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Local prey community composition and genetic distance predict venom divergence among populations of the northern Pacific rattlesnake (*Crotalus oreganus*)

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Abstract

Identifying the environmental correlates of divergence in functional traits between populations can provide insights into the evolutionary mechanisms that generate local adaptation. Here, we assess patterns of population differentiation in expressed venom proteins in Northern Pacific rattlesnakes (*Crotalus oreganus*) from 13 locations across California. We evaluate the relative importance of major biotic (prey species community composition), abiotic (temperature, precipitation and elevation) and genetic factors (genetic distance based on RAD-seq loci) as correlates of population divergence in venom phenotypes. We found that over half of the variation in venom composition is associated with among-population differentiation for genetic and environmental variables and that this variation occurred along axes defining previously observed functional trade-offs between venom proteins that have neurotoxic, myotoxic and hemorrhagic effects. Surprisingly, genetic differentiation among populations was the best predictor of venom divergence, accounting for 46% of overall variation, whereas differences in prey community composition and abiotic factors explained smaller amounts of variation (23% and 19%, respectively). The association between genetic differentiation and venom composition could be due to an isolation-by-distance effect or, more likely, an isolation-by-environment effect where selection against recent migrants is strong, producing a correlation between neutral genetic differentiation and venom differentiation. Our findings suggest that even coarse estimates of prey community composition can be useful in understanding the selection pressures acting on patterns of venom protein expression. Additionally, our results suggest that factors other than adaptation to spatial variation in prey need to be considered when explaining population divergence in venom.

Introduction

Functional traits that show genetically based divergence between populations reflect adaptations to spatially variable selection pressures (Hoekstra *et al.*, 2006; Rundell & Price, 2009). We can therefore gain insights into the generation and maintenance of local adaptation by investigating the biological and environmental correlates of the patterns of trait divergence (Nachman *et al.*, 2003; Thompson, 2005; Schluter, 2009). Venoms are complex biochemical secretions used in predation or defence by animals as diverse as cnidarians, arthropods, molluscs and vertebrates (Casewell *et al.*, 2012) and are prime examples of functional traits with strong genetic underpinnings. Predators use venom to disrupt aspects of prey physiology, and venom composition has a direct impact on the fitness outcome of the interaction for both participants (i.e. prey death or prey escape). Animal venom therefore represents an explicit phenotypic

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link between a predator and its prey, and provides the opportunity to elucidate the contribution of prey-specific effects, the abiotic environment and other evolutionary forces in the generation and maintenance of intraspecific divergence in a complex, functionally relevant phenotype.

Three factors are hypothesized to contribute to intraspecific divergence in venom composition: spatially variable selection related to different diets among populations ('biotic' factor; Daltry *et al.*, 1996; Gibbs & Mackessy, 2009; Holding *et al.*, 2016; Margres *et al.*, 2017), variable selection related to environmental factors such as temperature and elevation (abiotic factor; Mackessy, 2010; Gren *et al.*, 2017) and neutral evolutionary processes such as genetic drift that lead to divergence between populations (genetic factors; Williams *et al.*, 1988). Diet variation is thought to be the major cause of venom evolution, because venom is a primarily trophic adaptation. Different venoms show prey-specific toxic effects (Barlow *et al.* 2009; Bernardoni *et al.* 2014; Pawlak 2006), suggesting population divergence in venom composition reflects functional adaptation to different prey (Casewell *et al.*, 2012). In support of this hypothesis, population-level divergence in the venom composition of Malayan pit vipers (*Calloselasma rhodostoma*) was best predicted by differences in the relative abundances of birds, mammals, reptiles and amphibians in the diets of these snakes (Daltry *et al.*, 1996). Population divergence in venom composition has also been linked to diet in other snakes (Creer *et al.*, 2003) and cone snails (Duda *et al.*, 2009; Chang *et al.*, 2015). One limitation of previous work linking diet and venom evolution is inherent bias that can occur in field studies that are most commonly used to infer the diets of venomous predators (Glaudas *et al.*, 2017). For example, snakes are often collected as they cross roads. However, particularly large prey items will encumber snakes and induce them to remain hidden, affecting their probability of being sampled (Glaudas *et al.*, 2017). Alternative methods for inferring the potential availability of prey based on, for example, prey distributions could circumvent these biases and allow more complete sampling of variation in the entire local prey community at given locations (Kerr & Packer, 1997; Aranda & Lobo, 2011; D'Amen *et al.*, 2015).

Abiotic environmental variation that selects for specific predigestive and other nonkilling venom functions may also drive adaptive divergence in animal venoms (Mackessy, 2008, 2010). Ectotherms inhabiting areas with suboptimal ambient temperature regimes may be selected to maintain predigestive functions of venom (Mackessy, 2008, 2010). Indeed, Holding *et al.* (2016) found that venom metalloproteinase activity among Northern Pacific rattlesnake populations was associated with site elevation and thus potentially temperature. Furthermore, another study of Northern Pacific rattlesnake venom found that temperature and precipitation data outperformed genetic and geographic distance measures as predictors of venom variation (Gren *et al.*, 2017).

Finally, venoms have rarely been hypothesized to diverge through neutral evolutionary processes such as genetic drift. For example, among island populations of tiger snakes (*Notechis* sp.), the best predictor of venom differentiation was the depth of the seas between islands and thus their degree of isolation. Although increased isolation may facilitate local adaptation, Williams *et al.* (1988) interpreted the correlation between isolation time and venom divergence as evidence for neutral evolution of venom in allopatry. More recently, genome-scale data showed that venom genes in the Asian pit viper *Protobothrops mucrosquamatus* experience a combination of positive selection and relaxed selective constraints, suggesting both selection and drift can play simultaneous large roles in the evolution of snake venoms (Aird *et al.* 2017). Most population-level studies that reject neutral divergence as an explanation for venom divergence have used genetic distances based on a single mitochondrial gene to estimate divergence due to drift (Daltry *et al.*, 1996; Creer *et al.*, 2003; Gren *et al.*, 2017). Given the smaller effective population sizes, maternal inheritance and single-gene nature of mitochondrial markers (Palumbi & Baker, 1994), a multi-locus estimate of neutral genetic differentiation would be more appropriate to assess the degree to which genetic differentiation at neutral loci accounts for phenotypic divergence in venom.

Here, we simultaneously evaluate evidence for biotic, abiotic and neutral factors as putative causes of venom divergence. We studied populations of the Northern Pacific rattlesnake (*Crotalus oreganus*), a large rattlesnake species distributed along the west coast of the United States. The venom of this rattlesnake is known to be haemotoxic and myotoxic, and is largely comprised of type I and type III snake venom metalloproteinases (MP-I and MP-III), serine proteinases (SP) and small basic polypeptide myotoxins (Mackessy, 2008, 2010). A recent analysis of venom variation in snakes from across central California, spanning the distributions of *C. oreganus* and the closely related *Crotalus helleri*, showed that abiotic environmental variation outperformed genetic distance based on a single mitochondrial gene and geographic distance in predicting geographic variation in venom protein composition (Gren *et al.*, 2017). While their work supports population-level venom divergence due to adaptive mechanisms, the authors stated that abiotic environmental variation could exert selection on venom directly or through indirect impacts on the distributions of prey. Specifically, Gren *et al.* (2017) did not attempt to estimate local prey community variation, leaving the role of prey diversity as a possible cause of venom divergence unknown. Finally, genetic distance based on a single

mitochondrial gene may be a poor estimate of neutral differentiation, so the strength of genetic differentiation as a predictor of venom variation in these rattlesnake species merits further consideration using a more comprehensive measure of differentiation.

In terms of diet, *C. oreganus* is a small mammal specialist (90% of known diet items; Sparks *et al.*, 2015). A meta-analysis of several local diet studies revealed that at least 21 small mammal species are consumed, with each study reflecting diet differences associated with regional variability in the species present (Sparks *et al.*, 2015). This suggests that these snakes feed opportunistically on all suitably sized mammal taxa present in their local environment. Therefore, to assess diet as an explanation for venom divergence, we use a novel approach based on prey distribution data to estimate differences in the small mammal community across multiple populations of *C. oreganus*. These distribution data are further likely to reflect prey availability, at least in terms of the presence or absence of particular species, because habitat heterogeneity is high along the West Coast, leading to rapid changes in community composition over short distance (e.g. transitions from dry San Joaquin Valley Grassland to cooler conditions in the high elevations of the Coast Ranges and Sierra Nevada) (Kerr & Packer, 1997). Further, the presence of well-known phylogeographic breaks at the Transverse Ranges, San Francisco Bay and Cascades Ranges enhances the effects of habitat heterogeneity to produce rapid changes in mammal communities across the region (Calsbeek *et al.*, 2003; Davis *et al.*, 2008). The variable landscape of California also presents significant amounts of abiotic environmental variation across elevational and latitudinal gradients, which could directly impact venom through selection for predigestive function where temperature varies or act indirectly by influencing the prey community. However, if environmental variation is simply a proxy measure for prey community variation, then we would expect direct information on prey community composition to outperform abiotic environmental information as a predictor of venom divergence. Finally, our study sites are separated by a putative phylogeographic break in *C. oreganus* (Goldenberg, 2013), raising the possibility that isolation and subsequent genetic drift impact venom divergence across these variably isolated rattlesnake populations.

To study the correlates of venom variation in *C. oreganus*, we first analyse phylogeographic and population structure present in this snake species using large numbers of nuclear loci obtained through restriction site-associated DNA sequencing (RAD-seq), then describe the extent of population differentiation in venom composition and identify the proteins involved in differentiation. We finally use constrained ordination for an integrated test of three hypotheses regarding population differences in venom composition, namely that venom differentiation is associated with (i) geographic differences in prey community composition, (ii) geographic differences in the abiotic environment and (iii) the degree of genetic divergence between populations as measured by our large SNP data set.

