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Unique physiological and regulatory activity drives divergent toxin and non-toxin gene expression in rattlesnake accessory venom glands

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ABSTRACT

Understanding the mechanisms by which organs and tissues evolve new physiological functions is central to understanding the evolution of novelty. This is particularly interesting in the context of related tissues that evolve specialized, yet complementary, functions. Snake venom glands are an attractive system to test hypotheses related to the evolution and specialization of novel physiological function, as these modified salivary glands have evolved over ~60 MY to synthesize and store venom. Front-fanged venomous snakes (elapids and viperids) possess two types of venom glands: the main and accessory glands. The larger main gland produces greater quantities of venom toxins and has been studied extensively, while the smaller accessory gland has received less attention. Here, we explore gene expression differences between main and accessory venom glands across three rattlesnake species (*Crotalus cerberus*, *C. oreganus concolor* and *C. viridis*). Our findings indicate that accessory glands express most venom genes at significantly lower levels than the main gland, with a few exceptions that may represent biologically relevant contributions of accessory glands to venom. The two glands also exhibit distinct trans-regulatory environments that we link to key differences in their underlying physiology and secretory roles. Our results further suggest that two signaling pathways that regulate venom, the unfolded protein response (UPR) and extracellular signal-regulated kinase (ERK), show significantly lower activation in the accessory gland. These findings provide insight into the physiological and functional diversification of snake venom systems, highlighting how distinct glandular systems have evolved contrasting and complementary roles driven by distinct physiological and regulatory mechanisms.

1. Introduction

Snake venoms consist of complex mixtures of enzymes and peptide toxins that are produced and stored in specialized venom glands (Mackessy and Baxter, 2006). Front-fanged venomous snakes (viperids and elapids) possess paired maxillary glands on either side of the head, each of which includes a large primary (or main) venom gland, and a much smaller anterior gland referred to as the accessory venom gland (Kerkkamp et al., 2017; Mackessy, 2022). Given its relative size, the main gland is assumed to function as the primary site for venom production and luminal venom storage, and is by far the most well-studied of the two glands, while the accessory venom gland and its function has received less attention (Mackessy and Baxter, 2006). Several studies have documented distinctions in venom gene expression (Schield et al., 2019; Valente et al., 2018b; Vonk et al., 2013; Zancolli et al., 2022) and differences in glandular structure and morphology between main and accessory glands (Gans and Kochva, 1965; Mackessy and Baxter, 2006; Mackessy, 2022; Valente et al., 2018a). However, there remain major gaps in our understanding of the molecular basis of the accessory gland's distinct physiology, its functional relevance, and

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how these may differ from, or potentially complement, those of the main venom gland.

Considering the location of the accessory gland — between the main gland and the maxillary fang — early studies hypothesized that the accessory gland might produce a different, but complementary, subset of activators or/and venom toxins from the main venom gland (Gans and Kochva, 1965; Mackessy and Baxter, 2006; Mackessy, 2022; Valente et al., 2018b). Other studies have predicted that the accessory gland may play a role in activating venom before excretion, or increasing the overall toxicity of toxins produced by the main gland (Gans and Kochva, 1965). However, subsequent studies have largely refuted this, showing that venom collected directly from the main gland lumen, thereby bypassing the accessory gland, had an overall proteomic composition that was indistinguishable from whole venom extracted through the fang (Mackessy and Baxter, 2006). To explore differences in gene expression between the two glands, previous studies have measured differential gene expression for the genes that encode venom toxins (which we refer to as 'venom genes') between accessory and main venom glands and found evidence for divergent expression patterns of venom genes (Kerkkamp et al., 2017; Perry et al., 2022; Schield et al., 2019; Valente et al., 2018b; Vonk et al., 2013). For example, members of the C-type lectin toxin family were expressed 40 times higher in the accessory venom gland compared to the main gland in the elapid *Ophiophagus hannah* (Vonk et al., 2013). Similarly, the accessory and main venom glands of the viperid *Bothrops jararaca* also showed highly divergent patterns of venom toxin expression, with nearly all venom gene families being expressed at relatively lower levels in the accessory compared to the main gland (Kerkkamp et al., 2017; Valente et al., 2018b). These findings suggest that the accessory gland may have some role in producing toxins or conditioning venom as it leaves the secondary duct. However, the degree to which the accessory gland contributes to overall venom toxin production, and how consistent variation between the main and accessory gland venom expression may be across individuals and species, remains poorly understood.

Beyond the expression of venom toxins, prior studies have also provided evidence that the accessory gland may have additional mechanisms for neutralizing the auto-toxic effects of venom during storage and secretion. For example, Valente et al. (2018b) detected relatively higher expression of several phospholipase A_2 (PLA_2) and snake venom metalloproteinase (SVMP) inhibitors in the accessory venom gland, which may serve as a protective mechanism against the auto-toxic effects of these venom proteins (Mackessy and Baxter, 2006). Additionally, the epithelium of the anterior accessory gland is largely comprised of mucus-secreting cells (Mackessy and Baxter, 2006); therefore, the accessory gland may also use secreted mucus to protect the gland and duct epithelium. Similar to the main gland, additional protection from the bioactive effects of venom may also be coordinated through the activity of mitochondria-rich cells within the accessory gland (Mackessy, 1991; Mackessy and Baxter, 2006). These cells are thought to play a role in acidifying the lumen of the main venom gland via the activity of vacuolar ATPase proton pumps (V-ATPases), which effectively inhibits the proper folding (and thus function) of venom proteins (Mackessy and Baxter, 2006; Perry et al., 2020). However, the mechanisms used to neutralize venom activity in the accessory gland, and how these may differ from the main gland, have not been fully investigated.

Evidence for the distinct cellular structure and gene expression profile of the accessory venom gland suggests that the gland may represent a useful model for comparisons with the main venom gland to understand how venom gene expression is regulated in distinct cellular and glandular contexts. Recent work has substantially advanced our understanding of the gene regulatory mechanisms that control the expression of snake venom genes within the main venom gland (Gopalan et al., 2024; Hogan et al., 2024; Margres et al., 2021; Perry et al., 2022; Westfall et al., 2023). This has included the identification of cis-regulatory elements (e.g., enhancers and promoters) and trans-regulatory factors (e.g., transcription factors) involved in regulating snake venom gene expression, along with the higher-level signaling pathways that control these processes (Perry et al., 2022). Two key signaling pathways, the unfolded protein response (UPR) and extracellular signal-regulated kinase (ERK) pathway, are hypothesized to have been evolutionarily recruited to regulate the expression of rattlesnake venom genes within the main venom gland (Perry et al., 2022), and the involvement of these pathways has also been implicated in the regulation of venom in other systems (Kerchove et al., 2004; Zancolli et al., 2022). While evidence for the differential expression of venom genes between main and accessory glands inherently suggests that the two glands may recruit distinct sets of transcription factors, or differentially activate key signaling pathways, this hypothesis has not been investigated.

Here, we conduct a systematic comparison of gene expression between the accessory and main venom glands in rattlesnakes to address gaps in our understanding of the distinct physiological and functional roles of the accessory venom gland, and the regulatory mechanisms that may underlie these distinctions. We address these broad questions by analyzing mRNA-seq data derived from paired (i.e., from the same individual) main and accessory venom glands collected from ten individuals across three closely related rattlesnake species: *Crotalus cerberus*, *C. oreganus concolor* and *C. viridis*. With this comparative dataset, we examine patterns of venom gene expression between the two glands to test the hypothesis that accessory glands may contribute distinct toxin components to venom. We also test for differences in the expression of genes that don't encode venom toxins (which we refer to as "non-venom genes") between the two glands, including those related to mucus production, lumen acidification, venom inhibition, and other non-toxin proteins. To explore divergence in the trans-regulatory environments of both glands, we test for correlations between transcription factor and venom gene expression and compare these inferences to prior evidence for transcription factor binding sites present in predicted cis-regulatory elements of venom genes. Lastly, to test the hypothesis that divergent venom gene expression patterns are driven by differences in regulatory pathway activity in the accessory and main glands, we compare patterns of venom-associated transcription factor expression and ERK and UPR pathway activation between gland types. Together, our findings provide new insights into the distinct function of the snake venom accessory gland and new broad perspectives on how distinct and complementary functions of organs may arise through the divergent activity of key signaling pathways.

