Contrasting patterns of venom regeneration in a centipede (*Scolopendra viridis*) and a scorpion (*Centruroides hentzi*)

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Abstract

As biochemical traits with clear fitness consequences, venoms serve a critical ecological role for the animals that produce them. Understanding how venoms are maintained and regenerated after use will, therefore, provide valuable insight into the ecology of venomous animals. Furthermore, most studies on venomous organisms often require removing animals from the wild and waiting extended periods of time between venom extractions. Uncovering the patterns of venom regeneration across different species will likely lead to the development of more efficient venom extraction protocols, reducing both experimental time and the number of animals required. Using reversed-phase high-performance liquid chromatography, we identified asynchronous regeneration of venom protein component abundances in the centipede *Scolopendra viridis* but found no evidence for asynchronous venom regeneration in the scorpion *Centruroides hentzi*. We also observed high levels of intraspecific venom variation in *C. hentzi*, emphasizing the importance of testing for intraspecific venom variation in studies evaluating the synchronicity of venom regeneration. Although the regeneration of relative venom protein component abundances is an asynchronous process in *S. viridis*, we provide evidence that the presence-absence of major venom components is not an asynchronous process and suggest that studies relying on just the presence/absence of individual proteins (*e.g.* bioprospecting, drug discovery) could use catch-and-release methods of venom extraction to reduce the number of animals removed from the wild.

1 Introduction

With their conspicuous functional roles and genetic tractability, animal venoms are powerful systems that have historically provided unique insight into the fields of evolution (Fry et al., 2003; Undheim et al., 2014; Whittington et al., 2018; Holding et al., 2021), protein interaction and structural biology (Wang et al., 2005; Velasco-Bolom et al., 2018), and drug discovery (Cushman and Ondetti, 1991; Tcheng and OâĂŹShea, 2002; Miljanich, 2004; Cardoso et al., 2021). Venoms are complex protein–dominated, biochemical phenotypes that have evolved across numerous metazoan lineages for use in predation and defense (Casewell et al., 2013), microbiome regulation (Gao et al., 2007; Baracchi and Tragust, 2017), intraspecific conflict (Grant et al., 2007), and maternal care (Tragust et al., 2013). This diversity of function and clearly linked fitness implications underscore the importance of venoms for the animals that maintain them. As venoms are depleted after use, understanding how animals maintain and regenerate their venoms will not only have implications for the ecology of venomous animals and their communities, but may provide insight into the genetic regulatory mechanisms that produce a complex phenotype.

Research using animal venoms often requires either identifying and/or isolating individual venom components (*e.g.* novel drug discovery) or quantifying the relative abundances of such components (*e.g.* characterizing expression differences). Both of these strategies employ similar venom extraction and collection methods that require (1) removing animals from the wild or captive breeding and (2) waiting an extended period of time between venom extractions to allow for complete regeneration. Therefore, unraveling the dynamics of venom content regeneration will help refine and tailor venom extraction methods, which could ultimately reduce the time and number of animals required.

In snakes, one of the more comprehensively studied venomous lineages, it can take a few days to more than two weeks for venom from a depleted gland to be fully restored (Kochva, 1960; Schaeffer Jr et al., 1972; Brown et al., 1975; Luna et al., 2009), with this restoration coming at a metabolic cost (McCue, 2006; Pintor et al., 2010). Regeneration of venom content also seems to happen asynchronously in snakes (Oron et al., 1978; Taylor et al., 1986; Guo et al., 2009; Luna et al., 2013), although that may not always be the case (Pintor et al., 2011). In invertebrates, near-complete venom regeneration has been observed to take anywhere between a few days and up to several weeks in various lineages, such as spiders (Perret, 1977; Boevé et al., 1995; Kuhn-Nentwig et al., 2004), scorpions (Nisani et al., 2007, 2012; Carcamo-Noriega et al., 2019; Díaz-García et al., 2019), hymenopterans (Haight, 2012), and centipedes (Cooper et al., 2014). Even amongst species from the same lineage, venom regeneration may happen at different rates, as observed by the time required for near-complete venom regeneration in the scorpions *Parabuthus transvaalicus* (8 days; Nisani et al., 2012), *Centruroides limpidus* (13 days; Carcamo-Noriega et al., 2019), and *Rhopalurus junceus* (15-21 days; Díaz-García et al., 2019). After venom extraction, *P. transvaalicus* experience a significant

increase in oxygen consumption, supporting the hypothesis that venom regeneration in scorpions has a metabolic cost (Nisani et al., 2007, 2012). Asynchronous regeneration of proteinaceous venom components has also been observed in invertebrates, such as tarantulas (Perret, 1977; Boevé et al., 1995), scorpions (Pimenta et al., 2003; Nisani et al., 2012; Díaz-García et al., 2019; Carcamo-Noriega et al., 2019), and one centipede species (*Scolopendra polymorpha*; Cooper et al., 2014). In some cases, the regeneration of activity and toxicity, not just venom content, is asychronous. For example, Carcamo-Noriega et al. (2019) show that not only is the regeneration of venom components from the scorpion *C. limpidus* asynchronous, but the regeneration of this venom's toxicity against crickets and activity towards human voltage-dependent Na^+ channel Nav1.6 is also asynchronous.

These discernible differences in the rates of venom content regeneration and potentially the regeneration of venom toxicity among different species emphasize the importance of increasing our understanding of venom regeneration dynamics on a species specific level. Furthermore, although asynchronous regeneration of relative venom protein abundances has been observed in the previously discussed invertebrates, whether this asynchronicity translates to presence-absence differences in venom components at different regeneration intervals is unclear. Therefore, we analyzed venom protein content at five regeneration intervals using reversed-phase high-performance liquid chromatography to test for asynchronous regeneration of venom protein components in the centipede, *Scolopendra viridis*, and the scorpion, *Centruroides hentzi*.

2 Materials and Methods

2.1 Specimen collection

Scolopendra viridis centipedes and *C. hentzi* scorpions were collected from Leon County, Florida. Adult *S. viridis* were collected by flipping logs, peeling bark from dead trees, and pitfall trapping. Pitfall trapping activities were conducted under a US Department of Agriculture Forest Service Special Use Permit (Authorization ID: WAK9112018522). Adult *C. hentzi* were collected using UV-flashlights and peeling bark from dead trees after dark. All centipedes and scorpions were housed individually at the Florida State University Department of Biological Science. Unlike *C. hentzi*, which exhibit obvious sexual dimorphism in the size and length of metasomal segments (females have shorter, more rounded metasomal segments), *S. viridis* sex was determined using a microscope by the presence (male) or absence (female) of two genital gonopods (Bonato et al., 2010; McMonigle, 2014).

2.2 Venom collection and processing

We collected a total of 16 male *S. viridis* and 15 female *C. hentzi*. To test the synchronicity of venom protein regeneration over time in *S. viridis* and *C. hentzi*, individuals were

randomly divided into five groups per species with each group allowed a different interval of time to regenerate venom between an initial and second venom extraction. These time intervals were 1, 2, 4, 10, and 14 days post-initial venom extraction.

Centipedes and scorpions were fed and subsequently starved for 21 days prior to the initial venom extraction (*i.e.* day 0). To prepare for venom extraction, animals were anesthetized under CO_2 for 90 seconds. Venom was extracted from *S. viridis* by electrostimulation at the base of the forcipules and from *C. hentzi* by electrostimulation at the base of the telson, as previously described (Ward et al., 2018b; Ward and Rokyta, 2018). In several scorpion species, venom secretion has been observed along a continuum with an initial clear secretion defined as "prevenom" (Yahel-Niv and Zlotkin, 1979; Gopalakrishnakone et al., 1995; Inceoglu et al., 2003; Abdel-Rahman et al., 2009). Therefore, each *S. viridis* and *C. hentzi* were electrostimulated at least three times to ensure complete emptying of the glands and consistency between venom extractions. Extracted venom was suspended in 100 μL of LC/MS quality water, centrifuged at 12,000×G for three minutes, freeze-dried using a lyophilizer, and stored at –80°C. Immediately before use, lyophilized venom samples were re-suspended in LC/MS quality water and spun at 12,000×G for 30 seconds to pellet insoluble material. Total venom protein content was quantified using a Nanodrop 2000c (Thermo Scientific).

2.3 Reversed-phase high-performance liquid chromatography

To evaluate differences in the synchronicity of *S. viridis* and *C. hentzi* venom protein regeneration over time, we performed reversed-phase high-performance liquid chromatography (RP-HPLC) on both the initial and the second, time-dependent venom extraction from the 16 *S. viridis* (32 total venom samples) and the 15 *C. hentzi* (30 total venom samples). This was completed using the Shimadzu Prominence HPLC system. We used a standard solvent regimen consisting of solvent A (0.1% trifluoroacetic acid [TFA] in water) and solvent B (0.06% TFA in acetonotrile). Approximately 15 μg of venom protein from each sample was injected onto an Aeris 3.6μm C18 column (Phenomenex, 125 Torrance, CA). All samples were allowed to run with a flow rate of 0.2 mL/min over a 125–minute gradient. This gradient was initialized at 10% B for five minutes, gradually increased to 55% B over 110 minutes, increased again to 75% B over five minutes, held at 75% B for another five minutes, and finished with 15–minutes at 100% B to wash the column. Peak clusters in RP-HPLC chromotagraphic profiles were identified in single-blind fashion using the manual peak integration tools in the Shimadzu Lab Solutions software.

