ORIGINAL ARTICLE

The molecular basis of venom resistance in a rattlesnake-squirrel predator-prey system

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Abstract

Understanding how interspecific interactions mould the molecular basis of adaptations in coevolving species is a long-sought goal of evolutionary biology. Venom in predators and venom resistance proteins in prey are coevolving molecular phenotypes, and while venoms are highly complex mixtures it is unclear if prey respond with equally complex resistance traits. Here, we use a novel molecular methodology based on protein affinity columns to capture and identify candidate blood serum resistance proteins ("venom interactive proteins" [VIPs]) in California Ground Squirrels (*Otospermophilus beecheyi*) that interact with venom proteins from their main predator, Northern Pacific Rattlesnakes (*Crotalus o. oreganus*). This assay showed that serum-based resistance is both population- and species-specific, with serum proteins from ground squirrels showing higher binding affinities for venom proteins of local snakes compared to allopatric individuals. Venom protein specificity assays identified numerous and diverse candidate prey resistance VIPs but also potential targets of venom in prey tissues. Many specific VIPs bind to multiple snake venom proteins and, conversely, single venom proteins bind multiple VIPs, demonstrating that a portion of the squirrel blood serum "resistome" involves broad-based inhibition of nonself proteins and suggests that resistance involves a toxin scavenging mechanism. Analyses of rates of evolution of VIP protein homologues in related mammals show that most of these proteins evolve under purifying selection possibly due to molecular constraints that limit the evolutionary responses of prey to rapidly evolving snake venom proteins. Our method represents a general approach to identify specific proteins involved in co-evolutionary interactions between species at the molecular level.

KEYWORDS

adaptation, coevolution, mammals, molecular evolution, reptiles, species interactions

1 | INTRODUCTION

Understanding how interspecific interactions shape the molecular basis of adaptations in interacting species is a long-sought goal of evolutionary biology. Venom in predators and venom resistance in prey are molecular phenotypes widely considered to have diversified through co-evolution (for recent reviews see Arbuckle, Rodríguez de la Vega, & Casewell, 2017; Holding, Drabeck, Jansa, & Gibbs, 2016). Venoms are under strong selection in these systems, leading to functional specialization on particular prey, local adaptation, and highly variable venoms among populations and related species (Casewell et al., 2014; Casewell, Wüster, Vonk, Harrison, & Fry, 2012; Doley,

Mackessy, & Kini, 2009; Fry, 2005; Rokyta, Wray, McGivern, & Margres, 2015; Vonk et al., 2013). Functional variation in venoms implies functional variation in prey susceptibility, yet large-scale identification and characterization of proteins involved in venom resistance lags far behind similar work on venom proteins having only been investigated in a few mammals and venomous snakes (Arbuckle et al., 2017; Holding, Drabeck, et al., 2016; Neves-Ferreira, Valente, Perales, & Domont, 2010 for reviews).

Serum "resistance factors" mostly from mammals are usually identified using time consuming fractionation experiments (e.g., Biardi, Ho, Marcinczyk, & Nambiar, 2011). Serum protein-based resistance through binding to venom is the mostly widely investigated type of resistance to snake venom, and the abundance of such proteins in mammals that interact with snakes (Perales, Neves-Ferreira, Valente, & Domont, 2005; Pérez & Sánchez, 1999), make these natural targets for the application of high-throughput methods for characterizing the whole resistome. For example, affinity chromatography (Calvete, 2011) has been used to evaluate antivenin efficacy for snakebite treatment, resulting in protein-by-protein information on which venom components are immunorecognized (termed "antivenomics", Calvete, Rodríguez, Quesada-Bernat, & Pla, 2018). The antivenomic framework should be equally effective for isolating any molecular components responsible for venom resistance when interactions are mediated by binding to venom. In vitro tests of function involving single versus multiple components could follow and allow us to address a series of important questions about how these parts of the resistome might interact to confer organismal resistance (Holding, Drabeck, et al., 2016). Identification of the specific proteins involved in resistance also opens the door for comparisons of the relative rates of evolution of offensive (venom) versus defensive (resistance proteins) molecules allowing tests of whether evolutionary constraints related to function play a role in shaping evolutionary interactions between coevolving species (Endara et al., 2017; Feldman, Brodie, Brodie, & Pfrender, 2012).

A natural system where co-evolution has shaped trait variation at the molecular and behavioral levels is the interaction between the venomous Northern Pacific rattlesnake (*Crotalus o. oreganus*) (hereafter Pacific rattlesnake) and its main prey the California ground squirrel (*Otospermophilus beecheyi*) (hereafter ground squirrel) (Barbour & Clark, 2012; Biardi, Chien, & Coss, 2006; Biardi et al., 2011; Coss, Gusé, Poran, & Smith, 1993; Poran, Coss, & Benjamini, 1987; Putman, Coss, & Clark, 2015). Recent work by Holding, Biardi, and Gibbs (2016) used in vitro assays of venom enzymatic activity and inhibition of this activity by squirrel blood serum to examine key features of this system with respect to co-evolutionary dynamics. This research showed substantial geographic variation in both snake venom metalloproteinase (SVMP) activity and resistance factor effectiveness and demonstrated local adaptation in the ability of rattlesnake venom to overcome resistance in local squirrel prey. Rattlesnake local adaptation suggests that the snake-squirrel interaction is mediated by phenotype matching at the molecular level, whereby a local specific SVMP protein will be best inhibited by a local specific resistance protein (Holding, Biardi, et al., 2016). Detailed information is now

available for venom variation in the rattlesnakes involved in these interactions (Holding, Margres, Rokyta, & Gibbs, 2018), whereas the molecular basis of resistance in ground squirrels is still limited to the identification of a small number of blood sera proteins involved in interactions with SVMPs (Biardi et al., 2011). This is despite studies of serum resistance in related mammals that suggest that the presence of a diverse set of resistance proteins that target multiple venom proteins (Perales et al., 2005; Pérez & Sánchez, 1999).

We predict that investigating serum-based resistance through binding to venom proteins will be fruitful in ground squirrels. Not only do the inhibitor proteins identified to-date function via binding to venom (Biardi et al., 2011), but variation in serum-venom protein binding scores covaries with the lethality of Pacific rattlesnake venom across ground squirrel populations (Poran et al., 1987). This suggests that variation in binding reflects functionally-relevant variation in resistance among squirrel populations and species. To this end, we use an affinity chromatography approach adapted from antivenomics technology developed to assess the efficacy of antivenoms for the treatment of snakebite (Calvete et al., 2018; Pla, Gutiérrez, & Calvete, 2012) to conduct an analysis of blood serum proteins in ground squirrels that interact with snake venom proteins, hence representing candidate resistance proteins. We combine affinity column-based protein isolation methods with mass spectrometry-based proteomics to identify proteins in squirrel sera that bind to specific rattlesnake venom proteins. Our study represents an advance on previous work (e.g., Biardi et al., 2011) in that it spans scales of biological divergence from phenotypically distinct populations to species, and uses comparisons at the population-level in the form of reciprocal affinity profiles to test the hypothesis that serum-affinity for venom proteins has undergone local adaptation.

2 | MATERIALS AND METHODS

2.1 | Samples from squirrels and rattlesnakes at different levels of divergence

Previous work on the ground squirrel-rattlesnake system has emphasized the presence of geographic variation in venom resistance (Holding, Biardi, et al., 2016; Poran et al., 1987) and venom composition (Holding et al., 2018). We incorporate this variation into our comparisons by sampling Pacific rattlesnake venom and ground squirrel serum from geographically distinct populations, as well as the phylogenetically distant grey squirrel (*Sciurus carolinensis*). For ground squirrel populations, we focused on two sites described in Holding, Biardi, et al. (2016): Chimineas Ranch (CR) located in the Coast Ranges of Central California and Sutter Buttes (SB) located in in the Central Valley of California. Ground squirrels and rattlesnakes show significant differences in both resistance to snake venom metalloproteinases and venom composition across these sites (Holding, Biardi, et al., 2016; Holding et al., 2018). We also collected serum samples from five grey squirrels (*Sciurus carolinensis*) (hereafter grey squirrel) in Ohio. Grey squirrels are known to be resistant to

the venom of other species of rattlesnakes but only weakly resistant to Pacific rattlesnakes (Pomento, Perry, Denton, Gibbs, & Holding, 2016). At each site, blood sera and venom were collected using standard protocols as described in Holding, Biardi, et al. (2016) and stored at −80°C until analysis. From each locality, we pooled serum samples from five individual squirrels in equivalent volumes to produce a serum sample representative of the population instead of a single individual. Likewise, Pacific rattlesnake venom used in this study was an equal-volume pool of 10 individual snakes' venom collected from each population. Finally, we used rabbit serum (*Oryctolagus cuniculus*) (hereafter rabbit) as a nonresistant control.