2. Materials and methods

2.1. Tissue sampling and mRNA-seq data generation

Left and right main venom glands, right accessory venom glands, and venom were collected from a total of ten specimens from three rattlesnake species (*Crotalus cerberus* (n = 1), *C. o. concolor* (n = 1), and *C. v. viridis* (n = 8); Crother, 2012; Uetz et al., 2024). These glands were collected for a prior study, and the collection methodology is explained here in brief. Specimens were acquired from natural populations in Colorado, New Mexico, Arizona, and Utah under appropriate scientific collecting permits, and housed at the University of Northern Colorado under the approved IACUC protocol 2303D-SM-S-26. Venom extraction was conducted manually one day before harvesting venom gland tissues. The gland tissues were preserved by flash freezing in liquid nitrogen and stored at −80 °C. The details of the specimen's NCBI accession, sex, latitude, longitude, and other data are provided in Supplementary Table 1.

Total RNA was extracted from flash-frozen main and accessory gland tissues using TRIzol reagent (Invitrogen Life Technologies, No. 15596026). Poly-A selected mRNA libraries were prepared and sequenced on an Illumina NovaSeq 6000 platform using 150 bp paired-end reads by Novogene Corporation (Sacramento, CA, USA). Because

one of the 20 main gland samples failed post-sequencing quality control, only 19 main gland samples, along with all ten accessory gland samples, were analyzed further. Raw reads were trimmed using Trimmomatic v0.39 (Bolger et al., 2014) and were mapped to the *Crotalus viridis* reference genome (NCBI GCA_003400415.2, Schield et al., 2019) using STAR v2.7.9 (Dobin et al., 2013). Reads mapped to exons were counted and summarized by gene using featureCounts v1.6.3 to provide expression estimates (Liao et al., 2014). DESeq2 (v1.30.1; Love et al., 2014) was then used to perform differential gene expression analyses between main and accessory gland samples. This included producing estimates of $\log_2$ fold change in expression between gland types, as well as producing normalized count matrices (using the 'counts' command) and variance stabilizing transformed (VST) count matrices (using the 'vst' command) used for downstream analyses (Anders and Huber, 2010).

2.2. Analysis of differential gene expression

To explore gene expression differences between accessory and main gland tissues, we analyzed normalized counts of mRNAseq reads using DESeq2 (Love et al., 2014) from 10 accessory gland and 19 main venom gland samples using principal component analysis (PCA) with the package ggplot v3.5.1 (Wickham, 2016). We used the VST counts (from DESeq2) to generate a heatmap for venom gene expression. We then log-transformed the DESeq2 normalized counts for all venom genes across all samples. We then grouped samples based on gland type and calculated the average counts of each venom gene within accessory gland samples and main gland samples separately. Then, for each venom gene, we subtracted the average counts in the accessory gland samples from the average counts in the main gland samples. These between-gland differences in average log-transformed counts were then visualized using a bar graph. Log-transformed normalized counts were also used to visualize expression of genes in and adjacent to the SVMP gene array.

We used the Human Protein Atlas (proteinatlas.org) to identify genes that code for non-venom proteins within both gland types. Expression (VST counts) of mucin genes were visualized using a heat map, and the expression of select V-ATPases known to be involved in gland lumen acidification (Perry et al., 2020) were visualized using a volcano plot. Genes that were significantly more highly expressed in the accessory gland were categorized into functional categories using WebGestalt and organized into subnetworks using STRINGdb (Szklarczyk et al., 2023) and Cytoscape (Shannon, 2003).

2.3. Correlating expression of venom genes with relevant transcription factors

Correlations between transcription factors (TFs) and venom gene expression were performed following methods from a prior study (Gopalan et al., 2024) with minor modifications explained below. To obtain an unbiased assessment of TFs that may putatively regulate venom genes in the accessory gland, we began with a comprehensive set of genes that encode human transcription factors (Lambert et al., 2018), which does not rely on prior inferences of what TFs may be relevant for regulating venom genes. To avoid considering TFs with particularly low or inconsistent expression (and thus, likely not functionally relevant or that may produce spurious correlations), we filtered this list by only retaining TFs with expression observed in more than 80 % of samples from our combined accessory and main venom gland dataset and that had VST expression values greater than six. Correlations of expression levels between all retained TF and venom genes were calculated using Pairwise Pearson's rho in R v4.2.3 (R Core Team, 2023) using the "rcorr" function from the "Hmisc" package(https://cran.r-project.org/web/packages/Hmisc/index.html) to create a matrix of correlation coefficients for these comparisons. Correlations were performed on accessory and main gland expression datasets separately, as well as a combined dataset including both glands. These coefficient matrices were filtered for p-value and false discovery rate (both $<10^{-5}$) to produce significance-filtered TF-venom gene correlation matrices.

From the correlations predicted, we next identified which subset of these correlations may reflect direct regulatory interactions based on evidence that venom gene promoters or enhancers (i.e., cis-regulatory elements; CREs; Perry et al., 2020) contained a 'substantial' presence of binding sites for the TF. Of the 42 TFs with significant correlations from the combined dataset, 17 had known TF binding site (TFBS) motifs from the JASPAR 2022 core non-redundant vertebrate database (Castro-Mondragon et al., 2022) that were also located within venom gene CREs. Using these 17 TFs, we scanned for the presence of TFBSs in *C. viridis* CREs using Ciiider v0.9 with default settings (Gearing et al., 2019). Finally, to determine whether TFs had a 'substantial' presence in venom gene CREs, TFs were considered to have passed this threshold if the total number of TFBSs across all CREs for a given venom gene exceeded the median number of TFBSs per gene for the dataset (27 TFBSs per TF per gene). In other words, TFs with fewer than 27 TFBSs in the venom gene CREs for a given gene were not considered to have a 'substantial' presence. Based on this analysis, we identified seven TFs with significant venom-TF gene expression correlations that also had substantial presence in venom gene CREs.

2.4. Transcription factor expression and ERK/UPR pathway activity

To identify differentially expressed TFs between accessory and main glands, we compiled a list of 158 transcription factors that previous studies have suggested regulate venom gene expression in snakes. In brief, previous studies identified 81 TFs that were upregulated in the snake venom gland compared to non-venom tissues and had binding sites in venom gene CREs (i.e., promotors and enhancers) in the Prairie Rattlesnake (Perry et al., 2022; Gopalan et al., 2024). Additionally, 77 TFs were previously identified as high-level regulons (or regulators) of gene expression in the snake venom gland based on single-nucleus RNA-seq data (Westfall et al., 2023; Supplementary Table 2). Of these 158 TFs, 157 were expressed in one or both glands and 101 were significantly differentially expressed between gland types. We then used StringDB (Szklarczyk et al., 2023) to infer functional interactions between a subset of 83 TFs and visualized the interactions using Cytoscape (Shannon, 2003). We also used this interaction network to further compare patterns of individual TF expression based on mean expression levels observed in main versus accessory glands. Finally, we estimated the relative activation of ERK and UPR pathways using the 'gvsa' package in R (Hänzelmann et al., 2013). The analysis was performed using VST counts, filtering for genes predicted to be involved in each pathway (Westfall et al., 2023; Supplementary Table 3).