2.4 Statistical analysis

All statistical analyses were performed using the relative abundances of identified RP-HPLC peak clusters. Statistical analyses for *S. viridis* and *C. hentzi* were performed separately using R v. 3.6.3 (R Core Team, 2017) with figures generated using the ggplot2

package (Wickham, 2016). To test for variation in venom regeneration at different time intervals, we first performed an ilr (isometric log-ratio) transformation on the RP-HPLC relative peak cluster abundance data. We then ran a permutational multivariate analysis of variance (PERMANOVA) using the adonis function from the vegan package in R (Oksanen et al., 2013) on the ilr-transformed relative abundance data from the second, regenerated venom sample with individuals grouped by regeneration time interval. To determine which peak clusters contributed most of the variation in RP-HPLC profiles from both the initial and regenerated venom samples, we ran a variance matrix on clr (centered log-ratio) transformed peak cluster abundance data using the robCompositions package in R (Templ et al., 2011).

To visualize patterns of venom regeneration over time, we performed a robust principal component analysis (PCA) on the RP-HPLC profiles from the second, regenerated venom extraction using the pcaCoDa function from the robCompositions package in R (Templ et al., 2011). The pcaCoDa function transforms the data using an ilr transformation, performs a robust PCA, and backtransforms the resulting loadings and scores using the clr transformation. This PCA method demonstrates superior results for compositional data and can be easily interpreted (Filzmoser et al., 2009). We then quantified the relative impact of the top two principal components (*i.e.* PC1 and PC2) by fitting a linear regression model with the principal component as the dependent variable and venom regeneration interval as the independent variable using the inherent lm function in R.

3 Results

3.1 Venom protein content regeneration in male *Scolopendra viridis*

Venom protein content from initial venom extractions of all *S. viridis* averaged 27.26 μg (12.96–54.93 μg; Figure 1, Supplemental Data 1). Initial venom extraction yields for intervals 1, 2, 4, 10, and 14 days averaged 17.97, 33.21, 32.82, 23.44, and 28.47 μg, respectively (Figure 1, Supplemental Data 1). Venom yield from the regenerated venom samples in the 1, 2, 4, 10, and 14-day regeneration interval groups averaged 15.73, 9.11, 12.81, 10.29, and 28.64 μg, respectively (Figure 1, Supplemental Data 1). Although two of the four individuals from the 14-day regeneration interval group fully replenished the total venom protein content in their venom, the average percent regeneration of total venom protein content among all four individuals after 14 days was 82%, indicating that venom protein content regeneration in *S. viridis* takes at least 10–14 days.

3.2 Asynchronous venom regeneration in male *Scolopendra viridis*

After performing RP-HPLC on an initial and regenerated venom sample from 16 male *S. viridis* individuals, we identified 13 distinct peak clusters in chromatographic profiles (Figure 2, Supplemental Data 2). Figure S1 shows all 16 *S. viridis* initial venom sample RP-HPLC profiles for comparison of identified peak clusters. We identified a significant difference in relative peak cluster abundance across time interval of the regenerated venom samples (PERMANOVA; $p < 0.01$), providing evidence for asynchronous venom regeneration in *S. viridis*. Utilizing a variance matrix, we determined that the five RP-HPLC peak clusters that contributed the most variation in the initial venom samples were peak clusters 1, 2, 5, 9, and 13, which contributed 16.20%, 5.92%, 6.74%, 36.22%, and 6.99% respectively (Figure 4). However, the five peak clusters that contributed the most variation in the regenerated venom samples were peak clusters 1, 6, 8, 9, and 11, which contributed 27.35%, 22.97%, 5.99%, 13.27%, and 8.30% of the variation, respectively (Figure 4). We then took the resulting peak cluster variances from our variance matrix and performed a linear regression using the clr-tranformed variance of the initial venom sample peak clusters and the clr-transformed variance of the regenerated venom sample peak clusters. With this regression, we identified a weak correlation between the variance of the initial venom sample peak clusters and the variance of the regenerated venom sample peak clusters ($\rho = 0.48$, $R = 0.55$, $R^2 = 0.31$, and $p = 0.05$; Figure 4), indicating that the amount of variation each peak cluster was responsible for was similar across most peak clusters in both the initial and regenerated venom samples.

Our PCA analysis on the RP-HPLC peak cluster data from the regenerated venom samples (Figure 5) revealed a distinct separation between venom chromatographic profiles from individuals with shorter venom regeneration intervals (*i,e.* 1, 2, and 4 days) and individuals with longer venom regeneration intervals (*i.e.* 10 and 14 days) in PC1-PC2 space. The most variable peak clusters in PC1-PC2 space included four of the five most variable peak clusters in the variance matrix on the regenerated venom samples (*i.e.* peak clusters 1, 8, 9, and 11). However, instead of peak 6 (*i.e.* fifth most variable peak cluster identified in the variance matrix), our PCA identified peak cluster 12 as one of the top five peak clusters that contributed the most variation in PC1-PC2 space. Peak cluster 1 was the peak cluster responsible for the largest portion of the variation in both the variance matrix and PC1-PC2 space and was observed in higher abundance in venom from individuals with shorter regeneration intervals (*i.e.* 1, 2, and 4 days). Peak clusters 6 and 12 were observed at higher abundances in the venom from individuals with longer regeneration intervals (*i.e.* 10 and 14 days). Peak cluster 9 was observed in low abundances in the group with a one day regeneration interval, compared to the other four groups. Interestingly, peak clusters 8 and 11 were observed at the highest abundance in the one- and 14-day regeneration interval groups.

The top two principal components, PC1 (49.9%) and PC2 (22.7%), accounted for 72.6% of the total variation. To quantify the relative impact of PC1 and PC2, we fit

a linear regression between the top two principal components and venom regeneration interval. We identified a significant relationship between PC1 and venom regeneration interval ($\rho = -0.72$, $R = -0.83$, $R^2 = 0.69$, and $p < 0.01$; Figure 6), providing more convincing evidence for asynchronous venom regeneration in *S. viridis* between one and 14 days. However, we found no significant relationship between PC2 and venom regeneration interval ($\rho = 0.21$, $R = -0.02$, $R^2 = 0.00$, and $p = 0.94$; Figure 6).

3.3 Venom protein content regeneration in female *Centruroides hentzi*

Total venom protein content of initial venom extractions from all *C. hentzi* averaged 135.04 μg (22.89–251.7 μg; Figure 3, Supplemental Data 3). Initial venom extraction yields for intervals 1, 2, 4, 10, and 14 days averaged 56.04, 216.18, 163.53, 82.06, and 157.39 μg, respectively (Figure 3, Supplemental Data 3). Venom yield from the regenerated venom samples in the 1, 2, 4, 10, and 14-day interval groups averaged 8.04, 60.21, 47.82, 45.83, and 65.86 μg, respectively (Figure 3, Supplemental Data 3). Total venom protein content was, on average, only 64% and 42% regenerated at 10 and 14 days, respectively, suggesting that even after 14 days, total venom protein content in *C. hentzi* was not fully regenerated.

3.4 No detectable asynchronous venom regeneration in female *Centruroides hentzi*

We identified 21 distinct RP-HPLC peak clusters from the initial and regenerated venom samples collected from 15 female *C. hentzi* (Figure 2, Supplemental Data 2). Figure S2 shows all 15 *C. hentzi* initial venom sample RP-HPLC profiles for comparison of identified peak clusters. As the large number of identified peak clusters combined with our small sample size would limit further statistical testing (*e.g.* PCA), we grouped peak clusters into bins encompassing approximately 10-minute intervals along RP-HPLC profiles. We selected 10-minute intervals starting from peak cluster 1 at approximately 10 minutes, and excluded any 10-minute intervals that contained no identified peaks, resulting in eight distinct bins (Bin 1 = Peak 1; 10–20 minutes, Bin 2 = Peaks 2–3; 20–30 minutes, Bin 3 = Peaks 4–6; 30–40 minutes, Bin 4 = Peak 7; 40–50 minutes, Bin 5 = Peaks 8–11; 50–60 minutes, Bin 6 = Peaks 12–16; 60–70 minutes, Bin 7 = Peaks 17–19; 70–82 minutes, and Bin 8 = Peaks 20–21; 85–95 minutes)

After running a PERMANOVA on the binned relative peak cluster abundance data for the regenerated venom samples, we did not identify any significant difference in venom regeneration across time ($p = 0.17$), indicating a lack of any detectable asynchronicity in venom regeneration. Our variance matrix identified bins 1, 4, 6, 7, and 8, as those that contributed the most variation in both the initial and regenerated venom samples (Figure 7A). Bins 1, 4, 6, 7, and 8 contributed 13.27%, 22.74%, 12.33%, 15.52%, and 20.76% of the variation in initial venom samples and 26.33%, 11.84%, 16.33%, 9.14%, and

22.74% of the variation in regenerated venom samples, respectively. After performing a clr-transformation on the resulting initial and regenerated binned variances and a regression between these variances, we identified a significant correlation between the variance of the initial venom sample bins and the variance of the regenerated venom sample bins ($\rho = 0.67$, $R = 0.80$, $R^2 = 0.64$, and $p = 0.02$; Figure 7B). This indicates that the amount of variation each bin was responsible for was similar across bins in both the initial and regenerated *C. hentzi* venom samples.