2.2 | Affinity chromatography capture of rattlesnake venom components with affinity for squirrel blood serum proteins

To determine which squirrel blood serum proteins interact with the diverse toxin proteins present in whole snake venom, 300 μl chromatographic columns containing immobilized proteins of 100 μl of whole blood serum from each of CR and SB ground squirrels were generated as described (Pla, Rodríguez, & Calvete, 2017) and used to capture the components from 250 μg of Pacific rattlesnake venom with affinity for serum proteins from local (sympatric) and foreign

(allopatric) ground squirrels (Figure 1a). A fully reciprocal combination of sympatric and allopatric combinations of venom and serum at the population-level underlies the power of this study design, as it allowed us to determine if the suite of proteins identified or overall affinity strengths showed signals consistent with local adaptation. Columns containing immobilized proteins from of 100 μl of grey squirrel and rabbit blood serum were also generated to examine species-specificity, and a mock column was used as a matrix control column. Species-specificity and mock control binding was analysed using 300 μl affinity matrix, without (mock) or with 100 μl of grey squirrel or rabbit sera incubated with 250 μg of CR or SB Pacific rattlesnake venom and developed in parallel to the affinity columns (Pla et al., 2017). Eluates from three technical replicates were concentrated in a vacuum centrifuge to about 100 μl, and 40 μl aliquots were fractionated by reverse-phase HPLC (Pla et al., 2017). The relative amount of captured venom proteins was estimated using the equation $\%R_i = 100 − [(NR_i/(R_i + NR_i)) × 100]$, where R_i corresponds to the area of the same protein "i" in the chromatogram of the fraction retained and eluted from the affinity column (Ainsworth et al., 2018).

Pacific rattlesnake venom proteins eluted from ground squirrel serum affinity columns were matched to congeneric venom proteins (Holding et al., 2018) using the online form of the MASCOT Server (version 2.6) against the last update (Release 234 of October 15th,
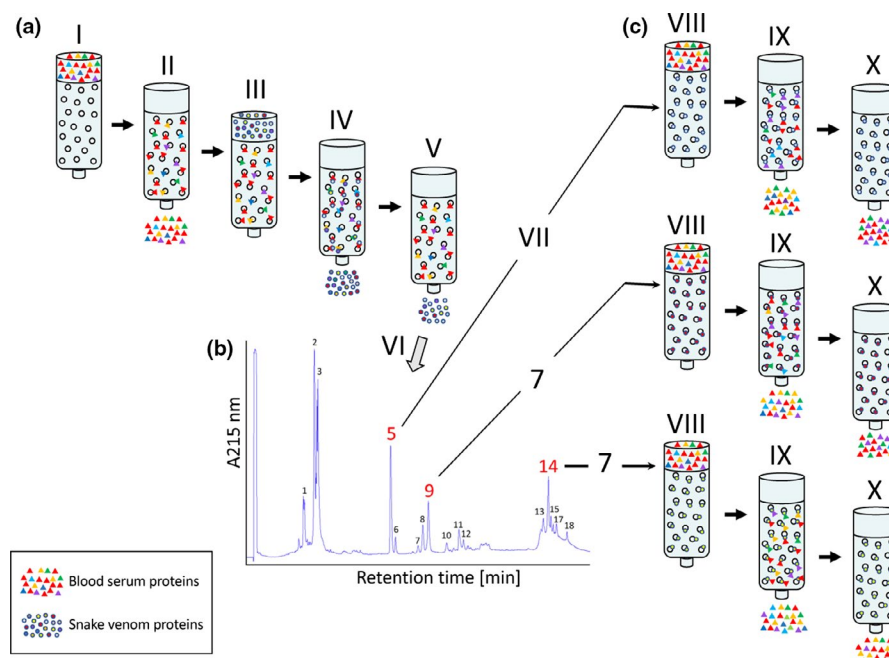

FIGURE 1 Scheme of the affinity chromatography workflow used to identify CR and SB ground squirrel blood serum proteins that interact with individual sympatric and allopatric Pacific rattlesnake venom proteins. (a) In a first step, affinity matrices were prepared, as described in detail in Pla et al. (2017), by incubating whole blood serum from SB and CR with CNBr-activated Sepharose 4B (I). After washing out the unbound serum proteins (II), the affinity columns were incubated with whole (CR or SB) rattlesnake venom proteins (III). The nonbinding proteins were washed out (IV) and the bound venom proteins eluted (V), purified by reverse-phase HPLC (VI) (b) and identified by MS/MS analysis. In a second step (c), the isolated venom proteins were used to generate toxin-specific affinity columns (VII). To this end, the venom protein-specific affinity columns were incubated with blood serum proteins from sympatric and allopatric California ground squirrels (VIII), the unbound proteins washed out (IX), and the serum proteins bound to the toxin-specific affinity columns (VIPs) were eluted (X), separated by 2DE, and identified by LC-MS/MS (see Dryad Data Depository—https://doi.org/10.5061/dryad.wm37pvmjz)

2019) of the NCBI nonredundant database by tandem mass spectrometry (MS/MS) or de novo sequencing, as described in Eichberg, Sanz, Calvete, and Pla (2015). To identify SB and CR ground squirrel blood serum proteins that bind to specific Pacific rattlesnake venom proteins, the venom proteins eluted from sympatric and allopatric ground squirrel serum affinity columns were purified by reverse-phase HPLC (Figure 1b) (Eichberg et al., 2015). Identical fractions eluted from technical replicates consisting of SVMPs, phospholipase A2s (PLA$_2$), serine proteases (SVSP) and disintegrins (DISI) were collected manually and dried in a vacuum centrifuge. The following amounts of specific venom proteins were redissolved in 100 µl of coupling buffer (0.2 M NaHCO$_3$, 0.5 M NaCl, pH 8.3): 6.4 mg SVMP13-14 (SB), 4.2 mg SVMP-10 (CR), 0.8 mg PLA$_2$-5 (SB), 1.5 mg PLA$_2$-9 (SB), 0.8 mg SVSP-10/11 (SB), 5.3 mg DISI-3/4 (SB). This material was then used to generate affinity columns (Figure 1c), as described above. Columns (350 µl) of protein-specific affinity matrices were incubated with 100 µg of total population-specific (CR, SB) ground squirrel and grey squirrel control (CON) blood serum proteins. The following combinations of venom (V)/serum (S) proteins were sampled: SVMP13-14 (SB)/CR-S; SVMP13-14 (SB)/SB-S; SVMP13-14 (SB)/CON-S; SVMP-10 (CR)/CR-S; SVMP-10 (CR)/SB-S; SVMP-10 (CR)/CON-S. Lots of three affinity columns, each containing PLA$_2$-5 (SB), PLA$_2$-9 (SB), SVSP-10/11 (SB), and DISI-3/4 (SB) baits, were incubated with SB-S, CR-S, and CON-S. The columns were then washed and eluted as described (Pla et al., 2017). Eluates of identical columns were pooled and 35 µg of blood serum proteins eluted from the protein-specific affinity columns, which we named "venom interactive proteins (VIPs)", were submitted to two-dimensional electrophoresis (2DE). The separated spots were identified by MS/MS as described in Eichberg et al. (2015).

2.3 | Rates of evolution in VIPs in mammals

Genes encoding proteins involved in co-evolution between predators and prey often show evidence of positive selection (Feldman, Brodie, Brodie, & Pfrender, 2009). To explore this possibility, we estimated evolutionary rates in homologous VIPs from other mammals with completed annotated genomes listed in the Ensembl genome browser (Zerbino et al., 2018) (species analysed are listed in Table S1). We analysed two sets of mammals: rodents, which are either reported, or based on ecology and range overlap, are likely to be in the diet of a viperid snake (M. Holding, unpublished data), and are potentially under strong selection for resistance to venom; and large bodied primates, which we assume were infrequently eaten by venomous snakes (but see Headland & Greene, 2011). We hypothesized that if positive selection was acting on prey VIPs, it would have a stronger signal in the rodents which experience heavier predation by snakes.

Except for California ground squirrel sequences, which were generated from liver transcriptomes reported in Hassinger (2020), complete protein and coding sequence databases were downloaded from the Ensembl Genome Browser (www.ensembl.org).

When genes were represented by alternative splicing isoforms, we retained only the longest gene isoforms. To identify homologous sequences across species, we used BLASTP (Altschul, Gish, Miller, Myers, & Lipman, 1990) and our selection of VIPs sequences (Table S2) as queries against each intraspecific protein database using an e-value cutoff of 1×10^{-5}. We used OrthoMCL v.1.0 (Li, Stoeckert, & Roos, 2003) to classify the resulting protein hits into gene families. The coding sequence databases and PRANK v.140603 (Löytynoja, 2014) were used to perform codon alignments to the identified putative single-copy orthologues.