Materials and methods

Sampling

We collected venom samples from field-caught adult snakes at 13 locations in California. At each site, we conducted visual searches of suitable habitat to locate rattlesnakes and captured them using metal tongs and cloth bags. Within 24 h of capture, the snakes were induced to bite a plastic-covered beaker to obtain a venom sample, which was immediately frozen in liquid nitrogen. Next, a blood sample was taken from the caudal vein for genetic analysis. We limited our collection to snakes greater than 60 cm snout-to-vent length to control for the significant ontogenetic shift in venom composition that occurs in this species as adult-size snakes shift towards a mostly mammalian diet (Mackessy, 1988). We characterized at least 10 adult venoms samples per site at all locations except San Joaquin Experimental Range ($n = 7$). We released all snakes at their site of original capture after samples were obtained. The Ohio State Institutional Animal Care and Use Committee (protocol #2012A00000015) approved our capture and sampling procedures.

Genetic data

We extracted genomic DNA from snake blood using the standard phenol–chloroform protocol and generated double-digest RAD-seq libraries for each individual following the methods of Sovic *et al.* (2016). Briefly, we digested genomic DNA with the *Eco*RI and *Sbf*I restriction enzymes (New England Biolabs, Ipswich, MA, USA) and electrophoresed each individual's genomic DNA on a single lane of a 2% (w/v) low melt point agarose gel to isolate fragments of between 300 and 450 base pairs. The size-selected fragments were extracted from the gel with MinElute gel extraction kits (Qiagen, Hilden, Germany), amplified via PCR and purified with AmPure beads. KAPA library quantification kits (KAPA Biosystems, Wilmington, MA, USA) were then used to quantify the number of molecules present in each sample for equimolar pooling into a final library. Each pooled library was then sequenced via 50-bp, single-end sequencing on HiSeq 2500 (Illumina, Inc., San Diego, California).

We assembled reads, identified SNPs and assigned genotypes to individual snakes using AftrRAD v.5.1 (Sovic *et al.*, 2015). Following Sovic *et al.* (2016), our analyses only included loci with no missing data across all samples to minimize the impact of unscored

genotypes on estimates of population-genetic parameters (Arnold *et al.*, 2013).

Assessing genetic structure

There is evidence for phylogeographic structure associated with the San Francisco Bay/Sacramento-San Joaquin River Delta for Northern Pacific rattlesnakes (Goldenberg, 2013). Therefore, we first determined whether each of our sampling localities was a member of the northern or southern 'genetic cluster'. We use the term 'genetic cluster' to refer to the phylogeographic units identified by Goldenberg (2013) to remain agnostic over debates about lineage designations in this group (Pook *et al.*, 2000; Ashton & de Queiroz, 2001). To assess whether populations formed genetic clusters concordant with a north/south split, we ran Bayesian clustering analysis using the program STRUCTURE (Pritchard *et al.*, 2000). Three separate iterations were run for $K = 2$ with sampling location (1 of 13 sites) as a prior and a burn-in of 100 000 iterations of 1 000 000 total Markov chain Monte Carlo iterations following Gilbert *et al.* (2012). Three separate iterations were run for each K value ranging from 1 to 14, with sampling location (1 of 13 sites) as a prior and a burn-in of 100 000 iterations of 1 000 000 total Markov chain Monte Carlo iterations following Gilbert *et al.* (2012). The best number of K clusters from the STRUCTURE analyses was determined using the delta K method (Evanno *et al.* 2005) as implemented in Structure Harvester (Earl & Vonholdt 2012).

To measure levels of genetic divergence at the level of the individual population, we calculated pairwise F_{ST} values among all pairs of sampling sites and between the two major genetic clusters detected by STRUCTURE (see below). We used the pairwise.WCfst function in the package hierfstat (Goudet, 2005) in R (R Development Core Team, 2015) to calculate F_{ST} and used the boot.ppfst function from the same package to test the significance of the observed values by calculating 99% confidence intervals from 10 000 bootstrap replicates and determining whether the lower bounds included zero.

Assessing venom composition

We analysed the protein composition of each venom sample using reversed-phase high-performance liquid chromatography (RP-HPLC) on a Beckman System Gold HPLC (Beckman Coulter, Fullerton, CA, USA) and used Beckman 32 Karat Software v.8.0 (Beckman Coulter, Fullerton, CA, USA) for peak abundance quantification. Sample preparation and chromatographic separation methods were identical to those of Margres *et al.* (2014). We identified 34 separate RP-HPLC peaks in the focal venom samples from among our entire set of 127 *C. oreganus* venom samples (Fig. 1). We quantified the area under each peak relative to the total area of all identified peaks, as a measure of the relative amount of protein (by weight) in a specific peak (Margres *et al.*, 2015b).

To identify the proteins present in our focal samples to toxin family, we loaded a NuPAGE 10% Bis-Tris precast gel (Invitrogen, Carlsbad, CA, USA) with each of the HPLC fractions collected from a pooled sample of six individual *C. oreganus* from across the species range, which were chosen to contain all of the 34 peaks quantified (details and image of the gel published in Claunch *et al.* (2017), corresponding peak IDs in Figs S2 and S3). We used the protein size ranges of Mackessy (2008) to assign bands in each peak to a particular toxin family.

Venom divergence between populations

We documented the presence and magnitude of geographic differentiation in venom composition by quantifying the amount of variation explained by genetic cluster (north or south) and individual population location (Daltry *et al.*, 1996; Gibbs & Chiucchi, 2011; Margres *et al.*, 2015b), while controlling for features of individual snakes such as body size (Mackessy, 1988; Wray *et al.*, 2015) and sex (Furtado *et al.* 2006). We used permutational multivariate analysis of variance (PERMANOVA) as implemented in the adonis function of the vegan package (Dixon, 2003) in R v. 3.2 (R Development Core Team, 2015) to test the significance and quantify the proportion of variance explained by each factor (clade identity, population identity, size and sex) on venom composition. Our PERMANOVA analysis used Euclidean distances on the isometric-log-ratio-transformed (ilr-transformed) venom peak abundances, which bring the relative abundance of each venom peak from the simplex into real space. We calculated statistical significance of each factor using 10 000 permutations of the raw data. The permutations were stratified across genetic groups (Wray *et al.*, 2015), because the individual sample localities are nested within the northern and southern genetic groups. After confirming that overall population differentiation in the venom phenotype existed, we determined which peaks differed among populations. To do this, we carried out separate two-way ANOVAS on the centred-log-ratio-transformed (clr-transformed) mean abundances of each HPLC peak, with clade (north vs. south) and locality of origin (nested within a clade) as factors.

We further visualized and assessed the differentiation in venom associated with geographic factors using ordination. Ordination was accomplished through principal component analysis of the compositional data (Filzmoser *et al.*, 2009) in R, using the pcaCoDa function in the package robCompositions (Templ *et al.*, 2011). We used linear regression to assess the association between clr-

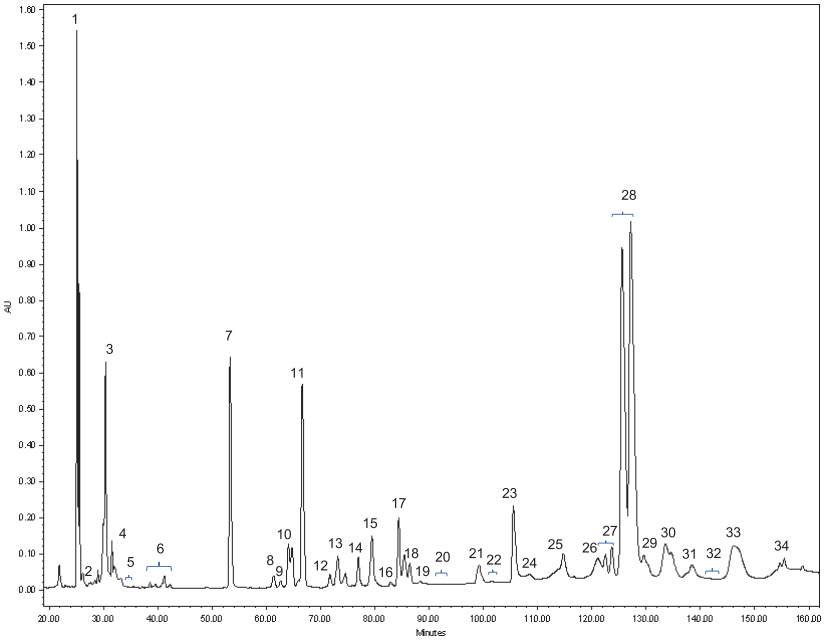

Fig. 1 Representative HPLC chromatogram showing the 34 venom protein peaks quantified in *Crotalus oreganus* venoms. Peaks that were defined as regions as opposed to individual peaks, or those that were missing in this venom sample, are marked with a number and bracket showing the elution region over which that particular peak was measured. Peaks 7–12 contain primarily PLA_2s, while peaks 23–33 contain MPs. These proteins are known to result in functional trade-offs between neurotoxic and haemorrhagic/haemotoxic venoms in rattlesnakes (Mackessy, 2008).

transformed abundance of each peak and the PC1, PC2 and PC3 scores of venom composition to identify significant contribution of a peak to each venom PC. We report the peaks with linear regression $P < 0.05$ and $R^2 > 20\%$ as contributing to a given PC (Gibbs & Chiucchi, 2011).