Our PCA analysis (Figure 8) did not reveal a distinct separation in PC1-PC2 space as did our PCA analysis on *S. viridis* venom. The top two principal components, PC1 (66.4%) and PC2 (15.1%), accounted for approximately 81.5% of the total variation in venom samples. After fitting linear regressions between our top two principal components and venom regeneration interval, we did not identify any significant relationship between PC1 and venom regeneration interval ($\rho = -0.38$, $R = -0.30$, $R^2 = 0.09$, and $p = 0.28$; Figure 9), or PC2 and venom regeneration interval ($\rho = 0.43$, $R = 0.5$, $R^2 = 0.25$, and $p = 0.06$; Figure 9), providing further evidence for a lack of any asynchronous venom regeneration in *C. hentzi*.

3.5 Presence-absence differences in venom RP-HPLC peak clusters across regeneration intervals are not the result of asynchronous venom regeneration

Although we identified evidence for asynchronous venom regeneration in *S. viridis*, we observed the presence of all venom RP-HPLC peak clusters in at least two individuals from each regeneration interval group (Supplemental Data 1). Furthermore, though we did not detect asynchronous venom regeneration in *C. hentzi*, we still observed the presence of all venom RP-HPLC peak clusters in at least two individuals from each regeneration interval group (Supplemental Data 3). In *S. viridis* venom, the only venom samples that did not contain a measurable abundance for every peak cluster were one 10-day regenerated sample (missing peak cluster 1) and one initial venom sample from each of the 1 and 10-day interval groups (both missing peak cluster 9). In the *C. hentzi* RP-HPLC profiles, the only venom sample that did not contain a measurable abundance for each of the 21 peak clusters was the initial venom sample from one individual in the 1-day interval group (missing peak cluster 19). Therefore, although the regeneration of relative venom RP-HPLC peak cluster abundance is an asynchronous process in *S. viridis*, the presence-absence of a particular RP-HPLC peak cluster at any point in the regeneration of *S. viridis* or *C. hentzi* venom does not seem to be asynchronous. As venom RP-HPLC peak clusters typically correspond to a general toxin type or family, the presence of all peak clusters at each regeneration interval provides evidence that a venom sample from *S. viridis* or *C. hentzi* at any point in the regeneration cycle would contain a measurable quantity of most, if not all, venom proteins.

4 Discussion

4.1 Asynchronous venom regeneration in *S. viridis* and comparison to venom regeneration in *S. polymorpha*

In this study, we identified evidence for asynchronous venom regeneration in the centipede *S. viridis*. Only one other study has attempted to study the timing of venom protein regeneration in centipedes (Cooper et al., 2014). Similar to our results in *S. viridis* after 14 days (*i.e.* 82% total venom protein regeneration), Cooper et al. (2014) found that regeneration of total venom protein content in *S. polymorpha* (sister species to *S. viridis*) took longer than 14 days and was still not fully regenerated after a 7-month follow up study (76% regenerated). Cooper et al. (2014) suggested that the inability for venom to regenerate to levels of the initial estimates could be the result of electrostimulation causing damage to the venom gland structure. Although we did not observe any fatalities or obvious harm to the animal after venom extraction, damage to the venom glands could have resulted in lower levels of venom regeneration.

Cooper et al. (2014) also observed an effect of extraction interval on the relative abundance of five of the ten RP-FPLC chromatographic regions in *S. polymorpha* venom, providing evidence for asynchronous regeneration of the relative abundance of venom protein components in *S. polymorpha*. The findings of Cooper et al. (2014) coupled with our evidence for asynchronous venom regeneration in *S. viridis* suggests that asynchronous venom regeneration may be widespread in centipedes of the genus *Scolopendra*, although studies on more species would be needed to confirm this hypothesis. Furthermore, our PCA and PC1-regeneration interval regression analyses (Figure 5, Figure 6; left) show a distinct separation between the 1, 2, and 4-day intervals, which loaded positively on PC1, and the 14-day interval, which loaded negatively on PC1, with the 10-day interval group loading in between. This suggests *S. viridis* venom is undergoing the most significant changes in relative protein component abundance between four and 14 days. Performing mass spectrometry on fractionated venom peak clusters would be needed to confirm the proteins present at particular peak clusters and the potential effects of asynchronous venom protein regeneration on the predatory and defensive capabilities of *S. viridis*.

Although incomplete expulsion of venom from glands is possible in our study, we expect that our consistent venom extraction procedures would still lead to venom-glands with similar states of venom expulsion. Nonetheless, incomplete expulsion of venom from glands could have resulted in an underestimation of the time needed for venom regeneration and, if it resulted in a venom sample dominated by one or a few proteins, could have confounded our ability to detect asynchronous venom regeneration. Cooper et al. (2014) suggested that dissection and examination of venom glands before and after venom extraction could provide information on the extent of venom gland depletion from techniques such as electrostimulation. Conversely, our venom extraction procedure was meant to completely exhaust the venom glands, a phenomenon that may not be common in wild centipedes, emphasizing that caution must be taken when interpreting results in

laboratory studies of venom regeneration.

4.2 Lack of detectable asynchronous venom regeneration in *C. hentzi* may be confounded by high levels of intraspecific venom variation

Here we also present evidence for a lack of detectable asynchronous venom protein content regeneration in *C. hentzi* scorpions. Interestingly, unlike in our study, asynchronous regeneration of relative venom protein abundances has been identified in four scorpion species to date, *Tityus serrulatus* (Pimenta et al., 2003), *P. transvaalicus* (Nisani et al., 2012), *C. limpidus* (Carcamo-Noriega et al., 2019), and *R. junceus* (Díaz-García et al., 2019). In the studies on *P. transvaalicus*, *C. limpidus*, and *R. junceus*, the authors noted that near-complete venom protein regeneration occurred after 8, 13, and 15–21 days, respectively. Although we did not observe near-complete regeneration of protein content in *C. hentzi* after our 14-day interval, this is not outside the regeneration times observed in the aforementioned studies.

Our ability to detect asynchronous venom regeneration in *C. hentzi* could have been hindered by our low sample size or high levels of intraspecific variation in *C. hentzi* venoms. To test for intraspecific variation that could confound our results in *C. hentzi*, we first performed an ilr transformation on the relative peak cluster abundance data from the initial venom samples. We then ran a PERMANOVA with samples grouped by the respective venom regeneration interval between the initial and second venom extraction. For consistency, we also repeated this test for intraspecific variation using our *S. viridis* data. Although we did not identify any significant intraspecific variation in our initial *S. viridis* venom samples (PERMANOVA; $p = 0.37$), we did identify significant intraspecific variation in our initial *C. hentzi* venom samples (PERMANOVA; $p = 0.02$). Intraspecific venom variation has been identified in *C. hentzi* before, with this variation being the result of differences between females and not males (Ward et al., 2018a). However, unlike our study, Ward et al. (2018a) observed intraspecific variation among female *C. hentzi* from different populations. The observed variation in *C. hentzi* of the same sex and population from our study underscores the importance of testing for intraspecific variation when assessing the potential for asynchronous venom regeneration. Further studies on venom regeneration in *C. hentzi* that employ larger sample sizes and account for intraspecific venom variation would be needed to confirm the observed lack of asynchronous venom regeneration.

4.3 Lack of presence-absence differences in RP-HPLC peak clusters across regeneration intervals and the impacts for designing venom-related experiments

Our RP-HPLC analysis of venom regeneration intervals in both *S. viridis* and *C. hentzi* revealed a measurable concentration of every RP-HPLC chromatographic peak cluster in at least two individuals from each venom regeneration interval group, providing evidence that a venom sample from *S. viridis* or *C. hentzi* taken after one day of venom regeneration would contain a measurable quantity of most, if not all, venom protein components. As individual chromatographic peak clusters may contain significant abundances of more than one type of protein, it is possible that presence-absence of some venom proteins from *S. viridis* or *C. hentzi* may not have been detected. However, we expect that larger patterns of overall toxin family presence-absence difference would have been identified in our analysis.