3. Results

3.1. Overall gene expression variation between accessory and main venom glands

When we analyzed expression across all genes, samples from the two gland types formed two distinct non-overlapping clusters in principle component space (Fig. 1A), with PC1 separating main vs accessory gland samples, and representing 65 % of the total gene expression variation. In total, we identified 4437 differentially expressed genes in both glands, with 3087 of the genes more highly expressed in the accessory venom gland, and 1350 more highly expressed in the main venom gland (Fig. 1B; Supplementary Fig. 1). When we evaluated venom gene expression only, we found that more venom genes had significantly higher expression in the main venom gland (39 genes) compared to those that had higher expression in the accessory gland (3 genes; Fig. 1B and C). Several venom genes that are often lowly expressed in the main venom glands of rattlesnakes, such as some C-type lectins (CTL4, CTL6) and CRISPs (CRISP3, CRISP4; full names for venom gene families

Fig. 1. Gene expression differences between glands. A) Principal Component Analysis (PCA) displaying clear distinctions between the accessory venom gland (AVG) and the main venom gland (MVG). Samples from different species are indicated by color. B) Volcano plot showing differentially expressed genes between accessory and main gland, with significance for differential expression (adjusted $p < 0.05$) shown by the horizontal dashed line. Venom genes are shown in blue, and yellow dots are differentially expressed non-venom genes within the two glands. C) Differential venom gene expression between accessory and main glands. Dot color distinguishes genes from the different venom gene families. D) Heatmap of normalized VST counts of venom gene expression across samples, and between accessory and main venom glands. Individual tissues are shown in columns, labeled by gland type (AVG = accessory venom gland; MVG = main venom gland), left or right side (L, R), and numbers that correspond to specific individual animals sampled. Venom genes are indicated at the right, and clustered by venom gene family with colored bars. Full names of the venom genes are provided in Supplementary Table 4. E) Difference in log-transformed normalized venom gene counts (averaged across all samples from the same gland) between AVG and MVG. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

provided in Supplementary Table 4), have higher average log fold change difference in the accessory gland compared to the main gland, suggesting that some venom genes that are typically more lowly expressed in the main gland may be more highly expressed in the accessory venom gland (Fig. 1D and E). We also found higher expression of several non-venom paralogs of the SVMP venom gene family (ADAM28 and ADAMTS; Supplementary Fig. 2) within the accessory venom gland.

3.2. Functional divergence of accessory vs. main venom gland secretory physiology

Based on inferences for which genes produce secreted proteins in humans (Uhlén et al., 2015), we compared expression of 'secretome-associated' genes between the main and accessory glands. We find that the accessory venom gland expresses a much greater number of upregulated secretome-associated genes (over four-times as many), compared to the main venom gland (305 vs. 69 genes, respectively; Fig. 2A, Supplementary Fig. 3). Several secretome genes more highly expressed in the accessory gland are gel-forming mucins (e.g., MUC5B, MUC5AC; Fig. 2B; Supplementary Fig. 3). Three paralogs of the secreted, gel-forming mucin MUC5B represent the three most strongly expression-biased genes with higher expression in the accessory gland (Fig. 2B). Other non-gel-forming, membrane-bound mucins, such as MUC1, MUC15, and MUC16, also show strong relative upregulation in the accessory gland (Fig. 2B). Further, we find evidence that V-ATPases, essential genes for the acidification of the main venom gland lumen, have distinct patterns of expression between the main and accessory glands, with most V-ATPases expressed at far lower levels in the accessory gland (Fig. 2C). Several proteins previously identified as venom inhibitors (e.g., SERPINE1, SERPING1, SERPINC1, ANXA1; Gibbs et al., 2020; Stuqui et al., 2015; Oliveira et al., 2024) are also relatively highly expressed in the accessory gland (Fig. 2D). Finally, several genes involved in immune system function are more highly expressed in the accessory gland, such as the pro-inflammatory matrix metalloproteinases (MMP2, MMP9, MMP25, MMP28), activators of innate immune cells (e.g., CXCL8, TNF, PIGR, SFTPD) and several members of the complement system (C2, C3, C4B, CF1, CFB, CFD; Fig. 2D; Supplementary Fig. 3). These findings indicate that the accessory gland has a distinct gene expression profile compared to that of the main venom gland, and that accessory gland physiology may primarily involve venom inhibition, mucus production and innate immunity.

3.3. Expression of venom-associated transcription factors

To investigate differences in transcription factor expression between the two glands, we first analyzed differential expression of 158 TFs that have been associated with snake venom gene expression from prior studies (Gopalan et al., 2024; Perry et al., 2022; Westfall et al., 2023; Supplementary Table 2). Of these 158 TFs, 157 were expressed in one or both glands, and 101 were significantly differentially expressed between gland types (Supplementary Fig. 4). Given evidence for substantially divergent venom-associated TF expression patterns between accessory and main gland tissues, we first investigated the relationships between venom gene expression and TF expression separately in these two tissue types.

To test for evidence of predictive relationships between expression levels of venom genes and all possible TFs (not limited to known venom-associated TFs), we calculated TF versus venom gene correlations by first analyzing the accessory and main gland data separately (Fig. 3A and B). These correlations identify TFs that are either positively and negatively correlated with venom gene expression in the accessory gland, including TFs involved in ERK and UPR signaling (e.g., XBP1, SP1, SMAD3 and NFKB1; full names provided in Supplementary Table 4). Of the 31 TFs negatively correlated with the expression of venom genes in the accessory gland, 17 are significantly upregulated in the accessory gland (relative to the main gland), suggesting these TFs may play roles as active repressors of venom gene expression (Fig. 3A). There are also several TFs that are positively correlated with expression in the accessory gland; for example, AEBP1 positively correlates with CTL4 expression, and CTL4 was expressed more highly in the accessory gland (Fig. 1E). To compare these relationships between main and accessory gland, we repeated these analyses using only the data from main gland tissues and find very different patterns of correlations (Fig. 3B). This contrast in correlations between the glands suggests that key TFs that modulate the expression of venom genes in the two gland types are remarkably different, and may indicate that the two glands have distinct trans-regulatory environments, as we find only three TFs (ZNF236, ZNF654 and PA2G4) correlate with venom gene expression in both glands.

Expression levels of transcription factors may correlate with expression of venom genes for a number of reasons, one of which is that they may directly bind venom cis-regulatory elements. Based on evidence from prior studies that identified TFs with binding sites (TFBSs) within venom regulatory elements, we focused on the subset of correlations that are significant and the TF has a substantial number of TFBSs in that gene's CREs (see Methods, Fig. 3C). For these correlations, we combined all expression data from both accessory and main glands (Supplementary Fig. 5), which is justifiable because almost no separate correlations contradict one another (Fig. 3A and B), and combining data substantially increases our power to detect correlations (Fig. 3C and D). A total of 7 TFs were associated with both highly abundant TFBSs and significant correlations with venom gene expression. These include MEIS1, OSR1, SMAD3 and VEZF1, which are more highly expressed in the accessory gland and negatively correlated with venom gene expression (Fig. 3C). This pattern contrasts with that of ELF5, which is relatively highly expressed in the main venom gland and positively correlated with venom gene expression. For example, ELF5 expression positively correlated with CRISP1 gene expression ($p < 10^{-9}$; $R^2 = 0.78$), whereas MEIS1 expression negatively correlated with CRISP1 gene expression ($p < 10^{-9}$; $R^2 = 0.76$; Fig. 3D). These findings suggest a subset of TFs appear to act as repressors of venom gene expression, potentially through their binding at venom cis-regulatory elements, and show greater repressive activity (and expression) in the accessory gland versus the main gland.

Considering evidence for relatively lower venom gene expression in the accessory versus main venom gland, we investigated the hypotheses that the trans-regulatory factors and associated pathways known to regulate venom expression may also be divergent between accessory and main venom glands. To address this, we visualized an interaction network of 83 TFs that are predicted to be regulated by the UPR and/or ERK pathways (Supplementary Table 2) along with their relative expression levels in main versus accessory glands and find evidence that 62 % of these TFs are significantly differentially expressed between the two glands. Indeed, 23 TFs are more highly expressed in the accessory gland and 30 are more highly expressed in the main gland (Fig. 4A). Many differentially expressed TFs that are associated with the ERK and/or UPR pathways, such as XBP1, ATF6, TBX3, and CREB3 (Fig. 4A), are also more highly expressed in the main venom gland, suggesting that these pathways known to regulate venom may be differentially activated in accessory versus main glands. To test this hypothesis, we compared the activation/enrichment of expressed genes predicted to be involved in the ERK and UPR pathways (Supplementary Table 3) between the different gland types and find evidence for significantly lower activation of both pathways in the accessory compared to the main gland (Fig. 4B).