We used CODEML, as implemented in PAML v.4.8 (Yang, 2007), and an input phylogenetic tree inferred from the TimeTree Web (www.timetree.org; Figure S1) to test for positive selection on the orthologous VIPs in the rodents in two ways. First, to detect episodic positive selection, we used branch-site models (null versus alternative) to determine if the rodent clade contains amino acid positions under positive selection (i.e., rate of nonsynonymous-to-synonymous substitutions (ω) > 1) that are otherwise under purifying (ω < 1) or neutral (ω = 1) selection in the primate clade. Second, to detect pervasive positive selection, we used site models (M8a versus M8) to determine if individual amino acid positions are under positive selection in the rodent clade alone. We used likelihood ratio tests followed by false discovery rate adjustments (Benjamini & Hochberg, 1995) to determine the significance of both episodic and pervasive positive selection operating on VIPs in rodents. We considered amino acid positions under positive selection if they exhibited a Bayes empirical Bayes posterior probability >.95 (Yang, Wong, & Nielsen, 2005). Sequence alignments and PAML input and output files are available in the Dryad Data Repository at https://doi.org/10.5061/dryad.wm37pvmjz

3 | RESULTS AND DISCUSSION

3.1 | Venom proteins with affinity towards mammal serum proteins and binding specificity across levels of divergence

Analyses of resistance based on the ability of serum affinity columns to bind venom proteins demonstrated a clear pattern of species-specificity in the binding patterns of ground squirrel serum proteins and rattlesnake venom proteins (Figure 2). All serum-based affinity columns bound toxin proteins found in whole Pacific rattlesnake venom, but the binding efficiency of ground squirrel-based columns was much higher than that of the grey squirrel or rabbit control serum columns (Figure 3).

We identified the toxins present in 8 and 13 scoreable protein peaks in the whole venom HPLC profiles of CR and SB venom, respectively (Table S1). These consisted of peaks with DISI venom proteins (SB, n = 2 peaks; CR, 2 peaks); cysteine-rich secretory proteins (SB, 1; CR, 1); PLA$_2$s proteins (SB, 5; CR, 2); SVSP proteins (SB, 3; CR, 2); and SVMP proteins (SB, 5; CR, 4). A comparison of the percentage of individual venom protein peaks retained (Figure 4) showed
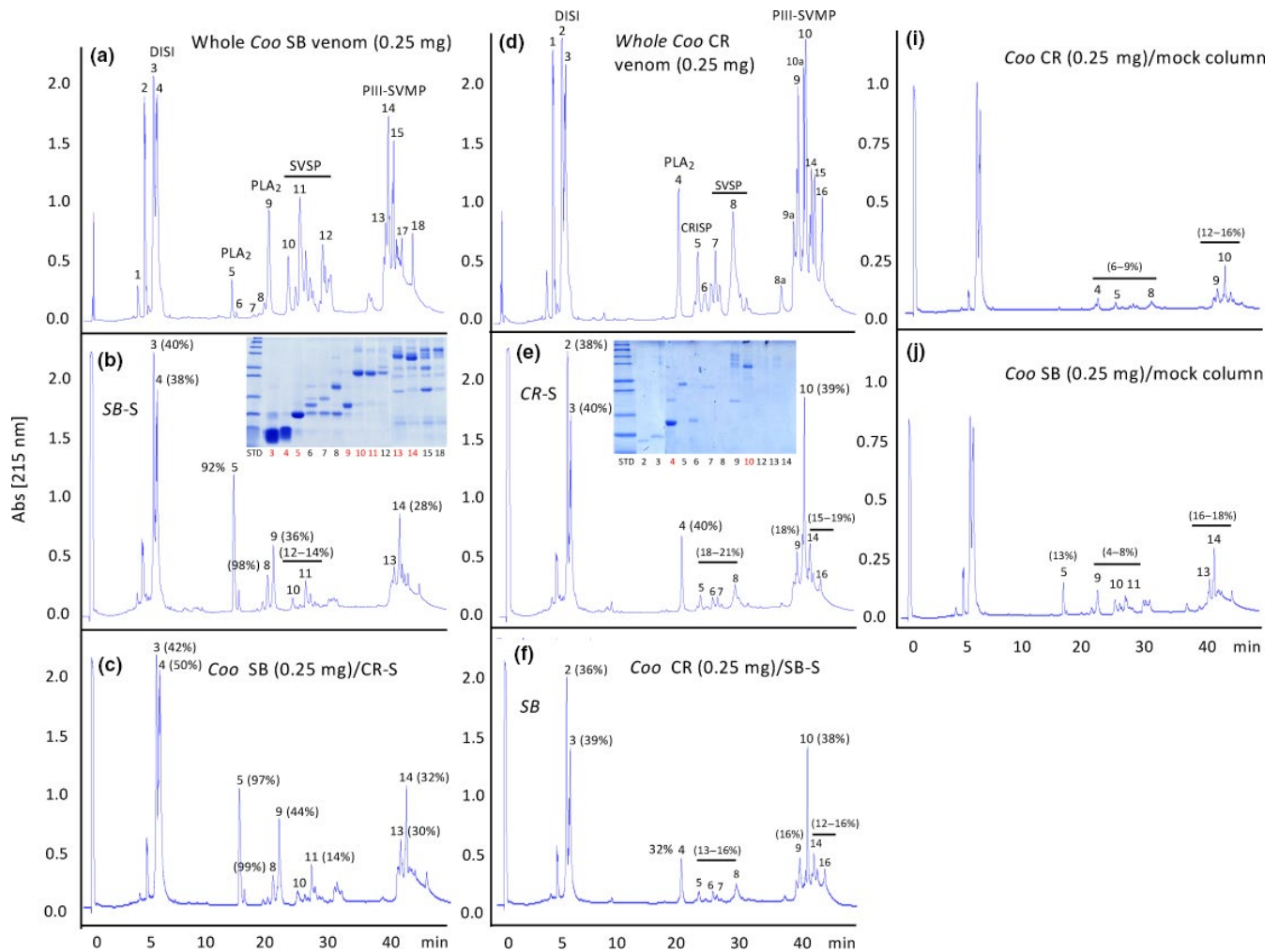
FIGURE 2 Identification of SB and CR Pacific rattlesnake venom proteins eluted from sympatric and allopatric ground squirrel serum affinity columns. Whole Pacific rattlesnake venom from Sutter Buttes (SB) (a) was subjected to affinity chromatography on immobilized blood serum proteins from SB (b) and CR (c). Similarly, whole Pacific rattlesnake venom from Chimineas Ranch (CR) (d) was subjected to affinity chromatography on immobilized blood serum proteins from CR (e) and SB (f). (i) and (ii) display specificity controls of Pacific rattlesnake CR and Pacific rattlesnake SB venoms run on mock columns. The identities of SB and CR venom proteins labelled in (a) and (d) were inferred by comparing the reverse-phase HPLC traces with those reported by Holding et al. (2018). SB and CR venom proteins with affinity for sympatric and allopatric squirrel serum were purified by reverse-phase HPLC and used to generate toxin-specific affinity matrices are highlighted in red in the SDS-PAGE gels shown in (b) and (e). Relative binding (% of total proteins incubated with the affinity matrix) of SB and CR venom proteins to sympatric and allopatric squirrel serum affinity columns are shown in parentheses

significantly higher values for each of the ground squirrel columns compared to the control grey squirrel and rabbit profiles. More CR venom was retained on the CR ground squirrel (mean across all peaks = 22.2%) column than on the grey squirrel (12.5%) or rabbit (13.5%) columns (paired Mann-Whitney U tests, both $p < .0027$). This result was replicated with SR venom, where more protein was retained on the SR ground squirrel column (31%) than on the grey squirrel (4.4%) or rabbit (2.7%) columns (paired Mann-Whitney U tests, both $p \leq .0087$). In contrast, there was no significant difference in the binding capacity between the grey squirrel or rabbit columns for each of the Pacific rattlesnake CR and SB venom experiments (both $p > .5$).

The lack of a difference in protein retention between grey squirrel and rabbit columns suggests that binding specificity between venom and serum rapidly decreases to baseline "nonspecific" levels whether or not prey are subject to predation by other venomous snakes. Grey squirrels are heavily preyed upon by a related rattlesnake (*C. horridus*) and are resistant to its venom while showing reduced resistance to Pacific rattlesnake venom (Pomento et al., 2016). In contrast, the wild ancestors of rabbits only occurred in the presence of European members of the genus *Vipera*, which do not feed on rabbits due to their small size (Street, 1979) yet both show similar low levels of serum binding to Pacific rattlesnake venom.