Biotic factors – prey community variation

The analyses described above showed a high degree of geographic variation in venom composition between populations (see below). To explore possible mechanisms that underlie this variation, we used redundancy analysis (RDA) to evaluate the power of variation in prey diversity, abiotic variation and genome-wide population divergence to explain the observed venom variation among our 13 sampling localities. The relationship between genetic differentiation and venom differentiation becomes nonlinear at the highest observed levels of genetic differentiation, violating the assumption of linear relationships between independent variables and responses in RDA. In contrast, correspondence analyses (CCA) do not make the assumption of linearity between variables but yield less interpretable measures of the variance explained by each factor in the model. Therefore, we also ran a CCA for each model tested via RDA. Results were qualitatively identical and quantitatively similar (i.e. within < 0.1% of variance explained). We therefore chose to only present the RDA results.

To quantify the variation in local prey community composition, we consulted published accounts of *C. oreganus* diet to establish which species to consider prey for rattlesnakes at a given site (Fitch & Twining, 1946; Wallace & Diller, 1990; Sparks *et al.*, 2015). Based on these studies, we included the following small mammal prey species in our analysis: two rabbits (genus *Sylvilagus*), five shrews (genus *Sorex*) and 37 small to medium-sized rodents (e.g. *Otospermophilus*, *Dipodomys and Microtus*; full list in Table S1).

We then classified each of these species as present or absent at each sampling locality based on range map overlap using detailed, georeferenced range maps for each prey which we obtained from the California Wildlife Habitat Relationships project (California Department of Fish and Wildlife). A mammal species was scored as present if its range included a given sampling locality. Range map overlap is a coarse measure of species presence and therefore can systematically overestimate biodiversity at small spatial scales due to habitat heterogeneity, but performs acceptably at coarser scales and produces estimates that are strongly positively correlated with true diversity across scales (Pineda & Lobo, 2012; D'Amen *et al.*, 2015). Finally, we obtained scores in multivariate prey space for each of our study sites by subjecting the matrix of binary presence/absence data for 44 small mammals to nonmetric multidimensional scaling (NMDS) using the metaMDS function in the vegan package (Dixon,

2003), and we recorded the NMDS axes 1 and 2 scores for each location.

Abiotic factors – environmental variation

To quantify variation in the abiotic environment at each location, we followed the approach used by Gren *et al.* (2017) on the same rattlesnake species by extracting principal component scores from a matrix of temperature, precipitation and elevation variables for each sampling locality. To obtain this data, we downloaded 30-arc-second raster grids of all 19 Bioclim temperature and precipitation variables from the WorldClim database (www.worldclim.org) and extracted the values at each site. The site elevation at the centroid of each of our sampling locations was added to the Bioclim data, to generate a 13 site × 20 abiotic environmental variable matrix. We summarized the variation in this environmental matrix using standard principal component analysis using the prcomp function in the R base package (R Development Core Team, 2015).

Genome-wide population differentiation

Finally, we used our SNP data to measure genome-wide genetic divergence between populations. Because including the average allele frequency at each locus in our RDA would overparameterize the model, we summarized the genetic data in our SNP matrix as principal components that explained the majority of genetic differentiation between the populations (Price *et al.*, 2006; Bryc *et al.*, 2010; François *et al.*, 2010; Ma & Amos, 2012). We used the dapc function in the R package adegenet (Jombart, 2008) to generate principal components of our ddRAD-seq data, and recorded the average scores of all snakes at each sampling locality on genetic PC1 and PC2, which collectively explained 33% of the total genetic variation present.

Assessing predictors of population differentiation in venom

Using the ordination scores from the first two axes of prey community, environmental and genetic variation, we tested the relative ability of each factor to explain variation in average venom composition among populations. For each population, we calculated the average abundance of each peak in ilr-transformed venom space and used conditioned RDA implemented through the rda function in the vegan package for the analysis. The conditioned RDA controls for the effects of one set of variables before performing traditional RDA on the residual matrix. In this case, we conditioned on genetic variation before testing for impacts of the biotic prey community or abiotic environmental data. Past studies have used Mantel tests to examine relationships between venom differentiation and attributes of populations (Gibbs & Chiucchi, 2011; Gren *et al.*, 2017), but this approach has been criticized as inappropriate when distance measures (e.g. genetic distance) are analysed instead of true dissimilarity measures (Legendre & Fortin, 2010; Legendre *et al.*, 2015). Conditional RDA has been suggested as a more appropriate approach to achieve the analytical goals of a partial Mantel test (Legendre *et al.*, 2011). Each independent variable was determined to have significant explanatory power compared to standard PCA of the venom data with 1000 permutations of the raw data, again using the vegan R package.

Exploratory analyses showed that the first two NMDS scores of the prey community strongly co-varied with the first two PC scores of environmental variation, which suggests they summarize similar underlying information (i.e. the abiotic environment predicts which mammals are present in a given location). To account for this covariation, we conducted two separate RDAs, one including the prey scores and a second that included the abiotic environmental scores, while conditioning each on the neutral genetic PCs. Comparing each result in terms of the amount of explained variance allowed us to compare biotic and abiotic data as models explaining venom variation and thus to determine whether prey community or abiotic environmental data is the better predictor of venom variation.

Results

Population-genetic structure

We generated a total of 64 596 311 reads with a mean read depth of 652 488 per individual across 105 individuals. Using AftrRAD, we identified a total of 22 709 nonparalogous loci, of which 5012 were polymorphic. The mean and median read depth per locus were 79.5 and 52 reads, respectively. The final SNP data set for downstream analyses consisted of 903 polymorphic loci scored in all individuals.

Bayesian clustering in STRUCTURE supports the existence of northern and southern genetic groups in these rattlesnakes, where the major break is coincident with the San Francisco Bay and Sacramento/San Joaquin River Delta (Goldenberg, 2013), and not the Transverse Ranges as previously suggested (Pook *et al.*, 2000). Comparisons of K values using the delta K approach (Evanno *et al.* 2005) indicate that $K = 2$ clusters are optimal (Fig. S1). Clusters identified using $K = 2$ show that five sampling localities fall into the northern cluster and seven localities fall in the southern cluster (Fig. 2).

Pairwise F_{ST} were > 0.05 for all pairs of populations (Table S2), and lower bounds of the 99% CI on these values were all > 0.03. The average pairwise F_{ST} value across all sampling localities is consistent with moderate amounts of genetic differentiation between populations

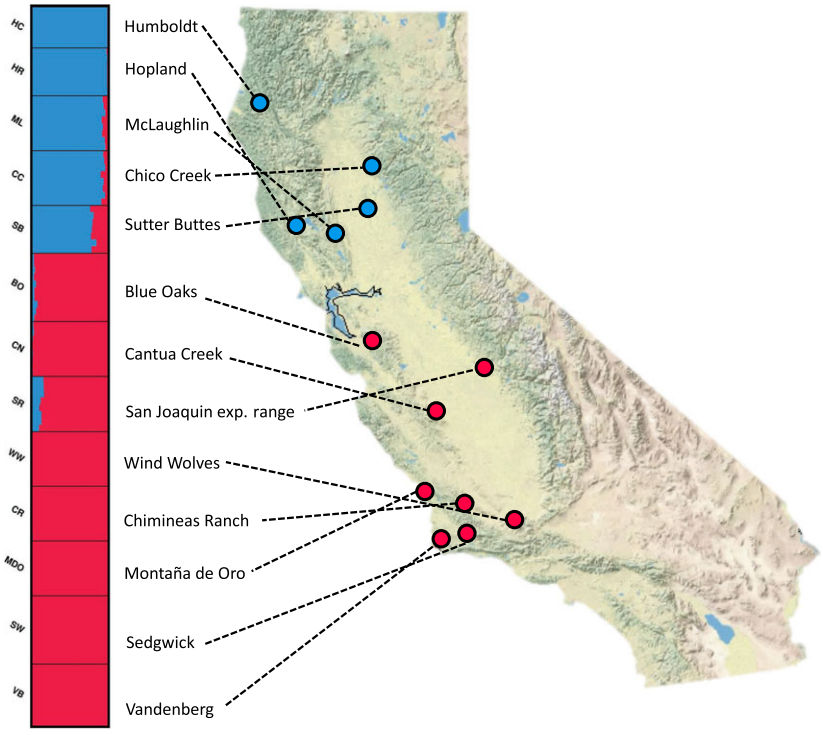

Fig. 2 Membership probabilities from STRUCTURE at $K = 2$ for 105 *Crotalus oreganus* and corresponding map of study localities. Populations on either side of San Francisco Bay form northern and southern genetic groups. Site abbreviations to the left of the STRUCTURE plot are used to identify individual populations.

(mean pairwise $F_{ST} = 0.18$, Table S2). Consistent with the presence of significant phylogeographic structure, there is higher genetic differentiation between the northern and southern genetic groups ($F_{ST} = 0.18$) than among sampling localities within the northern ($F_{ST} = 0.09$) and southern genetic groups ($F_{ST} = 0.10$).

Differentiation in venom composition

The venom composition of Northern Pacific rattlesnakes varied with respect to the geographic location of sampling and, to a lesser extent, the size of the snake, with location explaining over half of the variation in our data set. Our PERMANOVA analysis quantified the amount of multivariate venom variation explained by four factors: genetic group (north vs. south), specific sampling locality, snake size and snake sex (Table 1). Over 50% of the variation among individual HPLC venom profiles is explained by the two geographic factors: 31.9% is explained by the specific location from which the venom sample was collected while an additional 18.5% of the variation is associated with the northern and southern genetic groups present on opposite sides of San Francisco Bay. Despite including venom from only adult rattlesnakes, snake size also explained a small (2.9%) but significant amount of the variation in venom composition, suggesting that the ontogenetic shift in this species' venom may not be fully complete at the size (> 60 cm) that we used as our criterion for identifying adults. Rattlesnake sex was not a statistically significant predictor of venom variation ($P = 0.19$). Our post hoc analyses of each individual venom peak showed that all peaks showed significant differentiation between clades, between populations within each clade or both (Figs S2 and S3).