4. Discussion

Despite broad scientific and medical interest in snake venoms and venom systems, the accessory venom gland has received remarkably little attention or systematic study. Our work represents the first systematic comparison of accessory and main gland gene expression within and across species that looks beyond venom toxin expression to understand the accessory gland's distinct physiology and potential functional roles. We find that the majority of venom gene transcripts expressed in the main gland are also expressed in the accessory gland, but at lower levels. However, we also find evidence that a small set of venom genes are expressed at significantly higher levels in the accessory gland (including several CTLs and CRISPS), suggesting that the accessory gland may indeed contribute a small number of distinct toxins to venom. Our results also highlight evidence that the accessory gland appears to

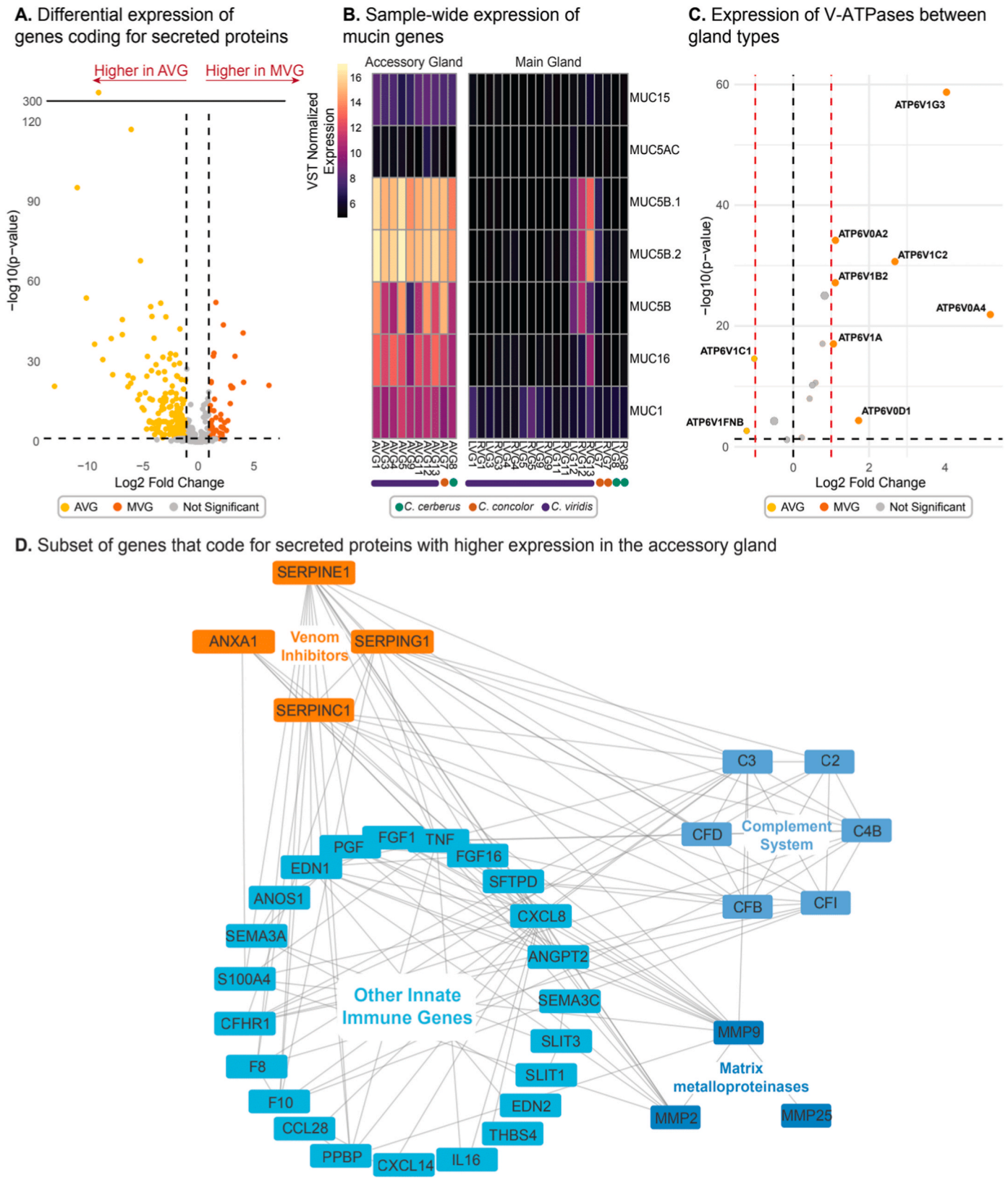
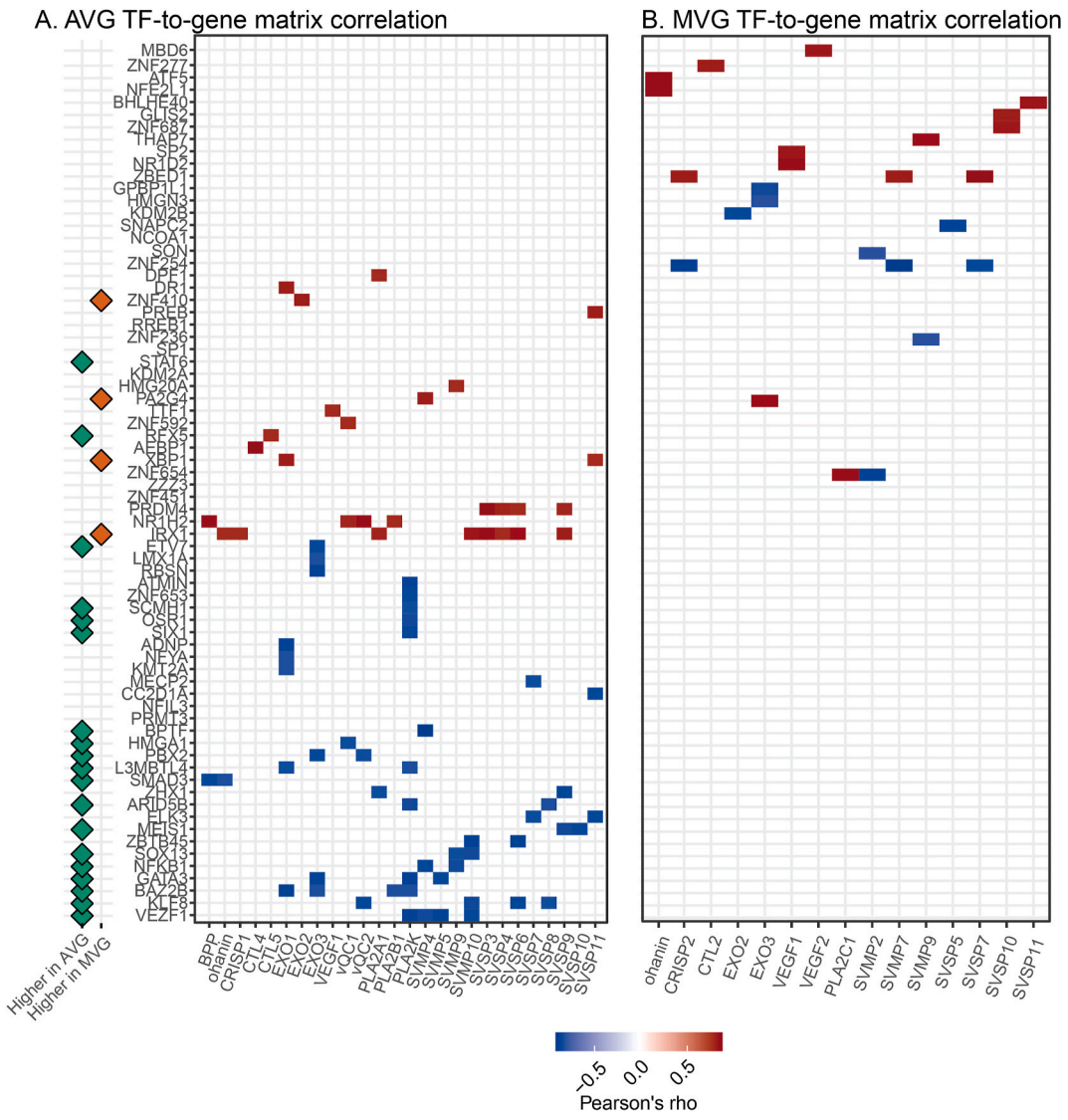
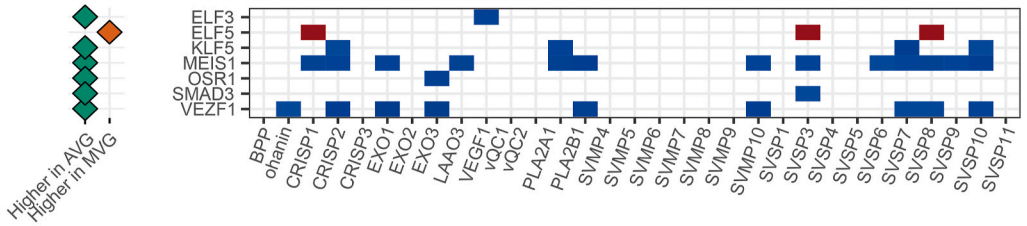