The strength of ground squirrel serum-to-venom binding appears to be toxin-specific (Figure 2). Among both the homologous and heterologous affinity interactions, DISI, PLA$_2$s, and some PIII-SVMPs exhibited the highest binding capacity. For example, SB and CR squirrel serum protein affinity columns showed capture efficacies of
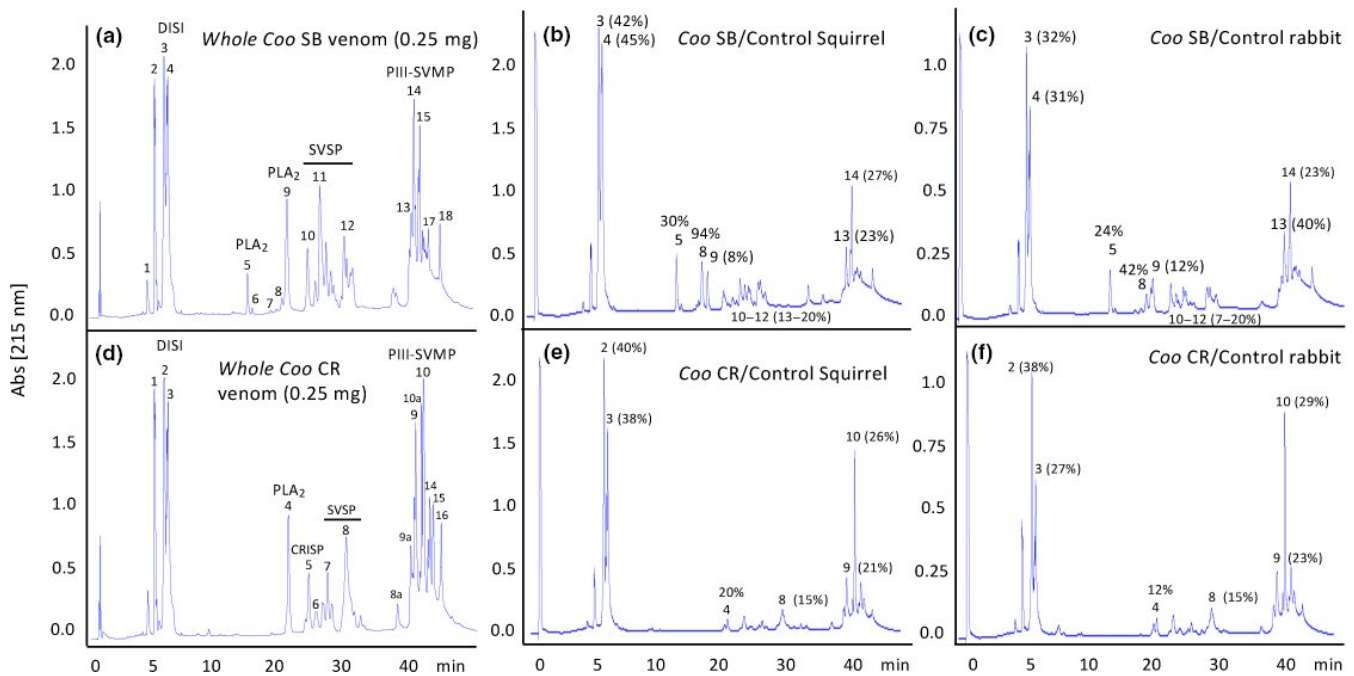
FIGURE 3 Identification of SB and CR Pacific rattlesnake venom proteins eluted from control grey squirrel and rabbit serum affinity columns. Whole rattlesnake venom from Sutter Buttes (SB) (a) and Chimineas Ranch (CR) (d) were subjected to affinity chromatography on immobilized blood serum proteins from control grey squirrel (b, e) and rabbit (c, f), respectively. Relative binding (% of total proteins incubated with the affinity matrix) of SB and CR venom proteins to sympatric and allopatric squirrel serum affinity columns are shown in parentheses

28%–44% (SB PIII-SVMP-13/14; CR PIII-SVMP-10; SB PLA$_2$-9; and CR PLA$_2$-4), 36%–50% (SB and CR disintegrins) and 92%–99% (SB PLA$_2$-5 and SB PLA$_2$-8) of the total toxin contained in the venom sample applied to affinity column (Table 1, Figure 2). Nonspecific binding to mock matrix ranged 12%–18% for PIII-SVMPs and 8%–13% for PLA$_2$s (Figure 2). On the other hand, 12%–14% of SVSPs SB-10 and 11, and 13%–21% of SVSPs CR-7 and 8 were captured in the affinity columns, with 4%–9% corresponding to nonspecific binding to mock matrix (Figure 2).

Finally, interactions between prey serums and predator venoms also showed population-specificity consistent with the hypothesis of local adaptation. Based on individual venom peak data (summarized in Figure 4), CR serum columns bind local (CR) venom proteins (mean percentage of peak bound = 22%) significantly more than foreign SB venom (17%; paired Mann-Whitney U, $p = .036$). Furthermore, SB serum bound more local SB venom (31%) than foreign CR venom (28.8%), but this difference was nonsignificant (paired Mann-Whitney U, $p = .567$). SB serum had high overall binding effectiveness but lacked a clear signal for population-specificity, whereas CR serum had lower binding efficiency but was more effective at binding local venom components.

Higher binding levels are assumed to be beneficial to the squirrel because of the positive relationship between overall ground squirrel serum-to-venom binding levels and resistance to venom injection (Poran et al., 1987), and therefore these binding values suggest ground squirrel local adaptation to rattlesnake venoms. Squirrel local adaptation appears to contrast with the enyzmatic serum inhibition analyses of Holding, Biardi, et al. (2016), who

documented rattlesnake local adaptation through the avoidance of SVMP inhibitors in squirrel serum. However, among PLA$_2$, SVSP, and SVMP binding levels in Figure 4, there is a clear departure from a signal of local adaptation for SVMP results in that CR serum was best at binding both CR and SB SVMPs. However, these results do not necessarily directly conflict with those of Holding, Biardi, et al. (2016) involving SVMPs. Instead, the current and previous studies suggest the possibility that rattlesnakes may have locally-adapted SVMPs while ground squirrels have locally adapted to SVSP and PLA$_2$ inhibition, raising the possibility that predator and prey have their highest adaptive potential along different dimensions of their molecular interactions. Future work that isolates the pairs of bound venom and serum proteins identified here will be required to determine whether the bound serum fractions eluting with PLA$_2$ and SVSP are venom inhibitors or venom targets, and thus confirm whether predator or prey are showing local adaptation regarding SVSP and PLA$_2$ function.

Since rabbit serum also captured some portions of all venom toxin classes tested (Figure 3), the results also suggest that the ground squirrel serum VIPs targeting disintegrins, SVSPs, and PIII-SVMPs may have originated before the divergence of Rodentia and Lagomorpha close to the Cretaceous-Paleogene boundary (Asher et al., 2005), approximately 66 million years ago (Renne et al., 2013). VIPs targeting major Pacific rattlesnake venom PLA$_2$ molecules (SB PLA$_2$-5, SB PLA$_2$-9, CR PLA$_2$-4) may have evolved in ground squirrels more recently, presumably through co-evolution between the squirrel and its main predator, extant Pacific rattlesnakes or an ancestor, during the last 3–6 million years ago (Alencar et al., 2016).
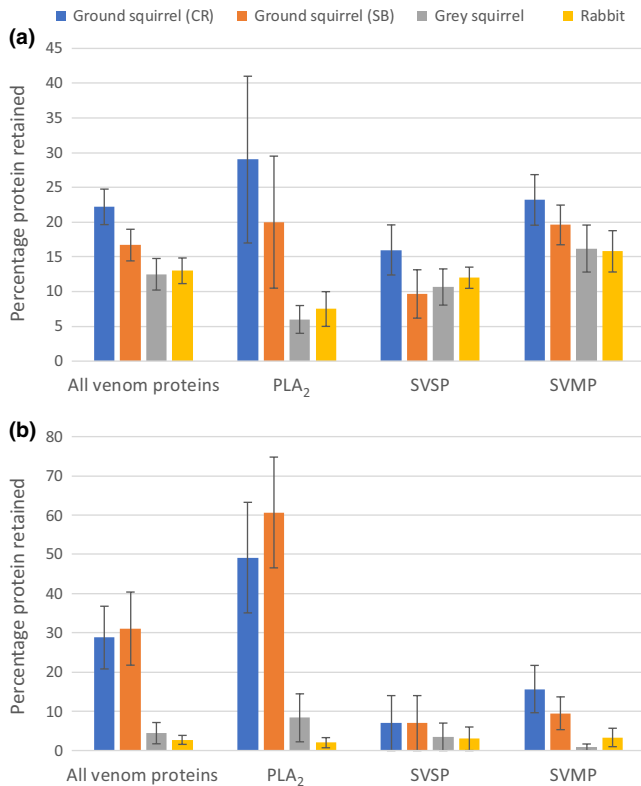
(a)

(b)

FIGURE 4 Retention values for Pacific rattlesnake venom proteins binding to ground squirrel and control serum protein affinity columns. Results based on a pooled sample of rattlesnake venom from (a) Chimineas Ranch (CR); and (b) Sutter Buttes (SB) individuals. Retentions values presented for whole venom ("All venom proteins") and specific major venom proteins (PLA$_2$, SVSP, and SVMP). Data are mean retention of all members of the focal protein class ± 1 *SE*

We recognize that our method does not evaluate the fine specificity of the interactions between the VIP proteins and specific venom proteins at the molecular level (e.g., see Bastos, Gomes-Neto, Perales, Neves-Ferreira, & Valente, 2016 for review), which is required to demonstrate that a given protein impedes venom function. Rather, our approach detects proteins that show a high degree of binding specificity to venom toxins, which is a required property of resistance proteins that act as inhibitors, and hence a reasonable criterion to identify candidates. However, we recognize that affinity binding could also identify prey proteins targeted by venom components and consider this possibility when discussing the potential function of individual serum proteins identified through proteomic analyses below.