Table 1 Results of PERMANOVA analysis explaining variation in the venom phenotype of Northern Pacific rattlesnakes from 13 populations in California.

Factor	d.f.	Mean squares	*F*	*P*-value	R^2
Clade	1	25 629.5	43.6	< 0.0001	18.5
Population	11	4018	6.83	< 0.0001	31.9
Snout-to-vent length	1	4097	6.97	< 0.0001	2.9
Sex	1	765.3	1.30	0.192	N/A
Residual	10	588			46.0

Next, we used principal components analysis to visualize the geographic differences in venom composition and to reveal specific venom peaks associated with this differentiation (Fig. 3). The first principal component (PC1) of venom variation accounted for 26% of the

variation present and delineates rattlesnakes from the north and south clades on either side of the San Francisco Bay, although there is also significant overlap with rattlesnakes from the eastern side of major river drainages in California's Central Valley (Sutter Buttes and San Joaquin Experimental Range). The second principal component (PC2; 17% of venom variation) separates individual populations from both the north and south regions. Peaks containing each of the major rattlesnake venom components (Disintegrins, SPs, MPs and PLA_2) loaded heavily and significantly onto these first two PC axes (Table 2), indicating that mixed sets of proteins contribute to these major axes of variation. This pattern of venom variation is consistent with results from another rattlesnake species, *Sistrurus catenatus* (Gibbs & Chiucchi, 2011). Both PC1 and PC2 were characterized by the previously documented trade-off between MP and PLA_2 venom proteins that is well-documented in rattlesnake venoms (Mackessy, 2010). Higher scores on PC1 were associated with higher amounts of four MP peaks and lower amounts of four PLA_2 peaks while higher scores on PC2 were associated with lower amounts of three MP-rich peaks and higher amounts of two PLA_2 peaks.

Predictors of population differentiation in venom

The redundancy analyses showed that both genetic differentiation among populations and local prey community composition are significant predictors of geographic variation in Northern Pacific rattlesnake venom composition (Table 3; Fig. 4). Genetic differentiation between populations was the best predictor of venom variation in the conditioned RDA analysis: the first two PCs that summarize population-genetic variation explained nearly half of the among-population variation in rattlesnake venom (Table 3). To visualize the relationship between genome-wide population-genetic divergence and venom differentiation, we plotted pairwise F_{ST} against pairwise venom dissimilarity for all pairs of sites (Fig. 5). This graph shows a positive relationship between genetic and venom differentiation that becomes nonlinear at high values of F_{ST}. Specifically, there is a positive association between pairwise F_{ST} and venom differentiation when comparisons are made within the north and south clades, but the relationship reaches an asymptote at $F_{ST} \sim 0.15$ such that in

Table 2 Loadings and amount of overall variation in venom composition explained for peaks with significant correlations with principal Components axes 1, 2 or 3 and which have more than 20% of their variance explained by that peak.

Axis (% Variance)	Peak I.D.	R^2	Axis loading
PC 1 (26%)	7 – PLA_2	59.7	−0.392
	8 – PLA_2	38.8	−0.239
	9 – PLA_2	48.9	−0.196
	11 – Disintegrin	30.5	−0.230
	12 – PLA_2	37.7	−0.355
	13 – SVMP, SP	42.4	0.124
	18 – SP	29.9	0.222
	19 – SP	24.2	0.198
	20 – PLA_2	38.9	0.277
	24 – SVMP	20.3	0.099
	25 – SVMP	25.1	0.193
	29 – SVMP	22.5	0.250
	31 – SVMP	35.2	0.269
PC 2 (17%)	8 – PLA_2	29.7	0.243
	11 – Disintegrin	23.5	0.243
	12 – PLA_2	28.1	0.406
	23 – SVMP	39.2	−0.360
	28 – SVMP, LAAO	22.6	−0.287
	33 – SVMP	52.0	−0.632
PC 3 (13%)	10 – PLA_2, Lectin	34.2	−0.120
	12 – PLA_2	21.3	0.428
	15 – SP	28.3	−0.246
	18 – SP	26.5	0.378
	27 – SVMP	56.6	−0.550

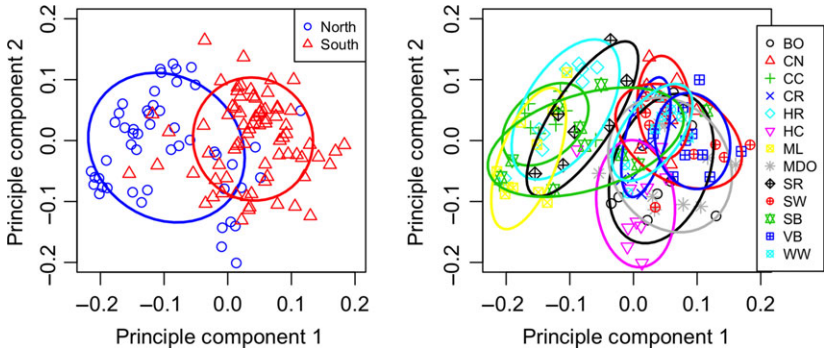

Fig. 3 Biplots of first two PCs from robust principal components analysis of venom variation. Left: Samples coded as northern (blue) or southern (red) genetic group. Right: Samples coded by sampling locality. Confidence ellipses show one standard deviation from the mean of each group.

Table 3 Results of redundancy analysis of population mean venom variation across 13 rattlesnake populations. The analysis assessed variance explained by the first two NMDS axes of prey community variation while conditioning the analysis on the first five principal components of RAD-seq genetic variation.

Factor	Inertia	Variance explained	*P*-value
Conditional (genetic variation)	14.9	47.6	
Constrained (prey community)	7.2	23.0	0.02
Unconstrained (standard PCA)	9.2	29.4	
Total	31.3		

between-clade comparisons, more genetically differentiated populations are not expected to be more or less different in their venom composition.

When we conditioned the RDA on genetic data, prey community variation explained a moderately large and significant portion of the remaining variation (R^2 = 23%, P = 0.02). Of the 23% of overall venom variation explained by the prey community, the first axis of prey community variation accounted for the majority (68%, P = 0.002). The second axis of prey community variation accounted for the remainder (32%), but it was marginally nonsignificant (P = 0.06). The main trends in each of these NMDS-derived prey community axes were the presence of xeric-adapted rodents at sites with smaller axis scores and mesic-adapted rodents at sites with high scores (Table S1), reflected by similar directions of greatest change when the prey axes are projected as eigenvectors into the RDA space (Fig. 4). Lower scores on the first prey community axis (Prey1 in Fig. 4) were associated with the presence of three kangaroo rats (*Dipodomys nitratoides, Dipodomys hermanni* and *Dipodomys ingens*), five mice (*Peromyscus eremicus, Peromyscus fraterculus, Peromyscus crinitus, Perognathus inornatus* and *Onychomys torridus*) and Nelson's antelope squirrel (*Ammospermophilus nelsoni*), whereas higher scores on this axis were associated with the presence of the chipmunk *Tamias sonomae*, the squirrel *Tamiasciurus douglasii*, two voles (*Myodes californicus* and *Arborimus pomo*) and several shrews, sciurids and voles that were found only at the Humboldt County (HC) site. Low scores on the second prey community axis (Prey 2 in Fig. 4) were associated with the presence of *D. nitratoides*, a fourth kangaroo rat species (*Dipodomys californicus*), and *P. inornatus*, whereas high scores were associated with the presence of a chipmunk, *Tamias merriami*, *Peromyscus californicus*, the woodrat *Neotoma macrotis*, and a fifth kangaroo rat, *Dipodomys agilis*. In summary, the significance of prey axis 1 as predictor of multivariate venom composition suggests a role for prey community variation in selecting for population-level venom divergence. Further, transitions from xeric-adapted rodents (particularly kangaroo rats) to mesic-adapted rodents (particularly voles) underlie the key prey community shifts that associate with venom variation.

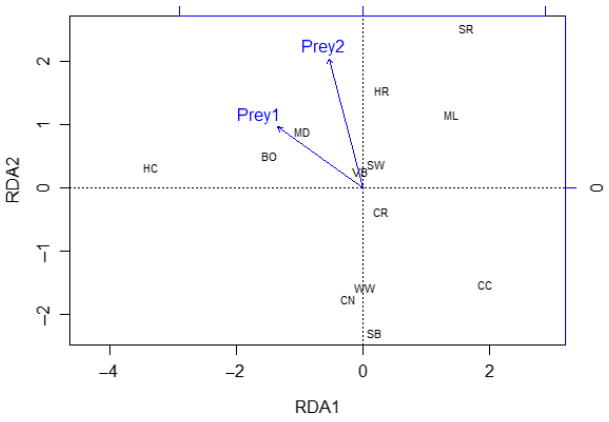

Fig. 4 Biplot of first two axes from constrained ordination of venom composition of Northern Pacific rattlesnakes from 13 sites (abbreviated site names on plot indicate site scores in a venom compositional space defined by the local prey community). Arrows are eigenvectors of the first two axes of prey community space. Sites in the top and left part of the graph tended to have more species of voles and other mesic-adapted rodents, whereas sites in the bottom and right have more kangaroo rat species and other xeric-adapted rodents. See map in Fig. 1 for population abbreviations.