Fig. 2. Differential expression and functional analysis of non-venom genes. A) Differential expression of genes that code for secreted proteins within the main and accessory venom glands. B) Sample-wide comparison of mucin (MUC) gene expression indicates that all mucin genes were more highly expressed in accessory compared to the main gland. C) Expression of V-ATPase genes (ATP) between gland types. D) STRINGdb interaction network (visualized using Cytoscape) of genes coding for secreted proteins with higher expression in the accessory gland, many of which are involved in innate immunity, inflammatory pathways, and pathogen defense. Full names of the genes are provided in Supplementary Table 4.



C. Combined AVG-MVG correlation matrix for TFs with TFBSs in venom CREs



D. Selected regressions from combined AVG-MVG correlation matrix

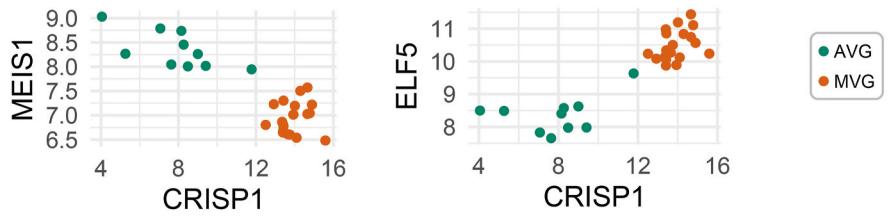


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Fig. 3. Transcription factor-to-gene correlations. A) Matrix of correlations (in Pearson's rho) between expression of transcription factors and expression of venom genes in the accessory gland only. Transcription factor expression data are shown as colored diamonds to the left, with green diamonds indicating that the TF has significantly higher expression in the accessory gland (AVG), and orange diamonds showing TFs with significantly higher expression in the main gland (MVG). Only correlations passing the significance threshold (FDR and p-value $<10^{-5}$) are shown as colored squares. B) Matrix of correlations (in Pearson's rho) between venom gene and TF expression in main gland samples only. C) Subset of the full correlation matrix which only displays transcription factors with evidence of binding sites at the promoters and enhancers of the venom genes they are predicted to regulate. The same functional annotations from panel (A) are shown to the left. Only correlations passing the significance threshold (FDR and p-value $<10^{-5}$) are shown as colored squares. D) Two selected regressions with strong correlations between TF expression (MEIS1 and ELF5) and venom gene expression (CRISP1) were chosen for illustration. Both the CRISP1 x MEIS1 correlation ($p < 10^{-9}$; $R^2 = 0.76$) and the CRISP1 x ELF5 ($p < 10^{-9}$; $R^2 = 0.78$) correlation are significant. Gland type for each sample is indicated in green (AVG) or orange (MVG). Full names of the transcription factors and venom genes are provided in Supplementary Table 4. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

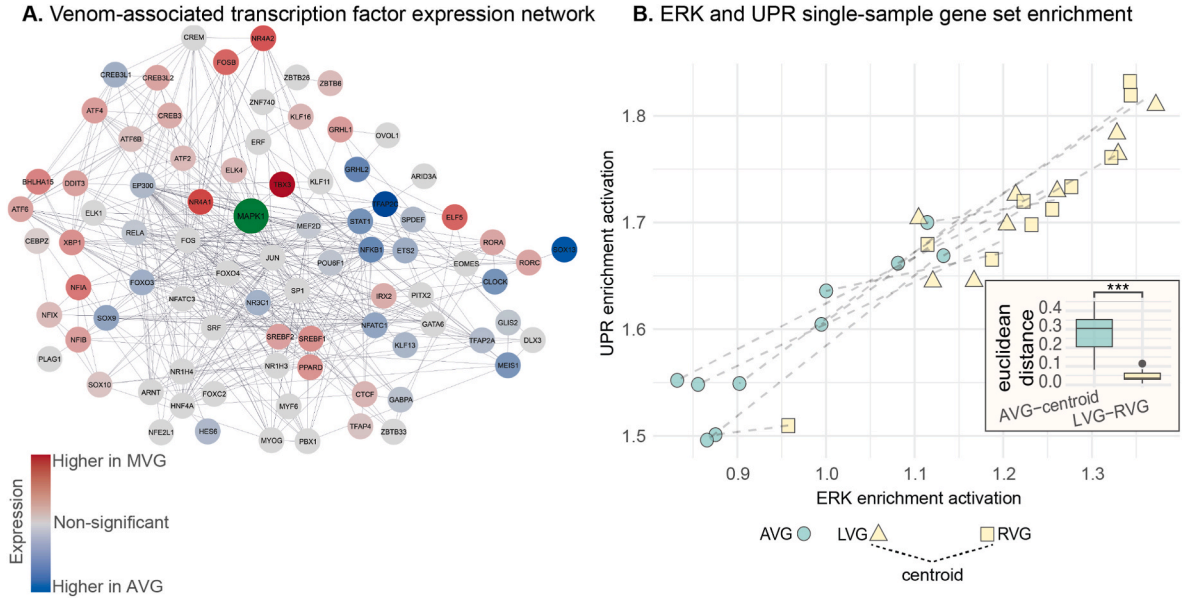

Fig. 4. Differential activity of transcription factors and ERK/UPR pathways across gland types. A) STRINGdb interaction network (visualized using Cytoscape) of select transcription factors that are known to regulate venom gene expression in rattlesnakes. Colors denote differentially expressed transcription factors, with those higher in the main gland shown in red and those higher in the accessory gland shown in blue. Transcription factors that are not significantly differentially expressed between the gland types are indicated in gray. A known ERK activator, MAPK1, was added manually to the network and is indicated in green. Full names of the transcription factors are provided in Supplementary Table 2. B) Enrichment scores of ERK and UPR for each sample are shown. Dotted lines connect accessory gland enrichment scores to the main gland centroid enrichment scores (mean of enrichment scores for left and right glands) for a given individual. Samples from the same gland type (main vs. accessory) are given the same color. The boxplot in the bottom right corner shows the Euclidean distance of enrichment values between the accessory gland and the centroid, as well as between the left and right main glands (***: 2-sample *t*-test p-value <0.001). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

utilize additional mechanisms for protecting the gland epithelium from venom toxicity by producing mucus and venom toxin inhibitors, in addition to lumen acidification (via V-ATPases) as in the main gland (Gopalan et al., 2024; Perry et al., 2022; Westfall et al., 2023). We find evidence that these two glands are also associated with very distinct trans-regulatory environments, including divergent activity of key venom regulatory pathways (ERK and UPR). Taken together, our findings raise broad questions about the distinct and complementary evolutionary trajectories of both glands and the degree to which the accessory gland may physiologically and functionally recapitulate key features of relatively ancient states in the development of the main venom gland from a more canonical salivary gland.

4.1. Divergent venom gene expression between the main and accessory gland

Our results provide new insight into the relative venom-producing role of the accessory venom gland including specific venom proteins that the accessory gland may contribute to the overall venom composition. While we detected the expression of all major venom genes in the accessory gland, nearly all venom toxins were expressed at far lower levels compared to the main venom gland. This suggests that the accessory gland may only make minor contributions to the overall venom composition, perhaps due to its small size. Despite these overall trends, we also identified a small set of venom toxins expressed at relatively higher levels in the accessory gland, all of which appear to show otherwise very low levels of expression in the main gland. These findings align with prior hypotheses that the accessory gland may produce a distinct, albeit relatively minor, source of venom proteins (Mackessy and Baxter, 2006; Valente et al., 2018b) and suggest that the expression of these toxins in the accessory gland may indeed alter the composition and function of secreted venom by contributing biologically relevant levels of some proteins to venom.