3.2 | Identification of SB and CR ground squirrel serum proteins captured in toxin-specific affinity columns

We used affinity chromatography on toxin-specific (SB PIII-SVMP-13/14; CR PIII-SVMP-10; SB SVSP-10/11; SB DISI-3/4; SB

PLA$_2$-5; and SB PLA$_2$-9) matrices to capture candidate SB and CR California ground squirrel serum VIPs. The affinity-retained proteins were separated by 2DE and identified by LC-MS/MS analysis. 2DE gels and their linked MS data are available in the Dryad Data Repository at https://doi.org/10.5061/dryad.wm37pvmjz as Proteomics Data Summary tables 1–11. Multiple proteins were recovered from each of the affinity matrices used, and many of the proteins captured in the same column were found in multiple 2DE spots, suggesting the possible presence of multiple VIP iso- or proteoforms (sensu Smith & Kelleher, 2013; Jungblut, Thiede, & Schlüter, 2016) within a given experiment. However, because the peptide sequences used to match databank entries represent only a small fraction of the entire parent protein sequence in most cases it was unclear whether the different peptide sequences gathered in the same 2DE spot belonged to iso- or proteoforms or represented different parts of the same protein and so these were grouped together.

The overall binding capacity of different toxin columns varied suggesting substantial differences in the degree that serum proteins bound to specific venom proteins (Table 1). Four of the six toxin-specific columns (SB SVMP 13/14. SB DISI ¾, SB PLA$_2$-5 and SB PLA$_2$-9) bound a similar percentage (~20%) of the 342 2DE protein spots identified (ground squirrel and grey squirrel combined). In contrast, the two other toxin-specific columns (CR SVMP 10 and SB SVSP 10/11) showed weaker affinity for serum proteins binding three-fold fewer of all proteins (~6% total 2DE spots for each). These patterns are similar when binding patterns for ground squirrel only proteins are compared. The differences in the binding capacity of the two SVMP columns demonstrates that venom protein class alone is not a good predictor of the degree to which venom proteins interact with these blood serum proteins. This is not surprising in light of the wide diversity of SVMP paralogues that can be present within a single rattlesnake species (Giorgianni et al., 2020).

Resistance proteins may be represented by the most abundant among the VIPs captured in the different toxin-specific affinity columns. To provide a broad semi-quantitative measure of protein abundance we scored the number of spots assigned to iso- or proteoforms of the same protein class, and used this measure in comparisons between affinity column experiments. Inspection of the data summarized in Table 1 showed that the number of specific serum protein 2DE spots identified in the different experiments is highly variable and show several patterns that are informative about the dynamics of binding between ground squirrel VIPs and venom.

First, each subset of ground squirrel VIPs recovered from the columns containing SVMP, SVSP, DISI, PLA$_2$-5 and PLA$_2$-9 baits exhibit distinct features (Table 1). For example, many of the candidate SB and CR VIPs recovered from the SB SVMP-13/14 affinity columns were also found, albeit in different numbers of spots, in the eluate of columns incubated with control grey squirrel serum. Major exceptions were kallikrein, α2-macroglobulin, and hibernation-associated proteins HP-20 and HP27 (exclusively recovered from ground squirrel serum from SB); hibernation-associated proteins HP-25 (found in both SB and CR ground squirrel serum) and clusterin (apolipoprotein J), inter-α-trypsin inhibitor H3, complement C4, haptoglobin,

TABLE 1 Number of 2DE spots identified in the serum protein fraction bound to the different toxin-specific affinity columns

Affinity matrix	SB SVMP-13/14			CR SVMP-10			SB SVSP-10/11			SB DISI-3/4			SB PLA2-5			SB PLA2-9		
Serum	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel
Capture effiiciency (%)	28	32	27	28	39	26	12–14	14	13–20	38–40	38–40	38–45	92	97	30	36	44	9
α1-antitrypsin	3	6	1	1	2			1					1	2	1	4	4	
Kallikrein	4			2														
Antithrombin-III	3	3	3		1						3		1	6	1	4	4	
α2-macroglobulin	4	2			1			1			1		1			2	3	
Serum albumin-like	2	2	1		1			2	2		1		2	1		2	2	5
Vitamin D-binding protein	1	1	1								1		2					
Hibernation-associated HP-20	1																	
Hibernation-associated HP-25	2	1				1											1	
Hibernation-associated HP-27	1																	
Apolipoprotein A-I	3	3	3	1	3		1	1	1	1	12	2	15	6	6	6	6	1
Apolipoprotein A-II													1					
Apolipoprotein A-IV		1	2										2				1	
Apolipoprotein J/Clusterin		4			1								1					
Apolipoprotein M																1		
α2-HS-glycoprotein		1	1															
Inter-α-trypsin inhibitor H3		1			1						1						1	
Inter-α-trypsin inhibitor H4		2		1	1						1		1				4	
Inter-α-trypsin inhibitor H1																	1	
Immunoglobulins			5	1				4	2	3	6	11	9	7	3	1	7	

(Continues)

TABLE 1 (Continued)

Affinity matrix	SB SVMP-13/14			CR SVMP-10			SB SVSP-10/11			SB DISI-3/4			SB PLA2-5			SB PLA2-9		
Serum	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel
α-1B glycoprotein							1	2									1	
Complement C1					1								1					
Complement C3											7	2					2	
Complement C4		1								1	3	1	1					
Complement C5											1					1	1	
Complement C6																1		
Complement C9											1		1					
Complement factor B											1		1				4	
Coagulation F IX										1					1			
Coagulation FX																		
Prothrombin										1								
Haptoglobin		1	1							1	3		3				1	
Paraoxonase/aryl estearase		1	3			1				1			1					
Transferrin								1					2	2			1	
Serotransferrin													2					
Hemopexin										1	1							
Transthyretin													1					
Vitamin K-dependent protein C													2					
Serum amiloid A4													1					
CD5 antigen-like										1	2		1					
Retinol-binding protein-4																	1	
Gelsolin																	1	
Vitronectin					1			1					1	1				
Glutathione-S-transferase							1											
Actin											1							

(Continues)

TABLE 1 (Continued)

Affinity matrix	SB SVMP-13/14			CR SVMP-10			SB SVSP-10/11			SB DISI-3/4			SB PLA2-5			SB PLA2-9		
Serum	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel	SB	CR	Grey squirrel
Total captured proteins	24	25	22	6	15	0	2	13	5	11	45	15	53	25	11	18	46	6
% of total protein spots	7.0	7.3	6.4	1.7	4.4	0	0.6	3.8	1.4	3.2	13.1	4.4	15.5	7.3	3.2	5.2	13.4	1.7

and paraoxonase/arylestearase, which were only captured from CR ground squirrel serum. On the other hand, inter-α-trypsin inhibitor H4 and immunoglobulins were uniquely found in the SB SVMP-13/14 affinity isolated proteins from grey squirrel serum. Of special note was the detection of inter-α-trypsin inhibitor H3 and hibernation-associated proteins as these are the two resistance factors previously identified from fractionated serum and shown to inhibit SVMP enzymatic activity (Biardi et al., 2011). Our capture of these proteins using SVMP baits confirms affinity chromatography as a way to isolate resistance factors, and supports the hypothesis of Biardi et al. (2011) that the inhibitory action of inter-α-trypsin inhibitor and hibernation-associated proteins occurs through binding to SVMPs.

For SVSP-based columns, ground squirrel (from CR squirrels) but not control grey squirrel VIPs α1-antitrypsin, α2-macroglobulin, α1B-glycoprotein and paraoxonase/aryl estearase bound to SB SVSP-10/11. Both ground squirrel and control grey squirrel serum shared two VIPs represented in two 2DE spots: albumin-like and immunoglobulins present in four (CR) versus 2 (control grey squirrel) spots.