Since a single venom extraction from a scorpion or centipede (>300 μg in this study) provides a much lower total protein content than a single extraction from many snakes (>10 mg in many cases; Morrison et al., 1982; Pe and Cho, 1986; Margres et al., 2014), studies that use or isolate individual venom components from invertebrates often require multiple venom extractions or the use of multiple animals. This results in an increase in experimental time and effort that often necessitates removing animals from the wild. However, our results show that although prolonged waiting periods between venom extractions seem to be necessary for complete regeneration of venom protein abundances, they are not necessary for studies that only require the presence of an individual protein in the venom, at least not for those involving *S. viridis* and *C. hentzi*. Therefore, studies on bio-prospecting, drug discovery, or the analysis of single proteins could perform venom extractions immediately upon capture and subsequently release the animal, decreasing the impacts on wild populations.

5 Conclusions

The results of our study build upon the growing literature detailing asynchronous regeneration of venom protein components in invertebrates, particularly centipedes. Future experiments utilizing mass spectrometry of invididual venom components is needed to confirm which venom components are experiencing asynchronous regeneration. Furthermore, our inability to detect asynchronous venom regeneration in *C. hentzi* provides evidence for a scorpion species that may not experience asynchronous regeneration of venom protein components. However, this lack of asynchronous venom regeneration could have resulted from our low sample size or the high levels of intraspecific venom variation identified in *C. hentzi*, indicating that further studies would be needed to confirm this finding. We also observed that the time required for complete venom regeneration in *S. viridis* and *C. hentzi* differed from the regeneration of other invertebrates, highlighting the need for designing species-specific extraction protocols. Finally, the presence of all

venom RP-HPLC peak clusters after one day of venom regeneration in both *S. viridis* and *C. hentzi* provides convincing evidence that studies relying on just the presence/absence of individual proteins (*e.g.* bioprospecting, drug discovery) could use catch-and-release methods of venom extraction, ultimately reducing the number of animals removed from the wild.

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Conflicts of Interest

The authors declare no conflicts of interest.

Availability of Data and Materials

All data are available within the manuscript and supplemental files.

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Figures and Figure Legends

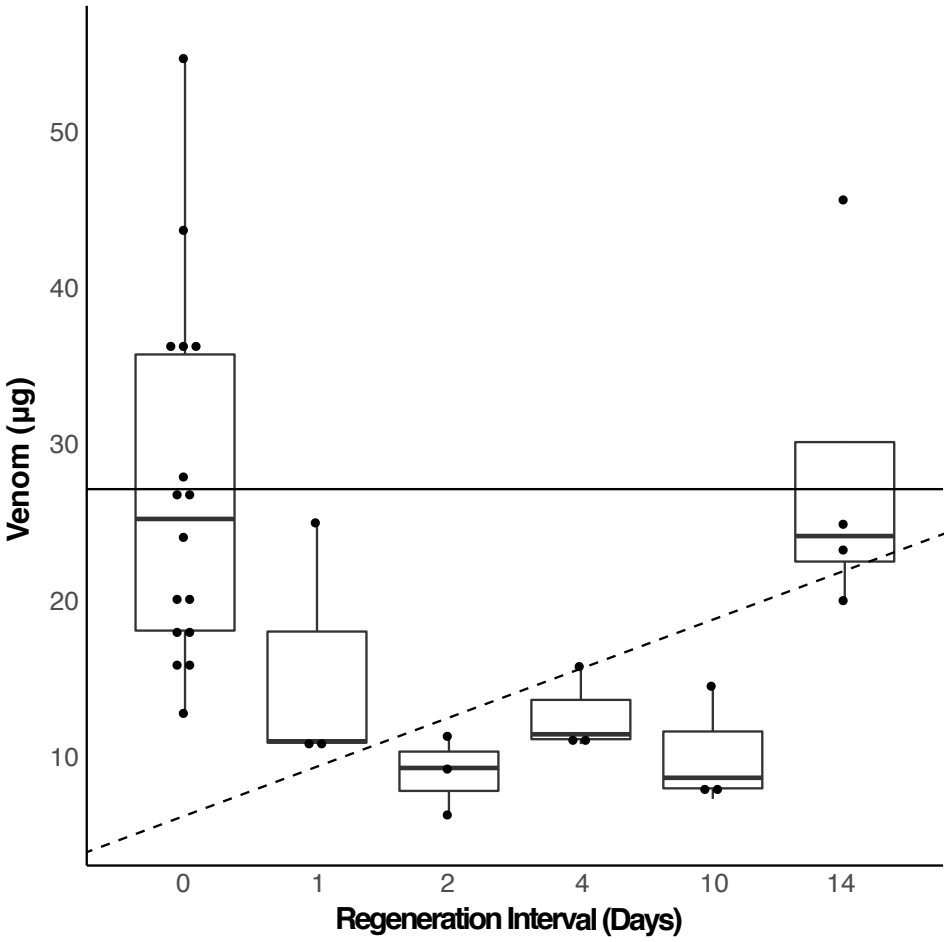


Figure 1. Boxplot showing the change in total venom protein content from between the initial venom samples and the 1–14 day regenerated venom samples in *S. viridis*. The solid horizontal line represents the mean quantity of venom in μg of the initial, non-regenerated venom samples. The dashed line represents a regression of venom quantity by regeneration interval, not including initial samples at Day 0 ($\rho = 0.39$, $R = 0.47$, $R^2 = 0.22$, and $p = 0.07$).

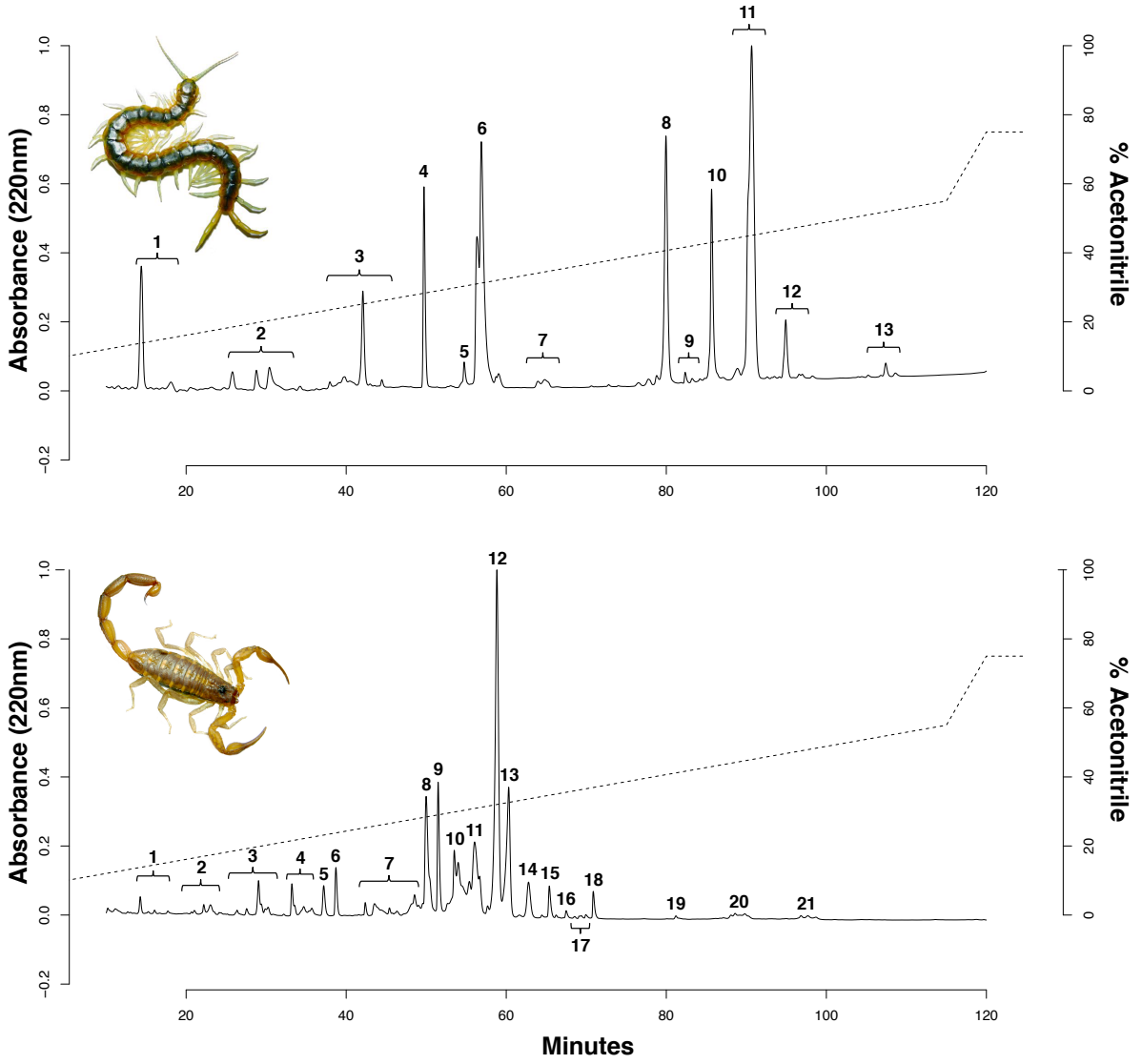


Figure 2. Representative RP-HPLC profiles with numbered peak clusters for *S. viridis* (top; 13 total peak clusters) and *C. hentzi* (bottom; 21 total peak clusters) venom.