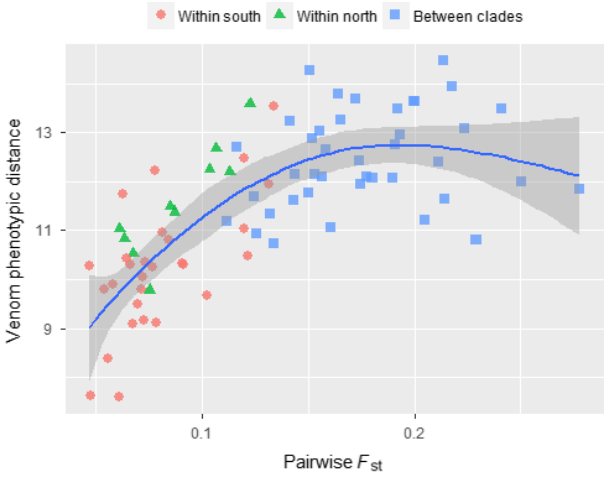

Fig. 5 Relationship between pairwise F_{ST} of rattlesnake populations and pairwise distance between the same populations in Euclidean distance in clr-transformed venom phenotype space. Comparisons are within the southern clade (red), northern clade (green) or between clades (blue).

The first three PCs of environmental variation explained 46%, 36% and 7% of environmental variation, respectively. Low scores on environmental PC1 were associated with warmer winters and stable climates, whereas higher scores reflected more variable temperature regimes (Table S3). Low scores on environmental PC2 were associated with hotter, drier climates, higher scores reflected cooler, wetter areas,

while scores of PC3 were positively associated with site elevation. Site scores on the first PCs of abiotic environmental data were significantly associated with our prey community NMDS axes (Fig. S4, $P < 0.05$), suggesting that the environmental (abiotic) and prey (biotic) matrices reflect much of the same information about each site and, not surprisingly, that prey community composition is related to abiotic environmental variation. Comparing the power of each data set to predict venom variation is therefore an important test to distinguish the effects of abiotic factors from the effects of variation in prey communities that are in turn influenced by the abiotic environment. To do this, we ran a separate RDA analysis conditioned on the genetic PCs but replacing the prey community NMDS scores with the environmental PC scores. The three environmental PCs were marginally nonsignificant as predictors of venom variation ($P = 0.08$), and account for slightly less of the variation in the data set (22%) compared with the prey community axes, despite the presence of one additional parameter (PC3; elevation). To ensure the weaker performance of environmental variation was not due to overparameterization (three environmental axes versus two prey community axes used in the analysis), we ran a second environmental RDA with only the first two environmental PCs. This RDA was significant ($P = 0.03$), which is expected given the association of these two PCs with the prey community axes (Fig. S4). However, the amount of venom variation predicted by the environmental PC scores is even lower (19%) than in the first analysis using the prey community scores directly (23%), suggesting that differences in available prey directly select for population-level venom variation.

We therefore found that genetic differentiation was the best predictor of venom differentiation among populations. When we controlled for this genetic differentiation, direct biotic information about the local prey community outperformed abiotic environmental data in predicting venom variation. Taken together, these results suggest significant roles for both the degree of population-genetic differentiation and variation in available prey in selecting for venom divergence over geographic space.

Discussion

Numerous studies of animal venoms have assessed whether diet variation, abiotic environmental variation or genetic distance best explain venom variation in their focal taxon with mixed results regarding the relative importance of each factor (Williams *et al.*, 1988; Daltry *et al.*, 1996; Creer *et al.*, 2003; Remigio & Duda, 2008; Duda *et al.*, 2009; Gibbs & Chiucchi, 2011; Chang *et al.*, 2015; Margres *et al.*, 2015b; Gren *et al.*, 2017). Here, we employed a novel method with regard to venom studies (constrained ordination), which allowed us to partition the variation associated with various factors to evaluate the relative importance of each. Our major findings are that while variation in the local prey community was associated with venom differentiation, the degree of genetic divergence explained more variation than either prey or abiotic environmental data. Below, we discuss the implications of these results for understanding the underlying evolutionary and ecological causes of population-level differentiation in venom.

Geographic variation in Northern Pacific rattlesnake venom

Population-level variation in snake venom has been repeatedly documented (Daltry *et al.*, 1996; Creer *et al.*, 2003; Gibbs & Chiucchi, 2011; Massey *et al.*, 2012; Margres *et al.*, 2016). Previous work on the same rattlesnake species with fewer individuals and over a smaller latitudinal range also sought to explain extensive venom variation over geographic space (Gren *et al.*, 2017). Gren *et al.* (2017) suggested that the venom of Northern Pacific rattlesnakes is more variable than any snake species previously studied although the quantitative basis for this claim is unclear. However, a study of congeneric Eastern diamondback rattlesnakes (*Crotalus adamanteus*) using similar methods to our own showed that population of origin only explained 11.6% of the observed venom variation (Margres *et al.*, 2015b) compared to over 50% of the variation explained by genetic group and location in *C. oreganus.* Additional comparisons of venom differentiation in other related species over similar geographic scales are needed to judge whether *C. oreganus* is in fact exceptional.

The clearest differentiation in the venom phenotype was between the northern and southern genetic groups that are present on either side of the best known phylogeographic break in the California Floristic Province: the San Francisco Bay and San Joaquin/Sacramento River deltas. This break coincides with phylogeographic divergence in several terrestrial vertebrates (Calsbeek *et al.*, 2003), including these rattlesnakes (Goldenberg, 2013), and coincides with the major axis of differentiation in the functional trait of venom. This pattern, along with the association between pairwise genetic and venom distances between populations, suggests a role for neutral evolution of venom during phylogenetic divergence in *C. oreganus*. Yet, phylogenetic impacts on venom variation are limited (Gibbs *et al.* 2013), while selection associated with isolation by environment (IBE) may lead to an association between genetic and venom distances. We discuss these possibilities in the following section.

High levels of geographic differentiation of venom in this species may reflect unusually high levels of habitat variation in this region compared with other areas where rattlesnakes are found in the United States. In particular, the California Floristic Province has been

called a 'hotbed of population and phylogeographic diversification' (Calsbeek *et al.*, 2003; Hickerson *et al.*, 2010), where steep environmental gradients of elevation and aridity are superimposed on longer-term drivers of diversification including mountain uplift, ocean embayment and glaciation (Matocq *et al.*, 2012). This is reflected in multiple studies of adaptive phenotypic variation which show high levels of variation in traits such as the mimicry colours of *Ensatina eschscholtzii* salamanders (Kuchta *et al.*, 2009), the toxicity of *Taricha* newts and resistance of garters snakes that feed on them (Hanifin *et al.*, 2008) and the corolla shape of *Lithophragma politella* plants that are both parasitized and pollinated by *Greya* moths (Thompson *et al.*, 2013). Mammals which are prey to snakes in this region also show considerable population and phylogeographic structure (Motocq, 2002; Conroy & Neuwald, 2008; Phuong *et al.*, 2014) and physiological adaptations to environmental gradients (Eastman *et al.*, 2012) that may result in selection for differences in venom efficiency in different rattlesnake populations (Holding *et al.*, 2016).

Population differentiation in venom composition has also been documented in two other rattlesnakes that occur west of the Rocky Mountains, namely the Southern Pacific rattlesnake *(C. helleri*; Sunagar *et al.*, 2014) and the Mojave rattlesnake (*C. scutulatus*; Massey *et al.*, 2012). Both species show trade-offs in the relative abundance of PLA_2 and MP proteins in their venom, which characterize a functional trade-off between neurotoxicity and haemotoxic/haemorrhagic mechanisms of killing prey. The Northern Pacific rattlesnake shows the same pattern, as the first three principal components of venom composition are characterized by a negative association between the signs of the loadings of PLA_2 vs. MP protein peaks. The abundance of several MP peaks increases with venom PC 1 scores, which means the venom of southern clade snakes is more MP-rich. Part of the functional significance of MP variation among these southern clade populations is tied to local adaptation to variable MP resistance in California ground squirrels (Holding *et al.*, 2016), but the fact that MPs also covary with broad-scale variation in the prey community composition suggests that MP proteins are under selection to adapt to additional mammal prey. Whether these other mammal prey also show evidence for a coevolutionary response in terms of resistance is an interesting question for future work.

Predictors of variation in venom composition

Our finding that genetic divergence between populations was the best predictor of venom divergence contrasts with results from other studies of intraspecific variation in snake venom (Daltry *et al.*, 1996; Creer *et al.*, 2003; Gibbs & Chiucchi, 2011; Margres *et al.*, 2015a,b), including a recent study of the same species (Gren *et al.*, 2017) that show a lack of correlations between venom and genetic divergence. A major difference between our study and others is that we used genome-scale data in the form of RAD-seq loci to estimate genetic differentiation between rattlesnake populations. This contrasts with all previous studies, which used either mitochondrial sequence data (Daltry *et al.*, 1996; Creer *et al.*, 2003; Gren *et al.*, 2017) or small numbers of microsatellite loci (Gibbs & Chiucchi, 2011) to estimate population-genetic differences. Our data set of over nine hundred loci should provide a more accurate measure of divergence among populations than estimates based on a single or few loci. Alternatively, there could be real biological differences in the strength of mechanisms driving the relationship between venom and genetic differentiation between studies (see below).

One interpretation of the strong relationship between genetic and venom divergence among populations is that neutral evolutionary processes such as genetic drift or founder effects have had a significant impact on functional venom variation (Aird *et al.* 2017). Changes in climate since the last glacial maximum likely mean that rattlesnakes are more widely distributed today than they were 15 000 years ago, particularly for our northern sampling localities. Hence, founder effects could possibly play a role in present day patterns of venom differentiation. However, we feel a significant role for neutral processes such as drift is unlikely because historical demographic analyses show that genetically effective population sizes in these snakes are large (> 10 000) suggesting that any effects of drift are probably weak (M. Holding, M. Sovic, and H. Gibbs, in preparation).