With respect to DISI venom columns, with the exception of immunoglobulins from control grey squirrel serum, which interacted more efficiently with the SB DISI-3/4 affinity matrix, all the other 14 VIP classes identified in serum of CR ground squirrel showed higher binding effectiveness towards SB DISI-3/4 than either SB ground squirrel or control grey squirrel serum. A distinct feature of the SB DISI-3/4 affinity matrix was the capture of a number of complement proteins captured exclusively (C3, C5, C9) or more efficiently (C4) from CR ground squirrel serum than from SB ground squirrel or grey squirrel control serum.

Finally, for experiments involving immobilized SB PLA$_2$s, there was a higher number and diversity of SB VIPs bound to the PLA$_2$-5 affinity matrix than for CR serum, while more and greater variety of CR serum proteins were captured in the PLA$_2$-9 affinity column (Table 1). Major SB and CR ground squirrel serum proteins retained in the PLA$_2$-5 and PLA$_2$-9 affinity columns varied both in qualitative and quantitative terms. Thus, major SB VIPs apolipoprotein A1, immunoglobulins, and haptoglobin were represented in the SB PLA$_2$-5 eluates by 15, nine and three spots, respectively, whereas in CR and control grey squirrel sera these VIPs were found, respectively, in (6, 6), (7, 3), and (0, 0) spots. Conversely, six antithrombin III iso or proteoforms were captured from CR ground squirrel serum, but only one antithrombin III spot was retained from SB and control grey squirrel sera. Eluates from the SB-PLA$_2$-9 affinity column contained two population (CR)-specific VIPs, α1-antitrypsin, complement factor B, each represented by four spots; and VIPs found in both (CR and SB), sera in equal (apolipoprotein A1, 6 spots; antithrombin III, four spots) or different (immunoglobulins, SB [1] versus CR [7]) number of spots (Table 1); the control grey squirrel serum fraction eluted from the SB PLA$_2$-9 column was enriched in albumin-like protein (five spots compared to two spots in both SB and CR sera), and depleted in ApoA1 (one spot versus six spots in both SB and CR sera) (Table 1).

In summary, two patterns provide evidence for a generalized tissue-wide resistance response with one-to-many mapping of ground squirrel serum proteins to snake venom proteins: First, single VIPs bind to multiple snake venom protein classes and, second, multiple VIPs bind to a single venom protein. When overlap in the binding of single VIPs across the three major classes of venom proteins that capture most VIPs (PLA_2, MP, and DISI) is characterized, almost a quarter (24%) of all identified VIPs bind to all three venom protein classes while 24% of all VIPs bind to >2 venom proteins (Figure 5). Likewise, multiple VIPs bind to single venom columns. For example, 9–23 VIPs bind to single venom protein columns (Mean $\pm$ SD: 17.0 $\pm$ 5.02) (Table 1). Overall, these results suggest that the squirrel serum resistome is broad-based and involves a complex molecular interface where multiple serum proteins interact with multiple venom proteins.

Reciprocal assessments of the binding dynamics of CR and SB ground squirrel serum to CR and SB SVMP columns also demonstrate complex population-specific patterns of VIP-venom interactions (Table 2). Broadly similar sets of VIP proteins bind to the same "local" and "foreign" SVMP proteins, while there were a small number of VIPs that were unique to sera from specific populations. Binding patterns of SB and CR ground squirrel sera suggest that that the CR sera shows population specificity. With CR venom, the local CR serum yield more bound spots (0.76 spots) than SB serum (0.24 spots; paired Mann-Whitney U test; p = .02). Meanwhile, there was no significant difference in the number of spots bound to SB venom between SB serum (1.1 spots) and CR serum (1.29 spots, paired Mann-Whitney U test; p = .42).

We emphasize that our characterization of proteins captured in toxin-specific affinity columns independently identified VIPs previously reported as proteins involved in resistance to venom in other mammals, confirming our method's utility in the identification of venom resistance factors. As mentioned above hibernation-associated proteins H25 and H27 and the type II
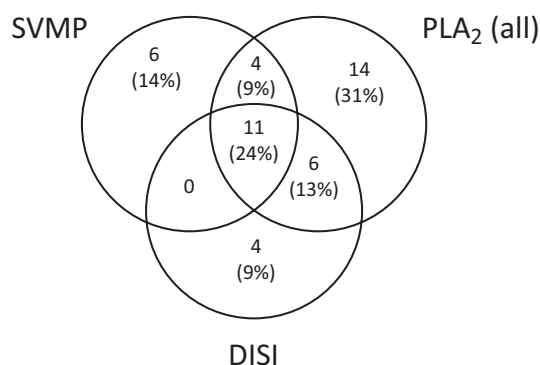
acute-phase protein Inter-α-trypsin inhibitor-H4 were described as part of the blood-based defences of California ground squirrel that reduces the metalloproteinase activity of Pacific and prairie rattlesnake (*C. v. viridis*) (Biardi et al., 2011). In addition, α-1B glycoprotein identified in SB and CR ground squirrel sera (Table 1) has 50% amino acid sequence identity with Oprin, a protein of the immunoglobulin supergene family isolated from serum of the opossum *D. virginiana* as a metalloproteinase inhibitor (Catanese & Kress, 1992), and 41% sequence identity with the venom metalloproteinase inhibitor DM43 characterized in *D. marsupialis* serum

TABLE 2 Binding of population-specific VIPs to population-specific SVMP columns

VIP Name	CR-S on CR-V	CR-S on SB-V	SB-S on SB-V	SB-S on CR-V
α-1-antitrypsin	2	6	3	1
Kallikrein	0	0	4	2
Antithrombin-III	1	3	3	0
α-2-macroglobulin-like	1	0	4	0
Serum albumin-like	1	2	2	0
Vitamin D binding protein	0	1	1	0
Hibernation-associated HP-20	0	0	1	0
Hibernation-associated HP-25	1	1	2	0
Hibernation-associated HP-27	1	0	0	0
Apolipoprotein A-I	3	3	3	1
Apolipoprotein A-IV	0	1	0	0
Apolipoprotein J/ Clusterin	1	4	0	0
α2-HS-glycoprotein	0	1	0	0
Inter-α-trypsin inhibitor H3	1	1	0	0
Interalpha-trypsin inhibitor H4	1	1	0	0
Immunoglobulins	0	0	0	1
Complement C1	1	0	0	0
Complement C4	0	1	0	0
Haptoglobin	0	1	0	0
Paraoxonase/amyl esterase	1	1	0	0
Vitronectin	1	0	0	0
Total	16	27	23	5
Mean/VIP	0.76	1.29	1.10	0.24
Sd	0.77	1.55	1.48	0.54

Note: CR-S and SB-S: ground squirrel serum samples from Chimineas Ranch and Sutter Buttes squirrels; CR-V and SB-V: Pacific rattlesnake SVMP columns generated using venom samples from CR and SB, respectively.

FIGURE 5 Numbers of specific VIP proteins that bind to different venom proteins. A specific protein was scored as binding to a specific venom protein if there were one or more identified spots representing that serum protein binding to a specific rattlesnake venom affinity column as shown in Table 1. SVMP, snake venom metalloproteinase; PLA_2, phospholipase A2; DISI, disintegrins

(Neves-Ferreira et al., 2002). The presence of oprin/DM43 in opossum sera may partially account for the resistance of these marsupials to the haemorrhagic effects of rattlesnake envenomation caused by SVMPs. Toxin-neutralizing serum proteins discovered so far in both opossums and mongooses are human α1B-glycoprotein homologues that inhibit either SVMPs (DM43) or PLA$_2$ myotoxins (DM64) (Bastos et al., 2016; Rocha et al., 2017; Voss & Jansa, 2012). Finally, alpha2-macroglobulin was previously isolated as the primary serum "antihaemmorhagic factor" conferring resistance to venom in the European hedgehog (*Erinaceus europaeous*, de Wit & Weström, 1987). These results validate the use of affinity chromatography techniques for the large-scale identification of resistance proteins from blood serum. On the other hand, the presence of a conserved class of SVMP inhibitors in sera of squirrel (Eutherian) and opossum (Marsupial) is consistent with convergent evolution of resistance of venom-induced haemorrhage and tissue damage in taxa which had a last common ancestor (*Juramania sinensis*) in the Jurassic, about 160 million years ago (Luo, Yuan, Meng, & Ji, 2011).

3.3 | Venom resistance serum proteins: Exaptation from host survival systems?