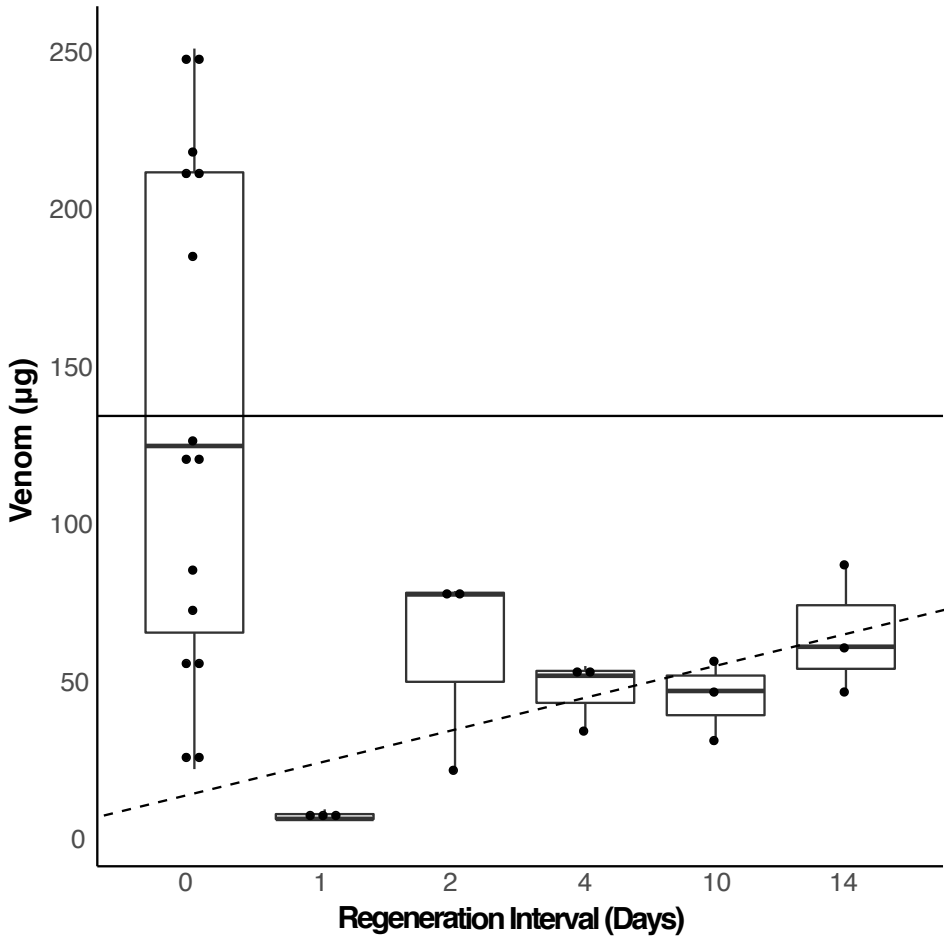


Figure 3. Boxplot showing the change in total venom protein content from between the initial venom samples and the 1–14 day regenerated venom samples in *C. hentzi*. The solid horizontal line represents the mean quantity of venom in μg of the initial, non-regenerated venom samples. The dashed line represents a regression of venom quantity by regeneration interval, not including initial samples at Day 0 ($\rho = 0.55$, $R = 0.57$, $R^2 = 0.32$, and $p = 0.03$).

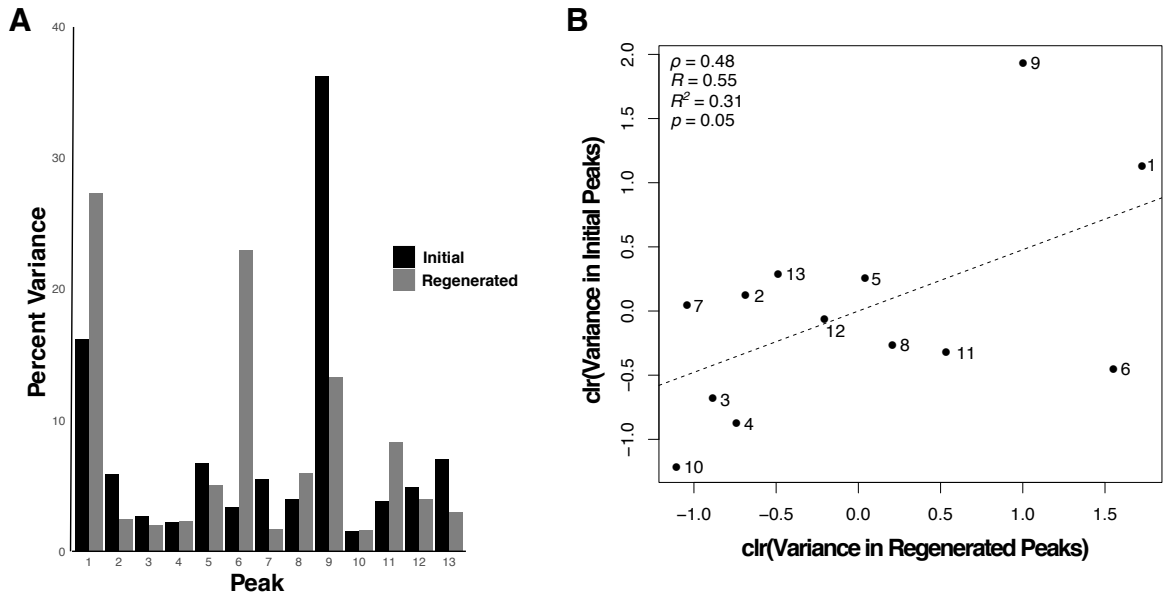


Figure 4. (A) Barplot showing the percent variance for each RP-HPLC peak cluster in both the initial and regenerated venom samples from *S. viridis*. The five peak clusters that contributed the most variation in the initial and regenerated venom samples were peak clusters 1, 2, 5, 9, and 13, and 1, 6, 8, 9, and 11, respectively. (B) Regression of clr-transformed variance in the initial venom peak clusters and clr-transformed variance in the regenerated venom peak clusters shows significant agreement.