We feel a more likely explanation for the close association between levels of divergence in venom and neutral genetic divergence is that it is due to an IBE mechanism (Wang & Bradburd, 2014). IBE occurs when local adaptation drives correlated divergence in adaptive traits and neutral markers through selection against maladaptive phenotypes in immigrants, with correlated impacts on neutral loci carried by such immigrants. This possibility is supported by the lack of a positive relationship between pairwise F_{ST} and distance in venom space when comparisons are on opposite sides of the major genetic break at San Francisco Bay, where venom divergence has likely occurred independent of the exchange of migrants. This hypothesis could be tested by taking advantage of California's Coast and Sierra Nevada ranges, which are oriented in a north-to-south direction, and sample populations at similar elevations along the mountain ranges as well as down into the Central Valley, where sampling could cover similar geographic distances but encounter large changes in species composition towards the Valley floor (Wang *et al.*, 2013; Wang & Bradburd, 2014). If IBE is the mechanism responsible for generating varying levels of neutral divergence between these rattlesnake populations, then environmental distance should outperform

geographic distance as a predictor of F_{ST} or similar measures.

This explanation also suggests that biological differences between species could also explain why genetic divergence was only a secondary (Daltry *et al.*, 1996; Creer *et al.*, 2003) or poor (Gibbs & Chiucchi, 2011) predictor of venom divergence in other snakes compared to the Northern Pacific rattlesnake. In particular, variation in the strength of an IBE effect among species or geographic locations could potentially explain these differences. The contribution of IBE to genetic divergence has been shown to vary among species. For example, among 17 Caribbean species of anole lizards (*Anolis sensu lato.*), the amount of population-genetic divergence explained by IBE ranged from 0.1 to 48% (Wang *et al.*, 2013). Lizards from the island of Hispaniola, where habitat heterogeneity was highest, showed the highest levels of IBE. Among rattlesnake venoms assessed for correlations between population-genetic and venom divergence, all occur in areas with lower levels of habitat heterogeneity than *C. oreganus*. For example, the Eastern massasauga (*Sistrurus catenatus*) occurs in areas of the upper Midwest, the Eastern diamondback rattlesnake in the coastal plain of the southeastern U.S. and the Southern Pacific rattlesnake (*C. helleri*) occurs in southern California (Conant & Collins, 1998) – all of which are areas that are less heterogeneous than central and northern California where our study took place (Kerr & Packer, 1997). Conditions across the range of *C. oreganus* may therefore produce local adaptation that is stronger and occurs at a finer scale on average in this rattlesnake, leading to stronger associations between population-genetic and adaptive venom differentiation than found in other species. This hypothesis could again be tested by studies of IBE in *C. oreganus*, where the degree of IBE in *C. oreganus* is compared to that of its close relatives, which are broadly distributed across more uniform habitats.

The second most important predictor of venom divergence was differences in prey community between sites. This result is consistent with the long-held hypothesis that variation in diet composition is an important selective force that moulds venom differences between snake populations (Daltry *et al.*, 1996; Wüster *et al.*, 1999; Casewell *et al.*, 2012). The first axis of prey community variation identified locations with prey communities consisting of rodents that are arid habitat specialists (kangaroo rats, desert mice and antelope squirrels) from sites with small mammals representative of wetter habitats at high elevation and/or latitude (chipmunks, Douglas squirrels and voles). While sciurids and mice in the genus *Peromyscus* occur among all locations, the dichotomy between kangaroo rats and voles as major diet items represents a major axis of taxonomic differentiation in rattlesnake prey and hence may impose distinct selection pressures on venom composition. These species are from divergent families within Rodentia, but are both medium-sized and locally abundant, and thus may be key components of prey that differ between xeric and mesic habitats.

The three published diet studies for this snake support the existence of a kangaroo rat-vole dichotomy in diet among locations. At sites in British Columbia and Washington, voles made up 50 and 57%, respectively, of the diet items found in the stomachs of Northern Pacific rattlesnakes (Macartney, 1989; Wallace & Diller, 1990), while on the San Joaquin Experimental Range in the central foothills of the Sierra Nevada, voles made up only 2% of the diet, whereas kangaroo rats were the second most abundant diet item (16% of prey taken). Reciprocal toxicity tests on voles vs. kangaroo rats using the venom of snakes from mesic vs. dry environments in California would provide a functional test of the role that these groups of rodents play in shaping the venom phenotype (Pomento *et al.*, 2016), which could be followed by investigating the physiological underpinnings of venom susceptibility in each rodent species.

Evidence that the prey diversity causes selection for venom divergence comes from the finding that abiotic environmental variables were correlated with prey community variables (Fig. S4) but were outperformed by the prey community as direct predictors of venom differentiation. Previous studies have suggested that the abiotic environment could impact venom directly through an increased requirement for predigestive functions of venom in more climatically variable environments (Mackessy, 2010), and abiotic environmental variation has predicted both functional and compositional differences in the venom of *C. oreganus* (Holding *et al.*, 2016; Gren *et al.*, 2017). However, comparative tests of whether these are direct effects or whether the environment acts indirectly by structuring prey community composition have been unclear. Our results help resolve this question by showing higher predictive power of a coarse measure of prey community variation to predict venom divergence, compared to a fine-grained (1 km^2 resolution) measure of abiotic environmental variation, by suggesting an overriding effect of the available prey species.

Assessing the availability of potential prey using presence–absence data to characterize local mammal community composition, instead of analysis of stomach or faecal contents, represents a new approach to evaluating prey diversity as a putative cause of population-level divergence in venom. Our finding that prey community differences predicted 23% of the variance in venom composition, and that prey community data outperformed abiotic environmental data, suggest that small mammal distribution data can be useful for studies of the adaptive significance of venom variation. While stomach content data provide direct information about snake diet, they can be

biased in terms of representing an accurate assessment of what snakes eat. For example, smaller prey items are more likely to be detected because snakes with large prey items are encumbered and therefore avoid movement and remain hidden. The intensive field study of Fitch (1949) reported 116 California ground squirrels in a total of 285 prey items (40%), with squirrels making up 70% of rattlesnake prey by weight. Yet, an assessment of 88 prey items in the guts of museum specimens from central California, which are often collected on roads, only included three ground squirrels and likely reflects bias against detecting these larger prey taxa (Sparks *et al.*, 2015). Community variation, using an assessment of which animals represent potential prey based on previous work, may circumvent the biases of stomach content studies. This approach could be refined by incorporating niche modelling to better predict localized absences and weight the presences based on habitat suitability indices (e.g. Aranda & Lobo, 2011; Pineda & Lobo, 2012; D'Amen *et al.*, 2015).

In some regions, *C. oreganus* feeds most heavily on one species, the California ground squirrel (Fitch & Twining, 1946; Fitch, 1949), which comprised the majority of videotaped encounters with potential prey at two sites (Putman *et al.*, 2016). Our previous work suggests that Northern Pacific rattlesnake venom is locally adapted to overcoming California ground squirrel MP resistance, suggesting adaptive divergence in venom specifically in response to variable resistance in a single species of prey (Holding *et al.*, 2016). Because here we show that broader prey community variation predicts venom divergence among these same populations, we conclude that population divergence in rattlesnake venom can be a simultaneous response to both broad taxonomic differences in prey communities and variable resistance in coevolving prey species in a geographic mosaic of coevolution (Thompson, 2005). In fact, responses to prey community differences and coevolving resistant species need not be mutually exclusive and instead highlight the complex nature of geographic variation in natural selection. Each distinct selection pressure could act on venom such that an optimum in venom phenotype space is reached by a population that is a balance between the relative abundance of available prey and the strength of coevolutionary selection between rattlesnakes and ground squirrels, which represents a continuum from opportunism to specialization on ground squirrels. Alternatively, functional modules that may evolve semi-independently have been proposed for snake venoms (Gibbs *et al.*, 2009; Gibbs & Chiucchi, 2011), which would allow separate sets of venom proteins to evolve in response to these various sources of selection and maximize a snake's chance of capturing different prey in its local environment.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Species scores from non-metric multidimensional scaling of presence/absence data for each species across 13 study sites in central California

Table S2 Pairwise Fst values between all sampled populations of *Crotalus oreganus* for 903 double digest RAD-seq loci

Table S3 Axis loadings from PCA for 19 Bioclim variables (Var.) and elevation (Elev.) across the 13 California study sites

Figure S1 Delta values based on the method of Evanno *et al.* (2005) for K = 2 through K = 13 clusters found by the program STRUCTURE (Pritchard *et al.*, 2000)

Figure S2 Population average of clr-transformed areas for peaks 1-17 in Northern Pacific rattlesnake venom HPLC chromatograms

Figure S3 Population average of clr-transformed areas for peaks 18-34 in Northern Pacific rattlesnake venom HPLC chromatograms

Figure S4 Relationships between site scores on the first two principal components (PC) of 19 Bioclim variables plus elevation and site scores on the first two non-metric multidimensional scaling axes of prey community variation

Data deposited at Dryad: https://doi.org/10.5061/dryad.rg098gq

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Table S1: Species scores from non-metric multidimensional scaling of presence/absence data for each species across 13 study sites in central California. Species marked with * only found at the Humboldt County (HC) site.