A number of candidate VIPs have been associated with the innate immune system, as has been noted previously (Arbuckle et al., 2017; Holding, Drabeck, et al., 2016). All animals and plants have innate immune systems that protect them from a diversity of pathogens by inactivating the pathogens themselves or promoting the inactivation and clearance of toxic products produced by the pathogens. Expected characteristics of a prey's defence mechanism against envenomation by a snake predator share functional features present in innate immunity: it is a generally nonspecific mechanism that does not require previous exposure, must be very rapid in response if the prey is to survive, is critical for the recognition of disease-causing agents, and is a major contributor to acute inflammation induced by microbial infection or tissue damage (Le Morvan, Troutaud, & Deschaux, 1998). It is also becoming increasingly clear that coagulation and innate immunity have co-evolved as an ancient survival strategy. The linkage between coagulation and inflammation can be traced back to the earliest events in eukaryotic evolution before the separation of plants and invertebrate animals (Opal & Esmon, 2003). At present these systems continue to function as a highly integrated unit for defence following tissue injury. As outlined below many of the major squirrel serum proteins identified as candidate VIPs have been reported to play a role in (innate) immunity and/or blood coagulation. Many have known functions that may facilitate their co-option in venom resistance (e.g., immunoglobulins) while a small number of others (e.g., coagulation factors) have functions that are easier to reconcile as potential targets of the toxic activity of venoms or as "sticky" proteins that incidentally bind to toxin-based columns (e.g., serum albumin-like proteins). We discuss the major classes of captured proteins in terms of their plausible functions below.

3.4 | Immunoglobulins

Immunoglobulins (Ig) are a diverse gene superfamily that are crucial in both innate and adaptive immunity (Marchalonis, Schluter, Bernstein, Shen, & Edmundson, 1998). In mice, IgE-dependent acquired immunity can increase the resistance of mice to exposure to the venom of viperied snakes (Starkl et al., 2016), and the historical basis for antivenom production is based on harvesting the Ig fraction from various mammal taxa previously inoculated with snake venom (Espino-Solis, Riaño-Umbarila, Becerril, & Possani, 2009; León et al., 2018). Hence, it is unsurprising that Ig family proteins were found bound to all columns and classes of venom tested herein (Table 1). As the ground squirrel and grey squirrel serum samples were all obtained from wild caught squirrels, we do not know whether these animals may have previously survived a bite, or whether Ig-mediated resistance can occur at first injection of venom in these species. But naïve rabbit Ig fractions are capable of venom inhibition (Souza et al., 2001), so both innate and acquired Ig-based venom resistance are probably present. A comparison of Ig affinity capture in wild caught and captive raised members of the same resistant species would be beneficial for highlighting the degree to which previous interactions with venomous snake predators influence venom specific Ig fractions in the serum of resistant prey.

3.5 | Serum albumin-like proteins

Serum albumin, the most abundant protein in serum (Fanali et al., 2012), and vitamin D-binding protein are both members of the albumin gene family. Their function as carrier proteins with both binding capacity and macrophage-activating activity make them potential resistance proteins (Yamamoto, Kumashiro, Yamamoto, Willett, & Lindsay, 1993). However, the promiscuity of their binding capabilities suggests they may be generally "sticky" proteins. In support of nonspecific capture of venom proteins, serum albumin-like proteins bound to every column and venom protein class we have tested herein. However, their potential role in venom resistance merits discussion because previous work showed that serum albumins can inhibit the lethal activity of rattlesnake venom (Clark & Voris, 1969). Future work comparing the inhibitory capacity of albumins across taxa may be worthwhile to elucidate whether and how a common protein with a "housekeeping" function might be co-opted as a means of protection against envenomation.

3.6 | Apolipoproteins

Apolipoproteins A-I, and to a minor extent A-II, A-IV, A-J, and A-M, are abundant serum proteins captured from SB and CR Pacific rattlesnake serum and ground squirrel serum in all (A-I) or particular venom toxin-specific affinity columns (Table 1). Apolipoproteins play an important role in innate immunity and produce inflammatory responses. Among all apolipoproteins, antimicrobial and

immunomodulatory roles of apolipoprotein A-I and A-II have been extensively studied in different fish (Sahoo, Mohapatra, & Jena, 2017). These apolipoproteins display bactericidal activity in vitro and are potentially important effectors of innate immunity in the teleost fish *Cyprinus carpio* (Concha et al., 2004) mediated by innate pattern recognition receptors (PRRs), including serum amyloid A, C-reactive protein, CD5 antigen-like, and mannan-binding lectin (Takeuchi & Akira, 2010). Serum amiloid A4 was found in the eluate of SB serum captured in SB-PLA$_2$-5 affinity column but not among the CR or ground squirrel VIPs; and CD5 antigen-like protein was recovered in the SB-DISI-3/4 eluates of ground squirrel SB and CR sera, and in the SB ground squirrel serum fraction bound to sympatric SB-PLA$_2$-5 affinity column (Table 1). Neither serum amiloid A4 nor CD5 antigen-like proteins were recovered from ground squirrel serum. Based on their previously established roles in both the direct inhibition of pathogens and the indirect destruction of foreign agents by flagging them for lysozyme (Concha et al., 2004), apolipoproteins merit additional research as potential inhibitors of venom.

3.7 | Complement proteins

Complement proteins C1, C3, C4, C5, C6, C9 and complement Factor B, a critical protease for complement activation, were captured in toxin-specific affinity columns incubated with both SB and CR Ground squirrel serum, but were notably absent from control grey squirrel serum (Table 1). The serum complement system (Müller-Eberhard, 1988) is an ancient mechanism of innate defence that includes opsonization of foreign agents, making them targets for phagocytosis and clearing by leucocytes (Ricklin, Hajishegallis, Yang, & Lambris, 2010). It is therefore possible that complement affinity for venom mediates leucocyte recruitment and subsequent clearing of venom.

3.8 | Alpha1-antitrypsin

Serpins function in the innate immune system as protease inhibitors, limiting damage caused by proteases at extracellular sites of inflammation. The expression in serum of serpin A1 (α1-antitrypsin; AAT), a potent extracellular inhibitor of the serine protease neutrophil elastase (Mangan, Kaiserman, & Bird, 2008), can be induced four- to six-fold (Guttman et al., 2015), and its roles extend beyond its serine protease inhibitory activity. ATT inhibits matrix metallopeptidase 9 (MMP-9) and membrane-associated ADAM metallopeptidase domain 17 (ADAM17) (Guttman et al., 2015), a close relative of PIII-SVMPs in snakes. ATT was identified in all the Pacific rattlesnake toxin-specific affinity matrices, except the SB DISI-3/4 column (Table 1). The fact that only one out of 21 2DE spots corresponded to ATT, indicated high (95%) binding specificity of ATT from SB and CR ground squirrel sera to sympatric and allopatric toxin-specific affinity columns, including SB-SVMP-10/14, CR-SVMP-10, and SB PLA$_2$-5 columns. Moreover, the binding of ATT to SB- PLA$_2$-9 and

SB SVSP-10/11 showed population (CR) specificity. This degree of species and population specificity in the binding of AAT to venom proteins, alongside evidence for positive selection on AAT in rodents (see below) make it a strong candidate for involvement in local co-evolution with rattlesnakes, and call for future work on the functional consequences of ATT binding to each of these protein classes.

3.9 | Alpha2-macroglobulin

Alpha2-macroglobulin represents an evolutionarily conserved arm of the innate immune system that functions by trapping and inhibiting a broad range of pathogen proteases (Armstrong & Quigley, 1999; Wong & Dessen, 2014), suggesting it could also play a role in venom protease capture. Indeed, the primary antihaemorrhagic factor in hedgehog serum was shown to be alpha2-macroglobulin (de Wit & Weström, 1987). Alpha2-macroglobulin from SB and CR ground squirrel sera bound to sympatric (SVMP and PLA$_2$) and allopatric (SVSP, DISI) toxin-specific affinity columns, suggesting it may play a role as a serum inhibitor in ground squirrels as well. Interestingly, despite its apparent recruitment in venom inhibition in distantly related hedgehogs and ground squirrels, it was not found in the eluates from control grey squirrel serum (Table 1). Lack of alpha2-macroglobulin bound to venom in the grey squirrel suggests that it is convergently recruited in resistance in only some taxa that interact regularly with venomous snakes.

3.10 | Haptoglobin, haemopexin, and transferrin

Haptoglobin (Hp) is the major haemoglobin-binding protein in the plasma of most vertebrates and all mammals (Wicher & Fries, 2006), whereas haemopexin binds free haeme in solution (Tolosano & Altruda, 2002). Transferrin is involved in conserved dual functions in vertebrates: the innate immune response to bacterial infection and iron metabolism. Iron sequestration is an ancient host defence mechanism against invading pathogens (Fearson & Locksley, 1996). Although a potential function of these proteins in venom resistance is difficult to speculate, iron sequestration is known to impact matrix metalloproteinase activity in humans (Zamboni et al., 2005). The iron-binding domains of these proteins may make them "sticky" to other protein types, resulting in incidental binding to venom proteins.