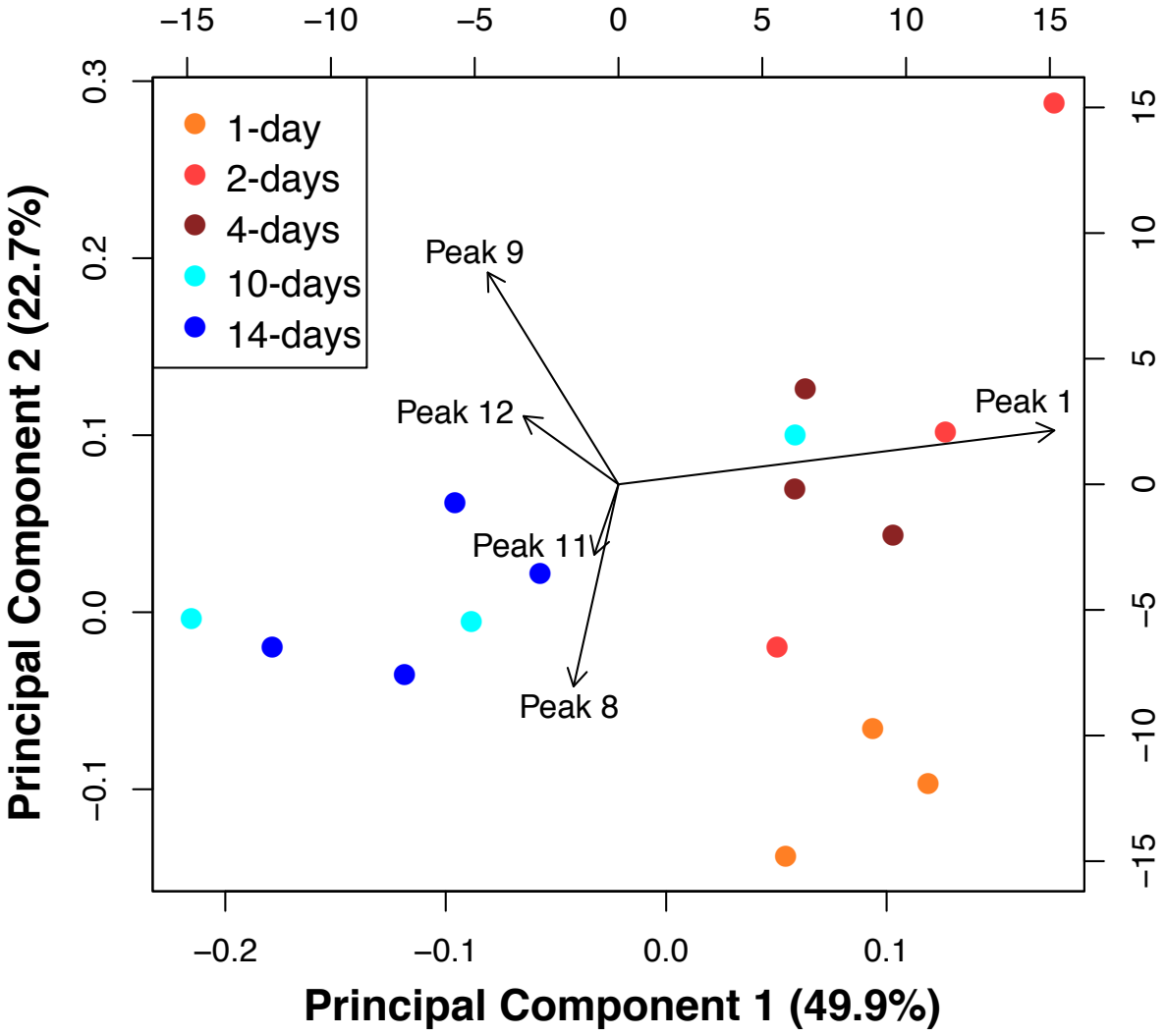


Figure 5. Principal component analysis of *S. viridis* extracted venom samples at each regeneration time interval (*i.e.* 1, 2, 4, 10, 14 days post-initial venom extraction) reveals a distinct separation between venom samples extracted at shorter regeneration intervals (*i.e.* 1, 2, and 4 days) compared to longer regeneration intervals (*i.e.* 10 and 14 days).

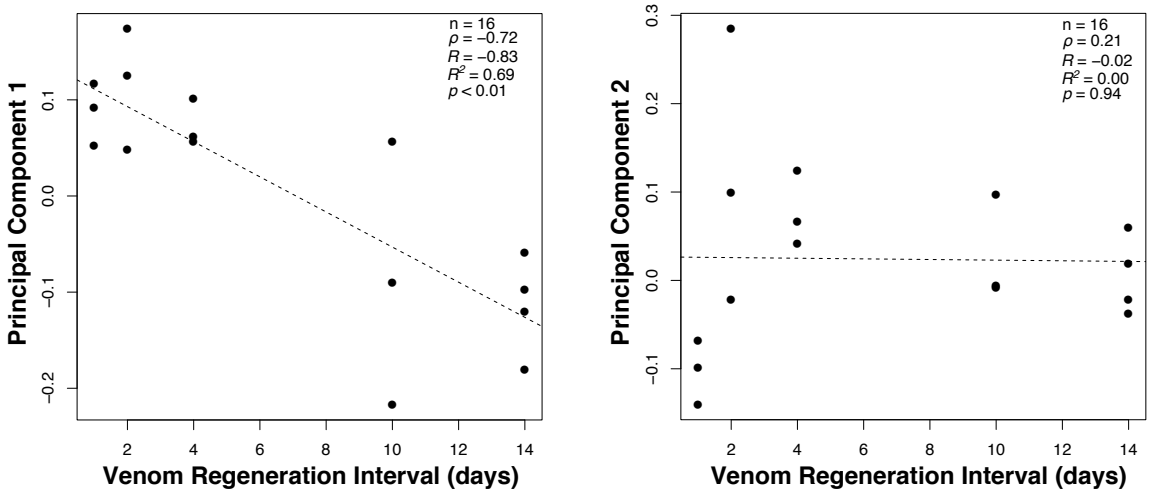


Figure 6. Regression of principal component 1 (left) and principal component 2 (right) by regeneration interval reveal a significant relationship between PC1 and venom regeneration interval, providing statistical evidence for asynchronous venom regeneration in *S. viridis*.

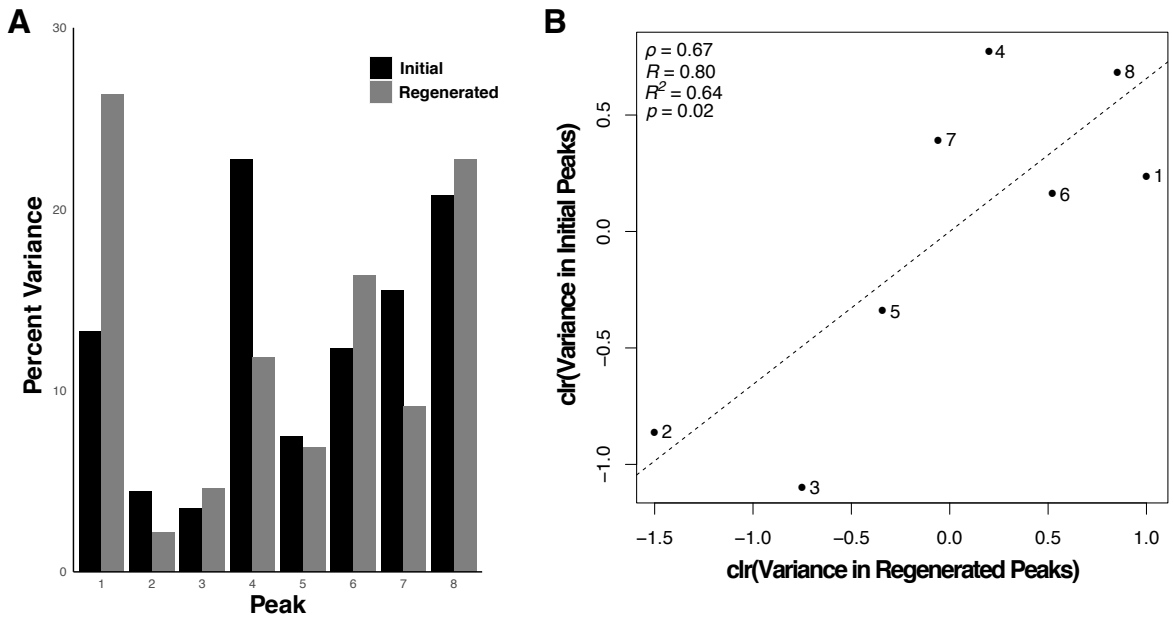


Figure 7. (A) Barplot showing the percent variance for each RP-HPLC binned peak cluster in both the initial and regenerated venom samples from *C. hentzi*. The five peak clusters that contributed the most variation in both the initial and regenerated venom samples were peaks 1, 4, 6, 7, and 8. (B) Regression of clr-transformed variance in the initial venom peak clusters and clr-transformed variance in the regenerated venom peak clusters shows a weak agreement.