Species	Axis 1	Species	Axis 2
Dipodomys nitratoides	-0.66	*Dipodomys californicus*	-0.55
Ammospermophilus nelsoni	-0.60	*Dipodomys nitratoides*	-0.39
Dipodomys ingens	-0.60	*Perognathus inornatus*	-0.36
Onychomys torridus	-0.54	*Ammospermophilus nelsoni*	-0.26
Peromyscus eremicus	-0.50	*Dipodomys ingens*	-0.26
Peromyscus fraterculus	-0.50	*Tamias sonomae*	-0.20
Peromyscus crinitus	-0.50	*Peromyscus truei*	-0.17
Perognathus inornatus	-0.45	*Neotoma fuscipes*	-0.16
Dipodomys heermanni	-0.38	*Onychomys torridus*	-0.12
Chaetodipus californicus	-0.27	*Tamiasciurus douglasii*	-0.09
Sorex ornatus	-0.26	*Myodes californicus*	-0.09
Tamias merriami	-0.26	*Arborimus pomo*	-0.09
Neotoma lepida	-0.26	*Microtus californicus*	-0.05
Sylvilagus audubonii	-0.25	*Spermophilus beecheyi*	-0.04
Peromyscus californicus	-0.20	*Thomomys bottae*	-0.04
Neotoma macrotis	-0.20	*Reithrodontomys megalotis*	-0.04
Dipodomys agilis	-0.18	*Peromyscus maniculatus*	-0.04
Microtus californicus	-0.13	*Sylvilagus audubonii*	-0.02
Dipodomys venustus	-0.09	*Dipodomys venustus*	0.00
Spermophilus beecheyi	0.00	*Peromyscus eremicus*	0.00
Thomomys bottae	0.00	*Peromyscus fraterculus*	0.00
Reithrodontomys megalotis	0.00	*Peromyscus crinitus*	0.00
Peromyscus maniculatus	0.00	*Sylvilagus bachmani*	0.06
Peromyscus boylii	0.00	*Sciurus griseus*	0.09
Sylvilagus bachmani	0.00	*Dipodomys heermanni*	0.11
Peromyscus truei	0.11	*Sorex vagrans*	0.13
Sciurus griseus	0.21	*Sorex pacificus*	0.13
Neotoma fuscipes	0.23	*Sorex bendirii*	0.13
Sorex trowbridgii	0.28	*Aplodontia rufa*	0.13
Dipodomys californicus	0.30	*Tamias senex*	0.13
Tamias sonomae	0.52	*Glaucomys sabrinus*	0.13
Tamiasciurus douglasii	1.05	*Arborimus albipes*	0.13
Myodes californicus	1.05	*Microtus longicaudus*	0.13
Arborimus pomo	1.05	*Microtus oregoni*	0.13
*Sorex vagrans**	1.44	*Zapus trinotatus*	0.13
*Sorex pacificus**	1.44	*Sorex ornatus*	0.16
*Sorex bendirii**	1.44	*Peromyscus boylii*	0.18
*Aplodontia rufa**	1.44	*Sorex trowbridgii*	0.25
*Tamias senex**	1.44	*Neotoma lepida*	0.28

*Glaucomys sabrinus**	1.44	*Chaetodipus californicus*	0.28
*Arborimus albipes**	1.44	*Tamias merriami*	0.47
*Microtus longicaudus**	1.44	*Peromyscus californicus*	0.59
*Microtus oregoni**	1.44	*Neotoma macrotis*	0.59
*Zapus trinotatus**	1.44	*Dipodomys agilis*	0.80

Table S2: Pairwise Fst values between all sampled populations of *Crotalus oreganus* for 903 double digest RAD-seq loci.

Population	**HC**	**HR**	**ML**	**CC**	**SB**	**BO**	**CN**	**SR**	**WW**	**CR**	**MD**	**SW**
HR	0.10											
ML	0.11	0.06										
CC	0.12	0.09	0.08									
SB	0.11	0.09	0.07	0.06								
BO	0.19	0.16	0.14	0.15	0.12							
CN	0.21	0.18	0.15	0.16	0.12	0.05						
SR	0.19	0.16	0.13	0.14	0.11	0.06	0.06					
WW	0.20	0.17	0.15	0.15	0.13	0.07	0.06	0.07				
CR	0.21	0.18	0.16	0.16	0.13	0.07	0.06	0.07	0.05			
MD	0.23	0.19	0.17	0.17	0.14	0.08	0.07	0.08	0.07	0.06		
SW	0.25	0.22	0.20	0.20	0.17	0.12	0.10	0.12	0.08	0.07	0.08	
VB	0.28	0.24	0.21	0.22	0.19	0.13	0.12	0.13	0.09	0.08	0.09	0.05

Table S3: Axis loadings from PCA for 19 Bioclim variables (Var.) and elevation (Elev.) across the 13 California study sites. PC1, PC2, and PC3 axes are shown, and each column is sorted in increasing order of loadings to facilitate environmental interpretation (Interp.) of each.

Var.	PC1	Interp.	Var.	PC2	Interp.	Var.	PC3	Interp.
		Warmer winters, Stable			Hot, Dry			Low Elev., Warmer
bio11	-0.31	climate	bio1	-0.30		bio1	-0.44	
bio3	-0.30		bio10	-0.23		bio6	-0.33	
bio6	-0.29		bio9	-0.22		bio10	-0.23	
bio8	-0.29		bio5	-0.20		bio8	-0.22	
bio15	-0.22		bio2	-0.16		bio9	-0.21	
bio18	-0.04		bio7	-0.16		bio11	-0.21	
bio1	0.06		bio4	-0.15		bio17	-0.15	
Elev.	0.13		bio15	-0.14		bio13	-0.12	
bio14	0.15		bio8	-0.11		bio14	-0.12	
bio16	0.15		bio11	-0.06		bio12	-0.11	
bio19	0.16		Elev.	0.01		bio19	-0.10	
bio12	0.16		bio6	0.04		bio16	-0.09	
bio13	0.16		bio3	0.09		bio4	-0.08	
bio17	0.17		bio13	0.29		bio5	-0.07	
bio10	0.23		bio14	0.29		bio18	-0.05	
bio2	0.24		bio17	0.30		bio7	0.04	
bio9	0.25		bio19	0.30		bio3	0.18	
bio5	0.27		bio16	0.30		bio15	0.21	
bio7	0.29		bio12	0.31		bio2	0.29	
bio4	0.30	Cooler winters, Variable Climate	bio18	0.34	Cool, Wet	Elev.	0.52	High elev., Cooler

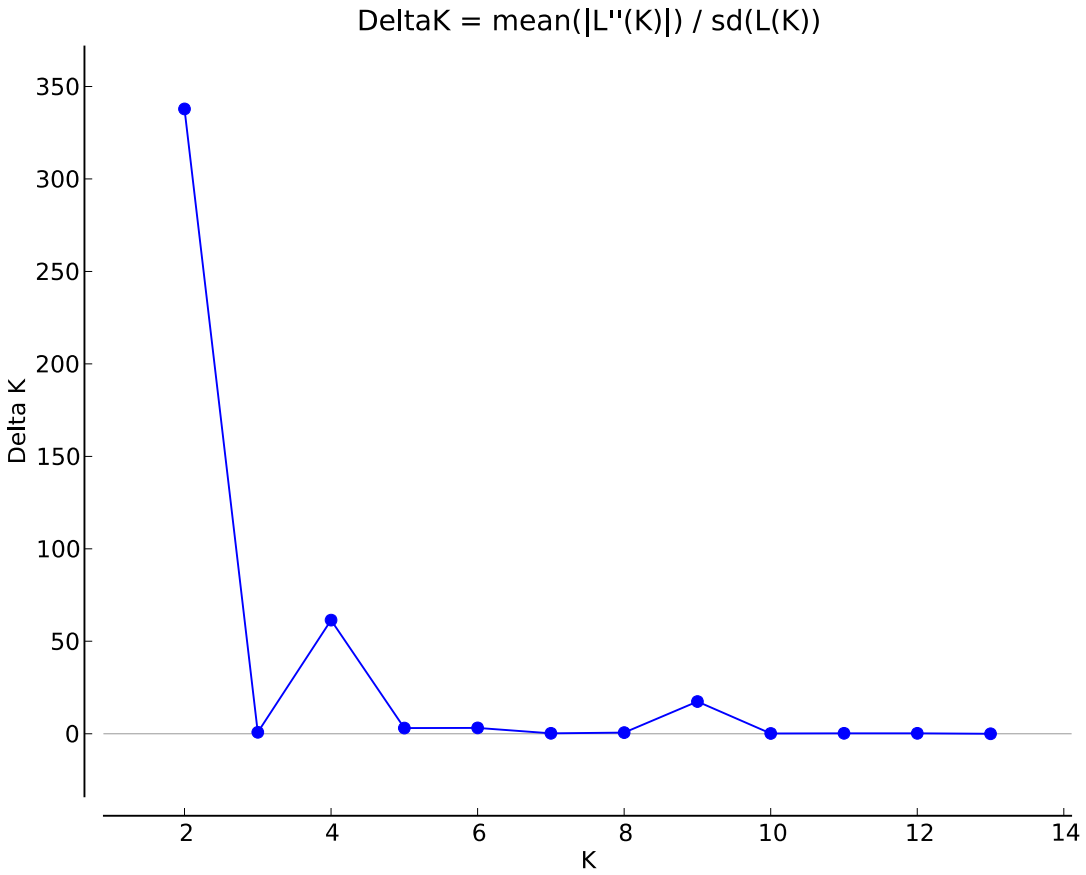

Figure S1: Delta values based on the method of Evanno *et al.* (2005) for K = 2 through K = 13 clusters found by the program STRUCTURE (Pritchard *et al*. 2000).