Venom gene families that show relatively higher expression levels in the accessory gland include CTLs and CRISPs, which have interesting notable associations with salivary gland secretions from both venomous and non-venomous vertebrate species. For example, these venom gene families are known to be expressed and secreted by salivary glands of toxicoferan reptiles with less specialized/complex venom systems than snakes, including helodermatid and varanid lizards (Fry, 2005; Fry et al., 2009; Morrissette et al., 1995). Proteins produced by these gene families are also thought to play various non-venom functional roles in salivary glands of other vertebrates (Gunput et al., 2016; Haendler et al., 1993). In addition to these select venom genes with higher expression in the

accessory gland, we also find evidence that several paralogs of the SVMP venom gene family (ADAM28 and ADAMTS), but which are not considered venom toxins *per se*, were more highly expressed in the accessory gland compared to the main gland (Casewell et al., 2012; Dowell et al., 2016; Sanz and Calvete, 2016). These findings further raise the possibility that the accessory gland possesses features that may resemble those of an ancestral, transitionary state in the evolution of salivary glands into specialized (main) venom glands.

4.2. Expression of distinct sets of non-venom genes highlights additional accessory gland roles

Beyond the expression of venom genes, our comparisons highlight broad physiological differences between the main and accessory venom glands. Our results suggest that the accessory venom gland may have additional and distinct functional and secretory roles from those of the main gland, including roles canonically associated with salivary glands. We identified over 200 non-venom genes predicted to produce secreted proteins with relatively higher expression in the accessory gland. Among the most highly expressed genes in the accessory gland are those related to the production of mucus (e.g., secreted mucins such as MUC5B and MUC5AC, and membrane-bound mucins such as MUC1, MUC15, and MUC16). Mucus is known to play critical roles in promoting the clearance of pathogenic agents and limiting bacterial contact with the epithelium (Cornick et al., 2015; Girod et al., 1992; Hansson, 2012; Taherali et al., 2018). Mucins also represent a major component of saliva and, in the mouth, salivary glands produce mucins to lubricate ingested food for passage through the esophagus (Taherali et al., 2018). In the context of the accessory gland, we expect that this high level of mucus production likely plays multiple roles, including acting as a protective barrier between the gland epithelium and bioactive venom that is stored in the lumen and sent through the duct. Increased mucus production may also serve as a lubrication mechanism for venom secretions to pass more easily from the main venom gland to the fang.

The acidification of the main gland lumen is coordinated by V-ATPases and is thought to play a critical role in reducing the enzymatic activity of many venom toxins during venom production and storage in the main gland (Fox and Serrano, 2008; Mackessy and Baxter, 2006). We find that the accessory gland shows distinct patterns of V-ATPase gene expression compared to the main gland, with most V-ATPase genes expressed at relatively lower levels, suggesting that glandular lumen acidification via V-ATPases may be significantly less relevant in the accessory compared to the main venom gland. However, our results suggest that the accessory gland may employ other distinct mechanisms for protecting its gland lumen from the harmful effects of venom, including high expression levels of protease inhibitors SERPINE1 and SERPINC1 (Chana-Muñoz et al., 2019; Gibbs et al., 2020; Oliveira et al., 2024) and phospholipase A_2 venom inhibitors ANXA1 and ANXA2 (Stuqui et al., 2015; Vecchi et al., 2021) in the accessory gland. These results suggest that the accessory venom gland may possess a distinct suite of specialized mechanisms for neutralizing the auto-toxic effects of venom during storage and expulsion that appear to include a combination of lumen acidification, mass production of protective mucins, and expression of venom inhibitors.

Our analyses indicate that several genes that code for secreted proteins involved in immunity are more highly expressed in the accessory gland compared to the main gland. Among these are multiple secreted matrix metalloproteinases (MMPs). MMPs are well-documented components of salivary secretions in non-venomous animals (Maciejczyk et al., 2016; Vanlaere and Libert, 2009), and their detection alone does not indicate that they play a relevant role in venom (e.g., role in prey immobilization or capture). However, MMPs make up a major fraction of the venom (secreted from the Duvernoy's gland) composition of several rear-fanged dipsadine colubrid snakes and are thought to function similarly to snake venom metalloproteinases (SVMPs) in prey capture and immobilization (Bayona-Serrano et al., 2020). Dipsadine venom MMPs are thought to have arisen from duplication of the non-venom paralog MMP9 (Bayona-Serrano et al., 2020; Junqueira-de-Azevedo et al., 2016) and interestingly we find that MMP9 (along with MMP25 and MMP2), is highly upregulated in our rattlesnake accessory glands. These findings raise broad questions about the biological relevance, physiological effects, and potential functional roles of MMPs or other secreted proteins (e.g., immune-associated proteins) not typically considered "venom toxins" that are produced by the accessory gland.

4.3. Gene-to-transcription factor correlation differences between gland types

We analyzed correlations between venom gene expression and transcription factor expression in the two glands to understand the regulatory mechanisms that may contribute to, and possibly explain, the relatively lower expression of venom genes in the accessory gland. We found that the expression of several TFs (e.g., MEIS1, NFKB1, SOX13, VEZF1) were negatively correlated with venom gene expression (Fig. 3A), suggesting that some TFs may be directly or indirectly acting to suppress venom gene expression in the accessory gland. This hypothesis is further supported by our finding that nearly all TFs that have negative correlations (and presumably repressive roles) with venom gene expression are more highly expressed in the accessory versus main gland. These findings broadly suggest that a set of transcription factors may function as repressors of venom gene expression within the accessory gland (and possibly in other tissues) to suppress venom expression, and that these TFs are distinct from those that serve as transcriptional activators in the main gland. In addition to these broad trends, we also identify specific TFs with significantly positive or negative relationships with the expression of distinct venom genes, which also provides new evidence for TFs not predicted to be relevant to venom regulation previously (e.g., IRX1, PA2G4, PRDM4). Collectively, these results indicate that venom expression is relatively reduced in the accessory gland through a combination of lower activation and higher repression compared to the main gland.

4.4. Variation in broader physiology and signaling among venom gland types

In viperids, previous studies have provided evidence that upon venom depletion, the venom production cascade is initiated via alpha- and beta-adrenergic receptors being stimulated within the secretory cells of the main venom gland (Kerchove et al., 2004). The stimulation of these adrenergic receptors activates the ERK signaling pathway, which drives the transcription of downstream targets, including venom genes (Kerchove et al., 2004; Perry et al., 2020; Yamanouye et al., 2000). It is predicted that this initial phase of venom production leads to endoplasmic reticulum (ER) stress, which activates the UPR pathway (Kim, 2024; Lin et al., 2008). UPR activation reduces ER and cellular stress while further upregulating venom gene expression because TFs from this pathway have been evolutionarily co-opted to regulate venom genes (Gopalan et al., 2024; Perry et al., 2022; Westfall et al., 2023). Together, the ERK and UPR pathways are hypothesized to regulate venom protein production within the main venom gland of rattlesnakes via their linked and cascading activation (Gopalan et al., 2024; Perry et al., 2022; Westfall et al., 2023). Our study is the first to evaluate the activity of these two pathways within the accessory venom gland, and we find that many key TFs involved in the UPR and ERK pathways are more highly expressed in the main venom gland (e.g., ATF6, BHLHA15, CREB3, CREB3L2, DDIT3, TBX3, XBP1; Fig. 4A). Further, we predict that the two pathways are consistently more highly activated in the main venom gland compared to the accessory gland (Fig. 4B). These findings suggest that additional alternative signaling pathways (possibly driven by distinct stimuli) may also coordinate gene expression within the accessory venom gland. While our current dataset may not readily enable the

identification of specific signaling pathways that regulate gene expression in the accessory gland, our findings do suggest that distinct pathways (and possibly distinct stimuli) may be primary drivers of the two glands' distinct activity, and further underscore the value of comparing these two glandular systems for dissecting the divergent regulatory mechanisms (e.g., gene repression, differential activation of high-level signaling pathways) underlying their distinct physiology and secretory functions.