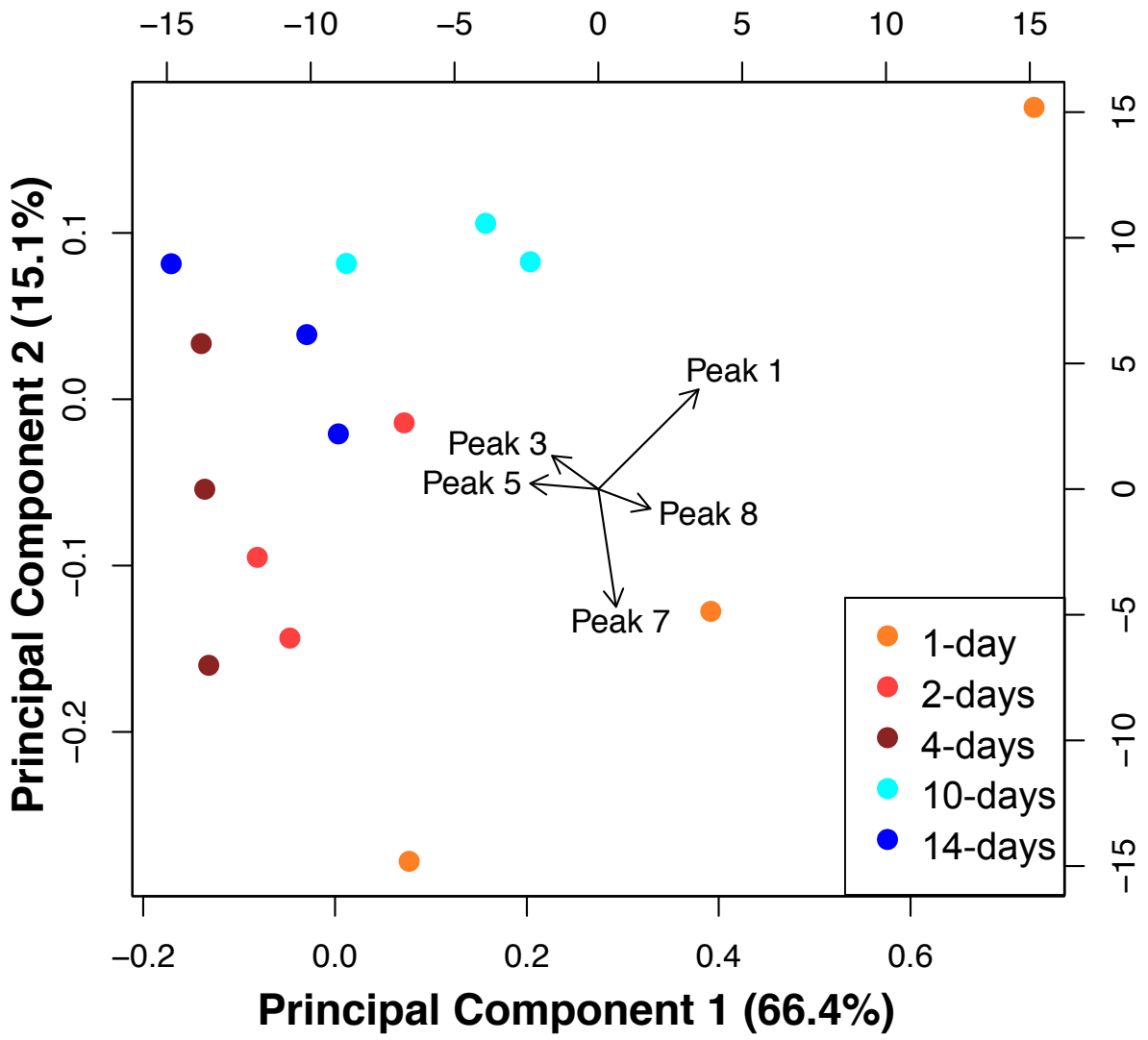


Figure 8. Principal component analysis of *C. hentzi* extracted venom samples at each regeneration time interval (*i.e.* 1, 2, 4, 10, 14 days post-initial venom extraction) does not reveal a distinct separation among regeneration intervals.

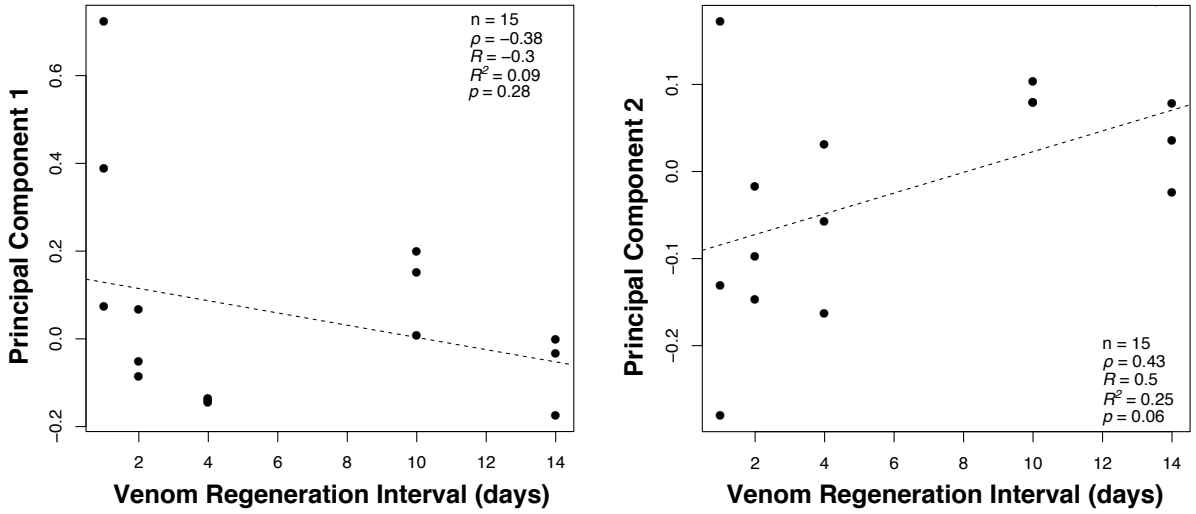


Figure 9. Regression of principal component 1 (left) and principal component 2 (right) by venom regeneration interval do not reveal any significant relationship between principal components and *C. hentzi* venom regeneration intervals.

Supplemental Figures and Supplemental Figure Legends

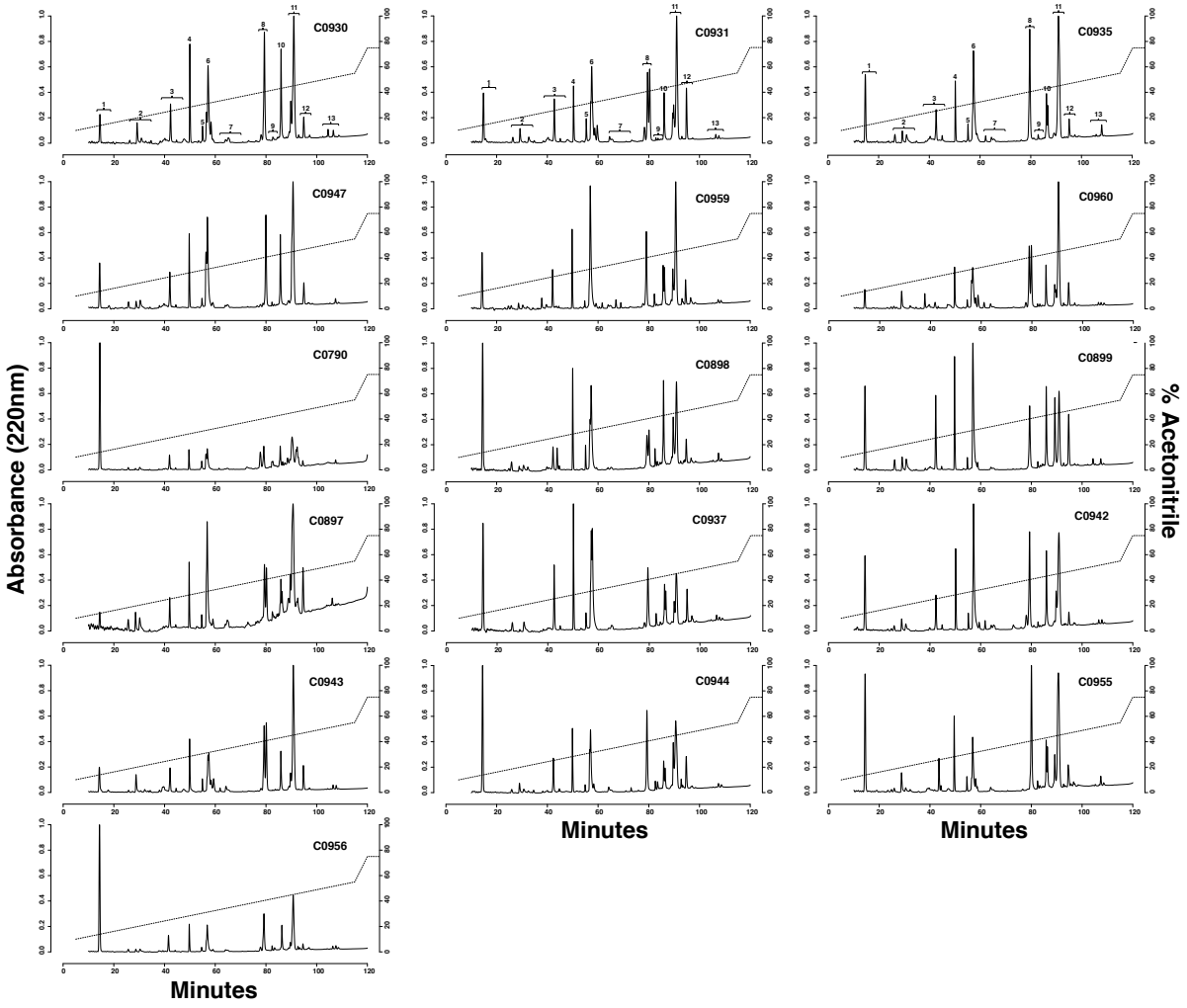


Figure S1. RP-HPLC profiles for all 16 *S. viridis* initial venom sample RP-HPLC profiles (labeled by specimen ID) with individual peak clusters labeled for the top profile in each column. RP-HPLC profiles are standardized to the highest peak cluster in each profile with peak cluster heights representing relative, not absolute, peak cluster abundances. Dashed lines represent the acetonitrile gradient.