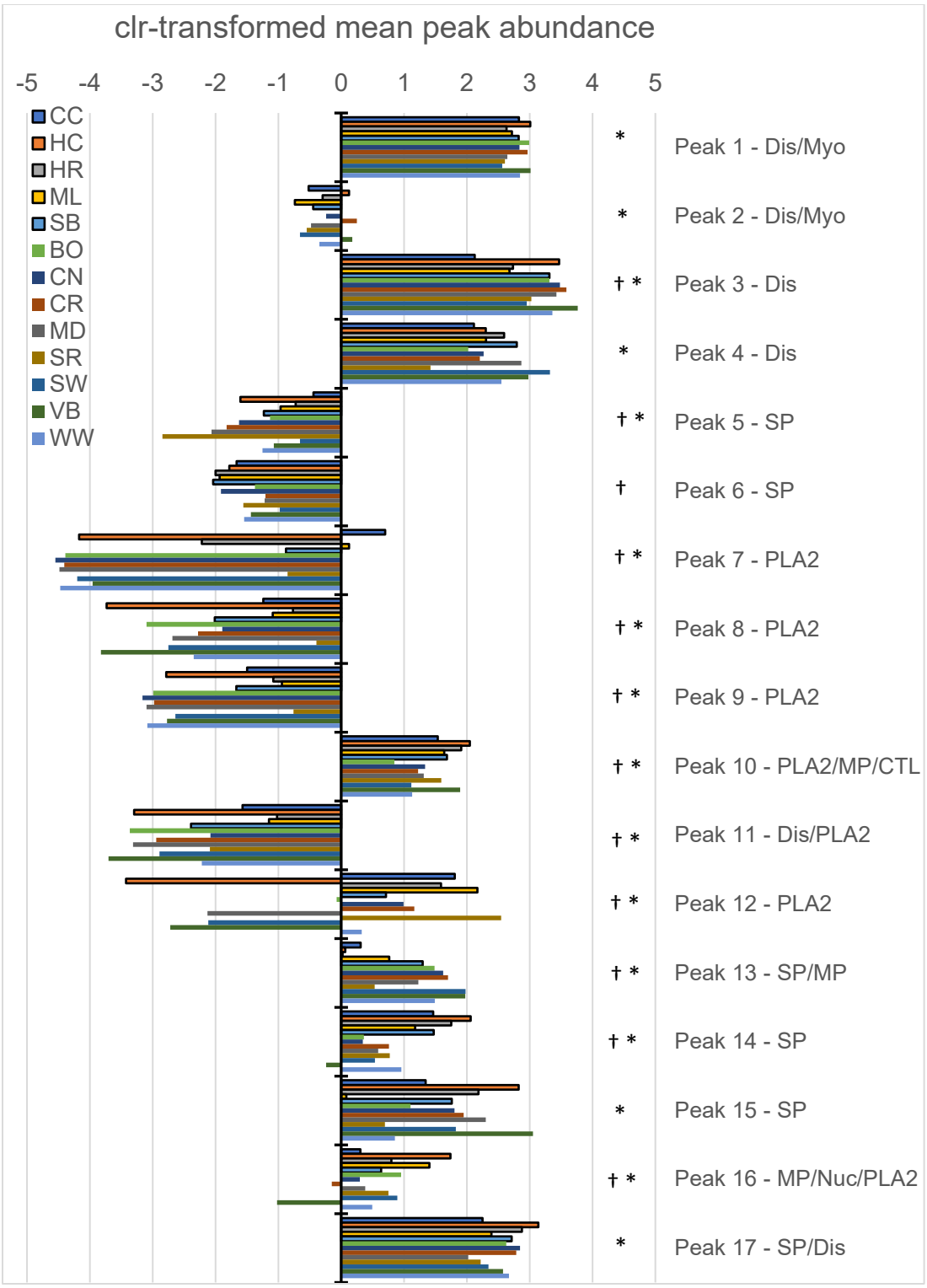

Figure S2: Population average of clr-transformed areas for peaks 1-17 in Northern Pacific rattlesnake venom HPLC chromatograms. North-clade populations have bars with dark borders. † indicates significant north/south variation, * indicates significant difference between populations, $\alpha = 0.05$.

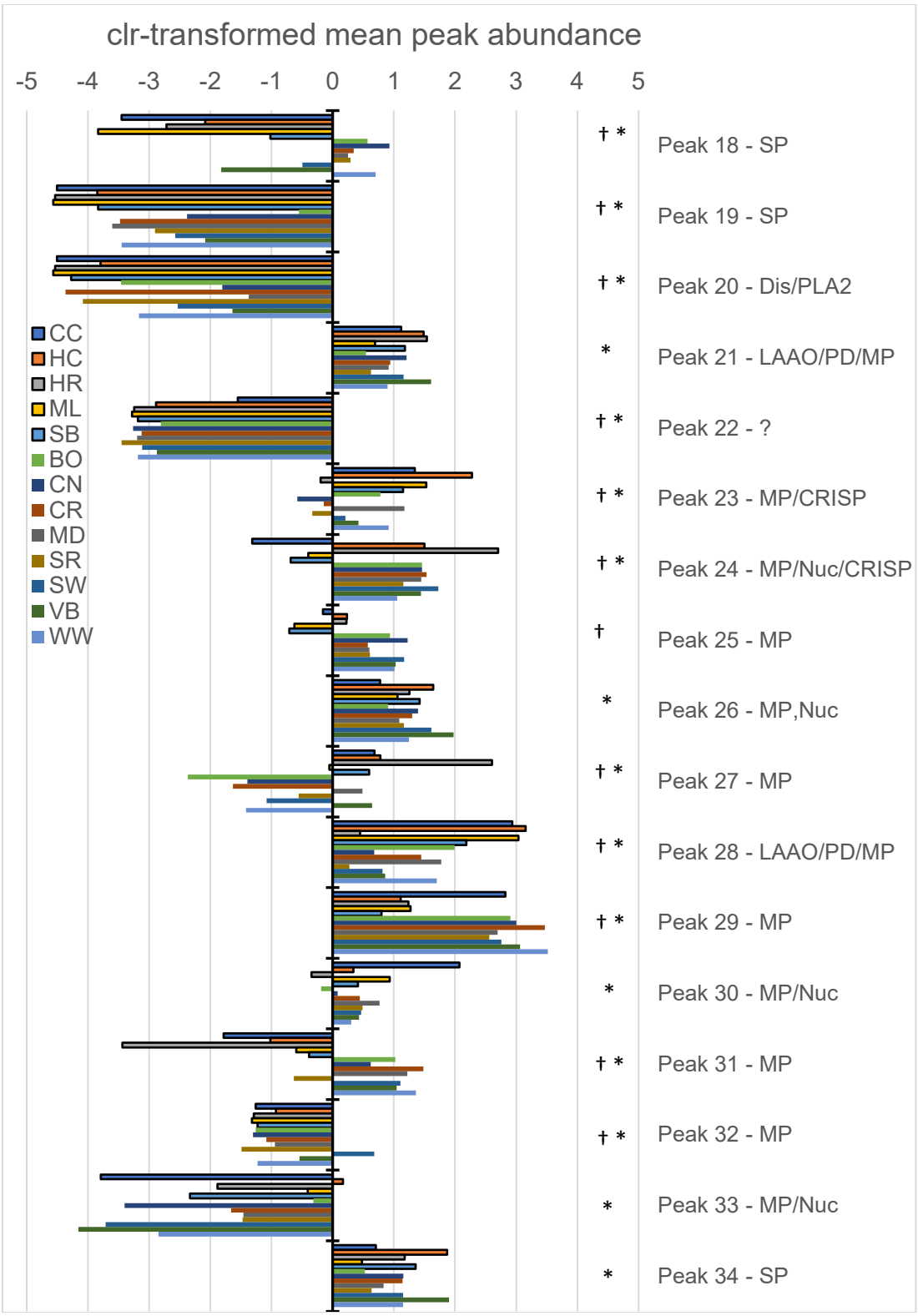

Figure S3: Population average of clr-transformed areas for peaks 18-34 in Northern Pacific rattlesnake venom HPLC chromatograms. North-clade populations have bars with dark borders. † indicates significant north/south variation, * indicates significant differences between populations, $\alpha = 0.05$.

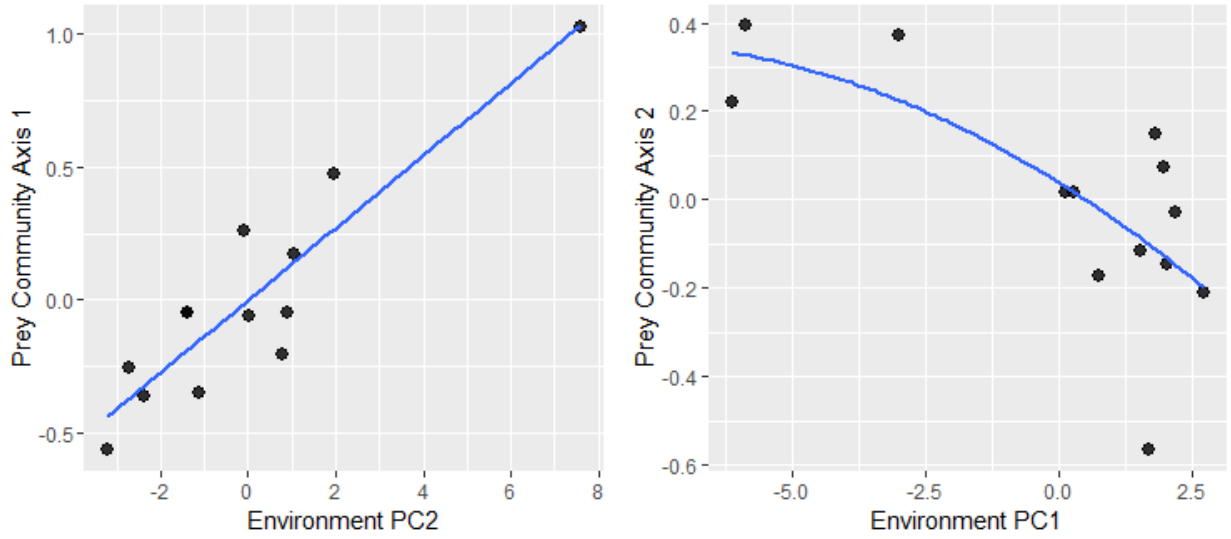

Figure S4. Relationships between site scores on the first two principal components (PC) of 19 Bioclim variables plus elevation and site scores on the first two non-metric multidimensional scaling axes of prey community variation. Associations between environmental and prey community site scores indicate that the two sets of variables carry much of the same information.