3.11 | Inter-α-trypsin inhibitor and hibernation-associated proteins

We discuss these two proteins (Bost, Diarra-Mehrpour, & Martin, 1998; Takamatsu, Ohba, Kondo, Kondo, & Shiba, 1993) together, as Biardi et al. (2011) provided evidence that they interact to provide ground squirrels with resistance to SVMPs. Inter-α-trypsin inhibitors (IαTI) but not hibernation-associated proteins (HP) inhibited SVMP activity directly, and Biardi et al. (2011) suggested that

HPs H25 and H27 might chaperone IαTI to bind and inhibit SVMPs. H25 and H27 have collagen-like domains (Takamatsu et al., 1993), which presumably form baits for SVMPs, as collagenase activity represents the ancestral state for SVMPs (Bernardoni et al., 2014). HPs (H20, H25 and H27) were found in the eluates of SB SVMP-13/14 affinity matrix incubated with SB and CR ground squirrel sera (Table 1), and H25 was, in addition, recovered in the eluates of CR SVMP-10 and SB PLA$_2$-9 column incubated with sympatric ground squirrel sera (Table 1). None of the HPs from grey squirrel serum were captured in any of the Pacific rattlesnake toxin-specific affinity columns tested, suggesting mediation of venom inhibition by this class of proteins may have evolved uniquely in ground squirrels (Table 1). H25 and H27 were found in conjunction with IαTI proteins in the CR serum fractions bound SB SVMP-13/14, but not in SB serum, where the HP proteins were found alone. Meanwhile, IαTI proteins were found when no HP proteins were bound for CR serum bound to SB DISI-3/4 and SB serum bound to CR SVMP-10, as well as in grey squirrel serum bound to SB PLA$_2$-5 and SB SVMP 13/14. The molecular complexity yielded by multiple possible IαTI/HP combinations should be investigated as a potential source of rapid local adaptation in venom toxicity and resistance in this system (Holding, Biardi, et al., 2016).

3.12 | Coagulations factors – venom targets?

Viperid snake SVSPs and SVMPs alter haemostasis in envenomated prey through enzymatic activation or deactivation of the targeted coagulation factors (reviewed in Serrano, 2013). Several identified VIPs are central components in the coagulation cascade, including vitamin K-dependent protein C, coagulation factor FIX, coagulation factor FX, prothrombin, kallikrein, and antithrombin III. Each of these proteins are known to be either mimicked or targeted by an

SVMP or SVSP (Bernardoni et al., 2014; Serrano, 2013). Binding of kallikrein and antithrombin-III to the CR SVMP-13/14 and SB SVMP-10 columns could be based on the affinity of these SVMP enzymes for their substrates. None of these coagulation factors were bound to the SVSP column, and the SB DISI-3/4 and SB PLA$_2$-5 columns captured protein C, factors FIX and FX, and prothrombin. Clearly, the possible dual function of coagulation factors as resistance proteins and key potential targets in the complex interface of venom and prey interactions, merits further investigation. Capture of these coagulation factors best demonstrates the potential for affinity capture to identify not only venom inhibitors, but also venom targets, which would provide a novel methodological approach to quantify the complex nature of in vivo venom function.

3.13 | Rates of evolution in VIPs in mammals

Our selection analyses show no evidence for episodic positive selection in rodent VIPs relative to homologous primate VIPs (Table 3). Gene-wide dN/dS values for VIPs in both types of mammals are all <1 suggesting that that in general VIPs evolve under purifying selection in mammals regardless of whether they are heavily preyed on by venomous snakes or not (Table 3). Similarly, the site model analyses in rodents showed similar results with the amino acid positions in most genes appearing to evolve under purifying or neutral constraints (Table 3). The notable exception was that we found evidence for pervasive positive selection in three amino acid positions in the VIP protein AAT (Figure S2; Table S3). As described above, AAT is a strong candidate for a resistance factor, as it functions as an inhibitor of both serine proteases and metalloproteinases (Guttman et al., 2015; Mangan et al., 2008). Future studies of how molecular variation in SVMP and AAT genes and proteins covary across

TABLE 3 Summary statistics of the branch-site models (null versus alternative) used for detecting episodic positive selection on venom interactive proteins (VIPs) in rodents relative to primates

Protein name	Null model (lnL)	Alternative model (lnL)	Likelihood ratio test (χ^2)	P (df =1)	Adjusted p	Rodents ($\overline{\omega}$)	Primates ($\overline{\omega}$)
Alpha-1-antitrypsin	−8,371.524	−8,371.524	<0.001	1.000	1.000	0.478	0.496
Alpha-2-HS-glycoprotein	−4,728.901	−4,727.295	3.211	0.073	.731	0.448	0.530
Antithrombin-III	−4,922.084	−4,922.084	<0.001	1.000	1.000	0.195	0.229
Apoliprotein A-I	−5,168.060	−5,168.046	0.028	0.868	1.000	0.369	0.246
Complement C4-A	−24,009.243	−24,009.243	<0.001	1.000	1.000	0.218	0.283
Hibernation-associated plasma protein HP-20	−2,516.247	−2,516.247	<0.001	1.000	1.000	0.129	0.229
Interalpha-trypsin inhibitor heavy chain H3	−5,230.023	−5,230.023	<0.001	1.000	1.000	0.156	0.169
Serum albumin	−9,483.005	−9,483.005	<0.001	1.000	1.000	0.281	0.295
Serum paraoxonase/ arylesterase 1	−5,463.027	−5,463.027	<0.001	0.992	1.000	0.239	0.282
Vitamin D-binding	−8,161.220	−8,161.151	0.138	0.710	1.000	0.280	0.262

Note: $\overline{\omega}$, mean rate of nonsynonymous-to-synonymous substitutions across sites for that lineage (obtained from model M8).

Abbreviation: *df*, degrees of freedom.

populations and species could be useful for understanding co-evolutionary interactions at the molecular level between ground squirrels and rattlesnakes.

Our results contrast with the observation that many snake venom genes generally evolve under diversifying selection (Malhotra, 2017; Rokyta, Wray, & Margres, 2013). Our findings suggest that evolutionary costs related to the physiological context of trait expression may also be important in accounting for differences in the evolutionary lability of resistance versus toxin molecules. Specifically, venom proteins are produced in a venom gland, and are therefore isolated from other tissues and proteins that circulate in blood and are used in other physiological processes. Molecular venom resistance must involve alterations to the physiological targets of venom, which are usually part of key homeostatic mechanisms, or the constant production of circulating inhibitors that can have pleiotropic effects on other normal function.

We suggest that in the greater potential for pleiotropic effects of mutations (Snell-Rood, Van Dyken, Cruickshank, Wade, & Moczek, 2010) in resistance proteins that circulate in blood serum versus isolated venom proteins may constrain the degree to which resistance genes can evolve in response to selection imposed by the evolution of toxins. This molecular constraint related to a cost of pleiotropy may provide a previously unappreciated explanation for why snakes are the locally adapted species in this interaction (Holding, Biardi, et al., 2016) despite demographic asymmetries imposed by the Life-Dinner principle (Dawkins & Krebs, 1979). It may also provide a general explanation for the continued success and diversification of venom as a predatory strategy despite the repeated evolution of resistance in prey. More broadly, differences in trait evolvability is increasingly recognized as important in understand evolutionary trajectories of coevolving traits in co-evolutionary systems (Endara et al., 2017; Feldman et al., 2012). We suggest that these evolutionary constraints related to functional consequences of mutations may play a more significant role in guiding the evolutionary trajectories of co-evolutionary interactions than has been previously appreciated.

3.14 | Implications for evolution of resistance

Mammals who are prey for venomous snakes possess serum-based toxin inhibitors that offer protection against envenomation by sympatric snakes (see Arbuckle et al., 2017; Perales et al., 2005; Pérez & Sánchez, 1999 for comprehensive reviews). Previous results (Rocha et al., 2002; Soares et al., 1997) suggested the existence of diverse arrays of serum resistance proteins that target multiple snake venom proteins. Our results confirm this suggestion and provide insights into how resistance has evolved as a complex, multitrait phenotype. One insight is that serum resistance to snake venom proteins involves single VIPs binding to multiple venom targets and single major venom proteins binding to multiple distinct VIPs. Thus, rather than the single resistance protein – single venom protein model of resistance, our results suggest that in general a more promiscuous model

of serum resistance involving multiple, possibly weak, interactions between VIPs and venom proteins is more appropriate. However, our detection of positive selection at a small number of amino acid positions in AAT sequences in rodents supports the previous idea that strong co-evolutionary interactions may drive the molecular evolution of this specific protein in rodents and therefore SVMP venom proteins in pitvipers. Like venom itself, prey resistance appears to be a complex molecular phenotype, which helps explain prey survival in the face of envenomation and underscores the potential to investigate the co-evolutionary dynamics of complex traits using venomous animals and their prey.

AUTHOR CONTRIBUTIONS

Designed research: H.L.G., J.J.C., and M.L.H. Contributed reagents: M.L.H. Performed research: L.S., A.P., and J.J.C. Analyzed data: H.L.G., A.O., A.T.B.H. and J.J.C. Wrote the paper: H.L.G., A.O., M.L.H., and J.J.C.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available in the Dryad Data Repository (https://doi.org/10.5061/dryad.wm37pvmjz).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.