5. Conclusion

Here we present one of the first comparisons of main and accessory snake venom gland gene expression across individuals from multiple rattlesnake species to identify distinct physiological and functional roles of the accessory venom gland. Our results provide new evidence that the accessory venom gland may contribute a small subset of proteins to venom, which raise additional questions about the functional significance of other secreted proteins and their potential roles in venom activity. We also find evidence that the accessory gland shares several functional and secretory similarities to both canonical salivary glands and Duvernoy's venom glands in mildly-venomous dipsadine snakes (i.e., mucus production, CTL production, high expression of matrix metalloproteinases). These similarities together suggest that the accessory gland may possess traits that recapitulate ancestral transitionary states between a salivary gland and a highly specialized main venom gland. We also present new correlative evidence for regulatory interactions between transcription factors and venom genes, including the identification of TFs that may act as transcriptional repressors of venom genes, further expanding our understanding of regulatory mechanisms that control venom gene expression. Lastly, our results reinforce evidence for the central roles of the ERK and UPR pathways in regulating snake venom genes and suggest that their activation is a primary axis that differentiates the physiology and secretory output of the main and accessory glands. Taken together, our findings highlight the rich potential of snake venom systems as a comparative model for understanding the evolution of physiological specialization, and its underlying gene regulatory basis, raising new questions about the biological roles of the accessory gland and its distinct secretory repertoire.

CRediT authorship contribution statement

Claire Kim: Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis. **Sierra N. Smith:** Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis. **Siddharth S. Gopalan:** Writing – original draft, Visualization, Formal analysis, Data curation. **Samuel R. Kerwin:** Writing – original draft, Methodology, Data curation, Conceptualization. **Kaas Ballard:** Writing – review & editing. **Blair W. Perry:** Writing – review & editing, Data curation. **Cara F. Smith:** Writing – review & editing, Data curation. **Anthony J. Saviola:** Writing – review & editing, Funding acquisition, Data curation. **Richard H. Adams:** Writing – review & editing, Funding acquisition. **Stephen P. Mackessy:** Writing – review & editing, Resources, Methodology, Funding acquisition, Data curation, Conceptualization. **Todd A. Castoe:** Writing – original draft, Validation, Supervision, Resources, Investigation, Funding acquisition, Data curation, Conceptualization.

Ethical statement

All samples were collected following approved protocols [IACUC protocol 2303D-SM-S-26]

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2025.108376.

Data availability

All RNA-seq data is available at NCBI under BioProject accession PRJNA1061517

References

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol 11, R106. https://doi.org/10.1186/gb-2010-11-10-r106.

Bayona-Serrano, J.D., Viala, V.L., Rautsaw, R.M., Schramer, T.D., Barros-Carvalho, G.A., Nishiyama, M.Y., Freitas-de-Sousa, L.A., Moura-da-Silva, A.M., Parkinson, C.L., Grazziotin, F.G., Junqueira-de-Azevedo, I.L.M., 2020. Replacement and parallel simplification of nonhomologous proteinases maintain venom phenotypes in rear-fanged snakes. Mol. Biol. Evol. 37, 3563–3575.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.

Casewell, N.R., Wüster, W., Wagstaff, S.C., Renjifo, C., Richardson, M.K., Vonk, F.J., Harrison, R.A., 2012. The origin and evolution of metalloproteinases in the venom of snakes. Toxicon 60, 119.

Castro-Mondragon, J.A., Riudavets-Puig, R., Rauluseviciute, I., Berhanu Lemma, R., Turchi, L., Blanc-Mathieu, R., Lucas, J., Boddie, P., Khan, A., Manosalva Pérez, N., et al., 2022. Jaspar 2022: the 9th release of the open-access database of transcription factor binding profiles. Nucleic Acids Res. 50, D165–D173.

Chana-Muñoz, A., Jendroszek, A., Sønnichsen, M., Wang, T., Ploug, M., Jensen, J.K., Andreasen, P.A., Bendixen, C., Panitz, F., 2019. Origin and diversification of the plasminogen activation system among chordates. BMC Evol. Biol. 19, 27.

Cornick, S., Tawiah, A., Chadee, K., 2015. Roles and regulation of the mucus barrier in the gut. Tissue Barriers 3, e982426.

Scientific and standard English names of amphibians and reptiles of North America north of Mexico, with comments regarding confidence in our understanding. In: Crother, B. I. (Ed.), SSAR Herpetological Circular 39, 1–92.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21.

Dowell, N.L., Giorgianni, M.W., Kassner, V.A., Selegue, J.E., Sanchez, E.E., Carroll, S.B., 2016. The deep origin and recent loss of venom toxin genes in Rattlesnakes. Curr. Biol. 26, 2434–2445.

Fox, J.W., Serrano, S.M.T., 2008. Insights into and speculations about snake venom metalloproteinase (SVMP) synthesis, folding and disulfide bond formation and their contribution to venom complexity. FEBS J. 275, 3016–3030.

Fry, B.G., 2005. From genome to "venome": molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. Genome Res. 15, 403–420.

Fry, B.G., Wroe, S., Teeuwisse, W., van Osch, M.J.P., Moreno, K., Ingle, J., McHenry, C., Ferrara, T., Clausen, P., Scheib, H., et al., 2009. A central role for venom in predation by *Varanus komodoensis* (Komodo Dragon) and the extinct giant *Varanus (Megalania) priscus*. Proc. Natl. Acad. Sci. U. S. A. 106, 8969–8974.

Gans, C., Kochva, E., 1965. The accessory gland in the venom apparatus of viperid snakes. Toxicon 3, 61–63.

Gearing, L.J., Cumming, H.E., Chapman, R., Finkel, A.M., Woodhouse, I.B., Luu, K., Gould, J.A., Forster, S.C., Hertzog, P.J., 2019. CiiiDER: a tool for predicting and analyzing transcription factor binding sites. PLoS One 14, e0215495.

Gibbs, H.L., Sanz, L., Pérez, A., Ochoa, A., Hassinger, A.T.B., Holding, M.L., Calvete, J.J., 2020. The molecular basis of venom resistance in a rattlesnake-squirrel predator-prey system. Mol. Ecol. 29, 2871–2888.

Girod, S., Zahm, J.M., Plotkowski, C., Beck, G., Puchelle, E., 1992. Role of the physiochemical properties of mucus in the protection of the respiratory epithelium. Eur. Respir. J. 5, 477–487.

Gopalan, S.S., Perry, B.W., Francioli, Y.Z., Schield, D.R., Guss, H.D., Bernstein, J.M., Ballard, K., Smith, C.F., Saviola, A.J., Adams, R.H., et al., 2024. Diverse gene regulatory mechanisms alter rattlesnake venom gene expression at fine evolutionary scales. Genome Biol. Evol. 16, evae110.

Gunput, S.T., Wouters, D., Nazmi, K., Cukkemane, N., Brouwer, M., Veerman, E.C., Ligtenberg, A.J., 2016. Salivary agglutinin is the major component in human saliva that modulates the lectin pathway of the complement system. Innate Immun. 22, 257–265.

