKMASISEVLKFTLF
HVFGKADRPEASRIALLLTASGEPFPMARNIGRYAQSLSEKKVTVIPVGLGPHVNLRQIR
NIEKVARENKAFLLSGVNELEQRRDDILGYFCDLVPDIPAPTIPS*QA*

Lutreolina_crassicaudata_Construct MRGSHHHHHHGSQE----
QLSPSFTTATPTTAYVEETPEPPLHSFHCSKMLDLAFLLDGSSKLSEAEFEVVKAFVVGV
MERLHISQKRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASSVRYPGSKMASISEVLKFA
LFHVFGKADRPEASRIALLLTASGEPLPIGRNIGRYAQSLKKKKVIVIPVGLGPHVNLRQIR
NIEKEARENKAFLLSGVNELEQRRDDILSYFCDLVPDIPAPIIPS*QA*
Philander_frenatus_Construct MRGSHHHHHHGSQK----
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MERLHISQKRIRVAVVEYHDGSHSYIELKDRKRPSELRRIASSVRYPGSEMASISEVLKFT
LFHVFGKADRPEASRIALLLMASGEPLPMGRNIGHYAQSLSEKKVTVIPVGLGPHVNLRQ
IRNIEKASRENKPFLLSGVNELEQRRDDILGYFCDLVPDIPAPTIPS*QA*
Philander_opossum_Construct MRGSHHHHHHGSQK----
QLSPSFTTATPTTAYVEETPEPPLHNFHCSKILDLVFLLDGSSKLSEAEFEVVKAFVVGVM
ERLHISQKRIRVAVVEYHEGSHSYIELKDRKRPSELRRIASSVRYPGSNMASISEVLKFTLF
HVFGKAKRPEASRIALLLTASGEPLPMARNIVRYAQSLSEKKVTVIPVGLGPHVNLRQIR
NIEKAARENKAFLLSGVNELEQRRDDILGYFCDLVPDIPAPTIPS*QA*

Appendix Material 4.6.

BLItz Pro software 1:1 binding model (ForteBio, Pall Corperation).

Curve Fitting

The BLItz Pro software version 1.2.1.3 subtracts a zero concentration curve from all curve data. Using a full-fitting model it optimizes for R_{max}, K_a, and K_d given each analyte (venom) concentration using the equations (ForteBio knowledgebase, Pall Corporation):

Association Phase:

$$Y = R_{max}\frac{1}{1+\frac{k_d}{k_a*[Analyte]}}(1-e^{-\left(k_a*[Analyte]+k_d\right)t})$$

Disassociation Phase:

$$Y = Y_A e^{-k_d(t-t_A)}$$

$$Y_A = R_{max}\frac{1}{1+\frac{k_d}{k_a*[Analyte]}}(1-e^{-\left(k_a*[Analyte]+k_d\right)t_A})$$

- Y is the BLI signal in nm, which indicates the level of binding as a nm shift.
- t is time in seconds.
- k_a is the association rate constant.
- k_d is the dissociation rate constant.

- [Analyte] refers to the provided concentration of the analyte in solution.
- R_{max} represents the fitted maximum achievable binding for an analyte to a given level of immobilized ligand on the biosensor surface.
- t_A represents the time at the end of association, which is also the time at the beginning of dissociation.
- Y_A represents the calculated nm shift at the end of association (when time is at t_A)

This full fitted model is used to optimize observed values for R_{max}, K_a, and k_d, which are directly observed from each analyte concentration curve.

Calculation of Binding Affinity

BLItz software subsequently uses these optimized values and the equations below calculate k_{obs}, R_{eq} and K_D for each analyte concentration.

$$k_{obs} = k_a * [Analyte] + k_d$$

$$R_{eq} = R_{max} \frac{k_a * [Analyte]}{k_a * [Analyte] + k_d} = R_{max} \frac{[Analyte]}{[Analyte] + K_D}$$

- k_a is the association rate constant.
- k_d is the dissociation rate constant.
- k_{obs} is the observed rate constant reflecting the overall rate of the combined association and dissociation of the two binding partners.
- [Analyte] refers to the provided concentration of the analyte in solution.
- R_{eq} (R equilibrium) is the fitted binding response value (nm shift) when the binding interaction reaches equilibrium between association and dissociation for a given analyte concentration.
- R_{max} represents the calculated maximum achievable binding for an analyte to a given level of immobilized ligand on the biosensor surface.