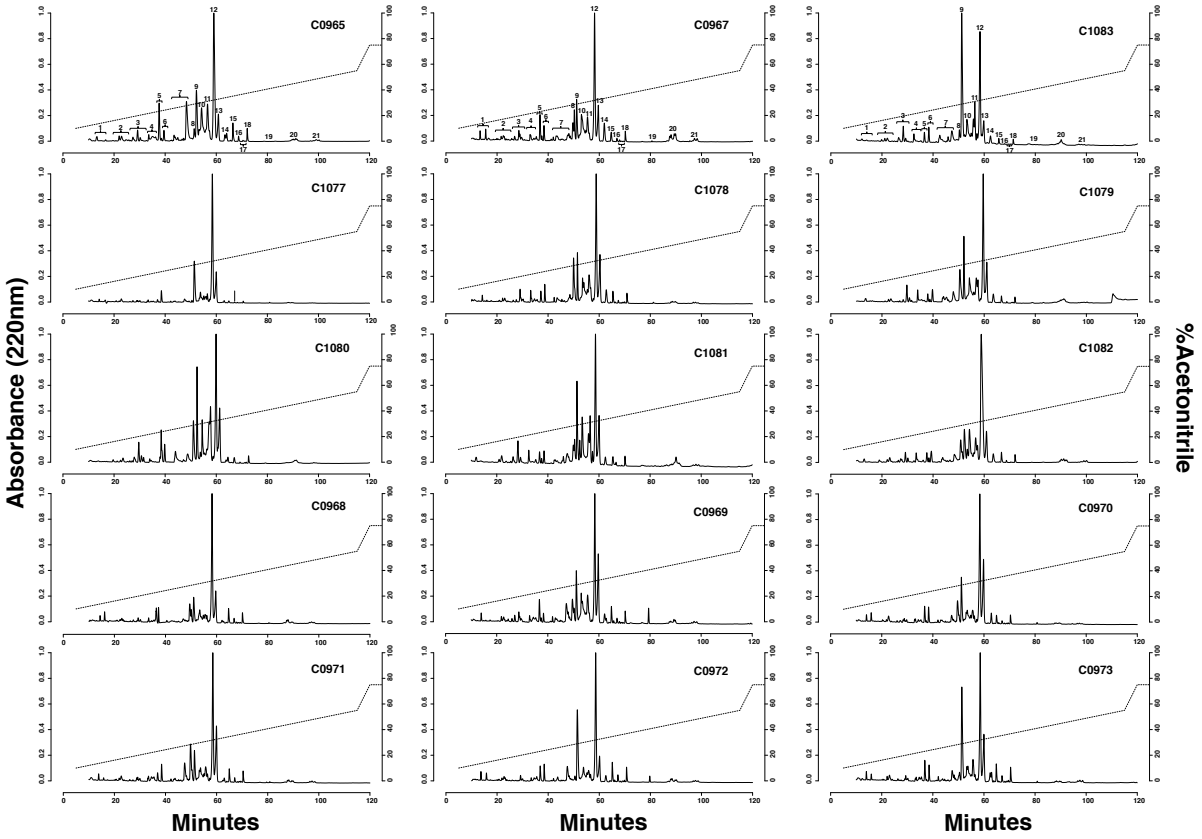


Figure S2. RP-HPLC profiles all 15 *C. hentzi* initial venom samples (labeled by specimen ID) with individual peak clusters labeled for the top profile in each column. RP-HPLC profiles are standardized to the highest peak cluster in each profile and differences in peak clusters across profiles represent differences in relative, not absolute, peak cluster abundances. Dashed lines represent the acetonitrile gradient.

- Asynchronous regeneration of venom protein content in the centipede, *Scolopendra viridis*.

- Lack of asynchronous venom regeneration in the scorpion, *Centruroides hentzi.*

- Venom regeneration in *Scolopendra viridis* takes at least 10-14 days.

- Venom regeneration in *Centruroides hentzi* takes at least 14 days.

- Presence-absence differences in *Scolopendra viridis* venom components across regeneration intervals are not asynchronous.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Darin R. Rokyta reports financial support was provided by National Science Foundation. Darin R. Rokyta reports a relationship with National Science Foundation that includes: funding grants.

Ethical Statement:

Reporting standards: The authors declare that our manuscript describes original research and every effort was made to ensure the accuracy of the results and the account.

Data Access and Retention: The authors declare that all data is reported and provided within the manuscript and that there is no additional data to make available.

Originality and Plagiarism: The authors declare that our manuscript is an original work with proper citations as needed.

Multiple, Redundant or Concurrent Publication: The authors declare that the data and work described in our manuscript has not and will not be submitted for consideration to another journal.

Acknowledgment of Sources: The authors have provided proper acknowledgment of sources to the best of their abilities.

Authorship of Paper: All authors of the manuscript made significant contributions, and no one making significant contributions was excluded from authorship. All authors have seen and read the submitted version of the manuscript and have approved submission.

Hazards and Human or Animal Subjects: This work did not involve the use of vertebrate animals.

Disclosure and Conflicts of Interest: The authors declare no conflicts of interest.

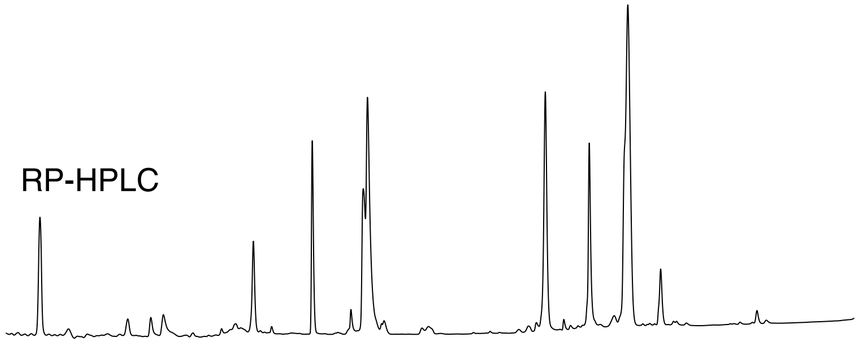
Fundamental Errors in Published Works: If a fundamental error or inaccuracy is discovered in the results described by our literature review, the authors will immediately notify the editor or publisher.

Contrasting patterns of venom regeneration in a centipede and a scorpion *(Centruroides hentzi)*

Scolopendra viridis
n = 16

Venom extraction

RP-HPLC



Venom regeneration interval

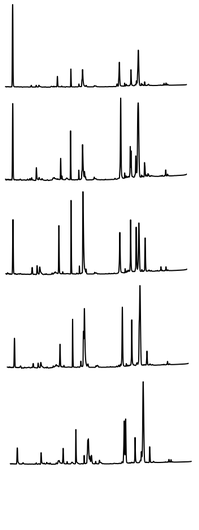
1 day

2 days

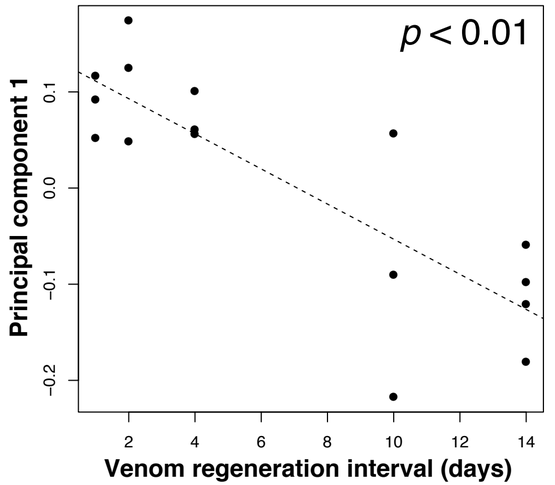
4 days

10 days

14 days



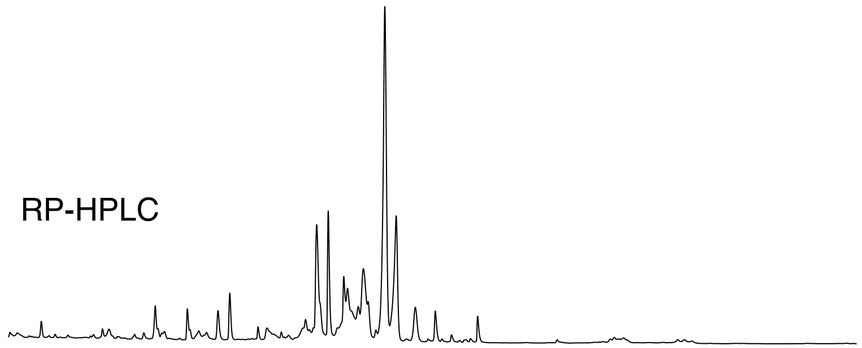
Asynchronous venom regeneration in *S. viridis*



Centruroides hentzi
n = 15

Venom extraction

RP-HPLC



Venom regeneration interval

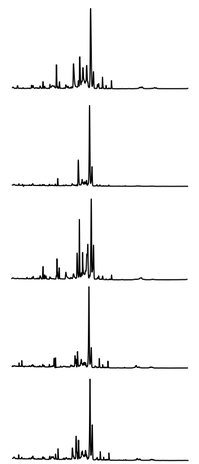
1 day

2 days

4 days

10 days

14 days



No asynchronous venom regeneration in *C. hentzi*