Haendler, B., Krätzschmar, J., Theuring, F., Schleuning, W.D., 1993. Transcripts for cysteine-rich secretory protein-1 (CRISP-1; DE/AEG) and the novel related CRISP-3

are expressed under androgen control in the mouse salivary gland. Endocrinology 133, 192–198.
Hansson, G.C., 2012. Role of mucus layers in gut infection and inflammation. Curr. Opin. Microbiol. 15, 57–62.
Hänzelmann, S., Castelo, R., Guinney, J., 2013. GSVA: gene set variation analysis for microarray and RNA-Seq data. BMC Bioinf. 14, 7.
Hogan, M.P., Holding, M.L., Nystrom, G.S., Colston, T.J., Bartlett, D.A., Mason, A.J., Ellsworth, S.A., Rautsaw, R.M., Lawrence, K.C., Strickland, J.L., et al., 2024. The genetic regulatory architecture and epigenomic basis for age-related changes in rattlesnake venom. In: Proceedings of the National Academy of Sciences of United States of America, vol. 121, e2313440121.
Junqueira-de-Azevedo, I.L., Campos, P.F., Ching, A.T., Mackessy, S.P., 2016. Colubrid venom composition: an -omics perspective. Toxins 8, 230.
Kerchove, C.M., Carneiro, S.M., Markus, R.P., Yamanouye, N., 2004. Stimulation of the α-adrenoceptor triggers the venom production cycle in the venom gland of *Bothrops jararaca*. J. Exp. Biol. 207, 411–416.
Kerkkamp, H.M.I., Casewell, N.R., Vonk, F.J., 2017. Evolution of the snake venom delivery system. In: Gopalakrishnakone, P., Malhotra, A. (Eds.), Evolution of Venomous Animals and Their Toxins. Springer Netherlands, Dordrecht, pp. 1–11.
Kim, P., 2024. Understanding the unfolded protein response (UPR) pathway: insights into neuropsychiatric disorders and therapeutic potentials. Biomol. Therapeut. 32, 183–191.
Lambert, S.A., Jolma, A., Campitelli, L.F., Das, P.K., Yin, Y., Albu, M., Chen, X., Taipale, J., Hughes, T.R., Weirauch, M.T., 2018. The human transcription factors. Cell 172, 650–665.
Liao, Y., Smyth, G.K., Shi, W., 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930.
Lin, J.H., Walter, P., Yen, T.S.B., 2008. Endoplasmic reticulum stress in disease pathogenesis. Annu. Rev. Pathol. 3, 399–425.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.
Maciejczyk, M., Pietrzykowska, A., Zalewska, A., Knaś, M., Daniszewska, I., 2016. The significance of matrix metalloproteinases in oral diseases. Adv. Clin. Exp. Med. 25, 383–390.
Mackessy, S.P., 1991. Morphology and ultrastructure of the venom glands of the northern Pacific rattlesnake Crotalus viridis oreganus. J. Morphol. 208, 109–128. https://doi.org/10.1002/jmor.1052080106.
Mackessy, S.P., 2022. Venom production and secretion in reptiles. J. Exp. Biol. 225, jeb227348.
Mackessy, S.P., Baxter, L.M., 2006. Bioweapons synthesis and storage: the venom gland of front-fanged snakes. Zoologischer Anzeiger - J. Comp. Zool. 245, 147–159.
Margres, M.J., Rautsaw, R.M., Strickland, J.L., Mason, A.J., Schramer, T.D., Hofmann, E. P., Stiers, E., Ellsworth, S.A., Nystrom, G.S., Hogan, M.P., et al., 2021. The tiger rattlesnake genome reveals a complex genotype underlying a simple venom phenotype. Proc. Natl. Acad. Sci. U. S. A. 118, e2014634118.
Morrissette, J., Krätzschmar, J., Haendler, B., el-Hayek, R., Mochca-Morales, J., Martin, B.M., Patel, J.R., Moss, R.L., Schleuning, W.D., Coronado, R., 1995. Primary structure and properties of helothermine, a peptide toxin that blocks ryanodine receptors. Biophys. J. 68, 2280–2288.
Oliveira, A.K., Rucavado, A., Escalante, T., Gutiérrez, J.M., Fox, J.W., 2024. A complex pattern of gene expression in tissue affected by viperid snake envenoming: the emerging role of autophagy-related genes. Biomolecules 14, 278.
Perry, B.W., Gopalan, S.S., Pasquesi, G.I.M., Schield, D.R., Westfall, A.K., Smith, C.F., Koludarov, I., Chippindale, P.T., Pellegrino, M.W., Chuong, E.B., et al., 2022. Snake venom gene expression is coordinated by novel regulatory architecture and the integration of multiple co-opted vertebrate pathways. Genome Res. 32, 1058–1073.
Perry, B.W., Schield, D.R., Westfall, A.K., Mackessy, S.P., Castoe, T.A., 2020. Physiological demands and signaling associated with snake venom production and storage illustrated by transcriptional analyses of venom glands. Sci. Rep. 10, 18083.
R Core Team, 2023. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
Sanz, L., Calvete, J.J., 2016. Insights into the evolution of a snake venom multi-gene family from the genomic organization of *Echis ocellatus* SVMP Genes. Toxins 8, 216.
Schield, D.R., Card, D.C., Hales, N.R., Perry, B.W., Pasquesi, G.M., Blackmon, H., Adams, R.H., Corbin, A.B., Smith, C.F., Ramesh, B., et al., 2019. The origins and evolution of chromosomes, dosage compensation, and mechanisms underlying venom regulation in snakes. Genome Res. 29, 590–601.
Shannon, P., et al., 2003. Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504.
Stuqui, B., Paula-Silva, M de, Carlos, C.P., Ullah, A., Arni, R.K., Gil, C.D., Oliani, S.M., 2015. Ac2-26 mimetic peptide of annexin A1 inhibits local and systemic inflammatory processes induced by *Bothrops moojeni* venom and the Lys-49 phospholipase A2 in a rat model. PLoS One 10, e0130803.
Szklarczyk, D., Kirsch, R., Koutrouli, M., Nastou, K., Mehryary, F., Hachilif, R., Gable, A. L., Fang, T., Doncheva, N.T., Pyysalo, S., et al., 2023. The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. Nucleic Acids Res. 51, D638–D646.
Taherali, F., Varum, F., Basit, A.W., 2018. A slippery slope: on the origin, role and physiology of mucus. Adv. Drug Deliv. Rev. 124, 16–33.
Uetz, P., Freed, P., Aguilar, R., Reyes, F., Kudera, J., Hošek, J. (Eds.), 2024. The Reptile Database. Available at: http://www.reptile-database.org.
Uhlén, M., Fagerberg, L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., et al., 2015. Tissue-based map of the human proteome. Science 347, 1260419.
Valente, R., Sakai, F., Portes-Junior, J.A., Godoy Viana, L., Mendes, Carneiro S., Perales, J., Yamanouye, N., 2018a. The primary duct of *Bothrops jararaca* glandular apparatus secretes toxins. Toxins 10, 121.
Valente, R., Luna, M.S., Oliveira, U.C., Nishiyama-Junior, M.Y., Junqueira-de-Azevedo, I. de L., Portes-Junior, J.A., Clissa, P.B., Viana, L.G., Sanches, L., Moura-da-Silva, A.M., et al., 2018b. *Bothrops jararaca* accessory venom gland is an ancillary source of toxins to the snake. J. Proteonomics 177, 137–147.
Vanlaere, I., Libert, C., 2009. Matrix metalloproteinases as drug targets in infections caused by gram-negative bacteria and in septic shock. Clin. Microbiol. Rev. 22, 224–239.
Vecchi, L., Araújo, T.G., Azevedo, FVPV, Mota, S.T.S., Ávila, V.M.R, Ribeiro, M.A., Goulart, L.R., 2021. Phospholipase A2 drives tumorigenesis and cancer aggressiveness through its interaction with annexin A1. Cells 10, 1472.
Vonk, F.J., Casewell, N.R., Henkel, C.V., Heimberg, A.M., Jansen, H.J., McCleary, R.J.R., Kerkkamp, H.M.E., Vos, R.A., Guerreiro, I., Calvete, J.J., et al., 2013. The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system. Proc. Natl. Acad. Sci. U.S.A 110, 20651–20656.
Westfall, A.K., Gopalan, S.S., Perry, B.W., Adams, R.H., Saviola, A.J., Mackessy, S.P., Castoe, T.A., 2023. Single-cell heterogeneity in snake venom expression is hardwired by co-option of regulators from progressively activated pathways. Genome Biol. Evol. 15, evad109.
Wickham, H., 2016. ggplot2: Elegant graphics for data analysis. Springer-Verlag, New York. https://ggplot2.tidyverse.org.
Yamanouye, N., Carneiro, S.M., Scrivano, C.N., Markus, R.P., 2000. Characterization of β-adrenoceptors responsible for venom production in the venom gland of the snake *Bothrops jararaca*. Life Sci. 67, 217–226.
Zancolli, G., Reijnders, M., Waterhouse, R.M., Robinson-Rechavi, M., 2022. Convergent evolution of venom gland transcriptomes across Metazoa. Proc. Natl. Acad. Sci. U.S. A 119, e2111392119.