Distinct regulatory networks control toxin gene expression in elapid and viperid snakes

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Running title: Distinct regulatory networks control toxin gene expression in front-fanged snakes

Abstract (250 words)

Venom systems are ideal models to study genetic regulatory mechanisms that underpin evolutionary novelty. Snake venom glands are thought to share a common origin, but there are major distinctions between venom toxins from the medically significant snake families Elapidae and Viperidae, and toxin gene regulation in elapids is largely unexplored. Here, we used high-throughput RNA-sequencing to profile gene expression and microRNAs between active (milked) and resting (unmilked) venom glands in an elapid (Eastern Brown Snake, *Pseudonaja textilis*), in addition to comparative genomics, to identify *cis-* and *trans-* acting regulation of venom production in an elapid in comparison to viperids (*Crotalus viridis* and *C. tigris*). Although there is conservation in high-level mechanistic pathways regulating venom production, there are histone methylation, transcription factor, and microRNA regulatory differences between these two snake families. Histone methyltransferases (KMT2A, KMT2C and KMT2D) and transcription factor (TF) specificity protein 1 (Sp1) were highly upregulated in the milked elapid venom gland, whereas nuclear factor I (NFI) TFs were upregulated after viperid venom milking. Sp1 and NFI *cis*-regulatory elements were common to toxin gene promoter regions, but many unique elements were also present between elapid and viperid toxins. microRNA profiles were distinctive between milked and unmilked venom glands for both snake families, and microRNAs were predicted to target different toxin transcripts. Our comparative transcriptomic and genomic analyses between toxin genes and isoforms in elapid and viperid snakes suggests independent toxin evolution between these two snake families, demonstrating multiple toxin genes and regulatory mechanisms converged to underpin a highly venomous phenotype.

Key words: front-fanged snakes, toxin expression, venom delivery system

Introduction

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73 Snake venom glands are highly specialized vertebrate secretory tissues, exhibiting elevated, tissue-specific

74 expression of 50-100 or more toxins. Based on structural similarity to cognate proteins involved in

75 physiological processes, snake toxins are hypothesized to have primarily evolved by gene duplication of

76 nontoxic genes (Vogel et al. 1984; Kochva et al. 1993; Hite et al. 1994; Joseph et al. 1999; Miwa et al.

77 1999; Rao and Kini 2002; Tamiya and Fujimi 2006) and subsequent neofunctionalization through

78 accelerated evolution of their exons (Deshimaru et al. 1996; Nobuhisa et al. 1996; Chang et al. 1997; Ohno

79 et al. 1998). Therefore, snake venom glands provide a unique opportunity to study epigenetic and genetic

80 mechanisms that regulate tissue-specific expression and gene neofunctionalization in multi-locus gene

81 families. Furthermore, as venom glands are a rich source of diverse bioactive proteins applicable to

82 bioprospecting and antivenom development, understanding how toxins are produced would also benefit

83 large-scale production *in vitro* (Post et al. 2020).

84 Snakebite annually leads to the death or disability of over 500,000 victims (Gutiérrez et al. 2017)

85 and medically significant species are predominantly from the families Elapidae and Viperidae. Venoms

86 from these two families cause debilitating effects and appear to share a common evolutionary origin, which

87 is supported by anatomical and developmental evidence (Vonk et al. 2008), and phylogenetic analysis of

88 toxins suggests venom evolved once, at the base of the advanced snake radiation (Fry and Wüster 2004).

89 Although elapids and viperids both have tubular fangs positioned anterior on the maxilla, elapid and viperid

90 delivery systems and venom components are distinct in several ways. Elapids have a less mobile maxillary

91 bone, and relatively smaller fangs and venom gland lumen, while viperids have a highly kinetic maxillary,

92 long fangs and a wide lumen (Kardong 1982; Kochva 1987). Different external adductor muscles are also

93 used to compress the venom gland in elapids and viperids (Jackson 2003), as well as likely convergent

94 evolution of accessory glands (Kerkkamp et al. 2015). After venom is expelled, venom gland cells do not

95 exhibit any marked size differences in elapids (Kochva et al. 1982; Lachumanan et al. 1999); while in

96 viperids, secretory epithelial cells dramatically increase in size from cuboidal to columnar with proliferation

97   of the rough endoplasmic reticulum (ER) (Oron and Bdolah 1973; Mackessy 1991). Further, elapid venoms

98   are largely dominated by non-enzymatic three-finger toxins (3FTxs), and enzymatic group I phospholipases

99   (PLA$_2$s) of pancreatic origin (Mackessy 2010; Tasoulis and Isbister 2017), while viperid venoms have

100   primarily enzymatic toxins, including snake venom metalloproteinases (SVMPs), serine proteinases

101   (SVSPs), and group II PLA$_2$s with structural features similar to non-pancreatic inflammatory PLA$_2$s

102   (Heinrikson et al. 1977; Seilhamer et al. 1989). Chromosomal locations of dominant toxins also vary

103   between these two snake families, with most located on macrochromosomes in elapids (Suryamohan et al.

104   2020; Zhang et al. 2022) and microchromosomes in viperid snakes (Shibata et al. 2018; Schield et al. 2019;

105   Margres et al. 2021).

106   In both elapids and viperids, toxin gene transcription and translation are upregulated when the

107   venom gland is emptied by venom extraction or "milking" (Rotenberg et al. 1971; Paine et al. 1992;

108   Lachumanan et al. 1999; Currier et al. 2012). Studies investigating mechanisms regulating the expression

109   of elapid toxins have focused on a small number of genes. These studies have identified *cis*-regulatory

110   elements (CRE) that regulate the expression of genes encoding 3FTx and group I PLA$_2$ expression in the

111   Javan Spitting Cobra (*Naja sputatrix*) (Jeyaseelan et al. 2000; Ma et al. 2001; Ma et al. 2002), the pseutarin

112   C catalytic subunit in the Eastern Brown Snake (*Pseudonaja textilis*) (Kwong et al. 2009), and silencer AG-

113   rich motifs in the first intron of the pseutarin C catalytic subunit gene (Han et al. 2016). Investigations of

114   viperids have found α_1- and $\beta-$adrenoceptor signaling as a mechanism activating transcription factors

115   (TFs) NF$-\kappa$B and AP-1 to initiate toxin synthesis in the Jararaca (*Bothrops jararaca*) (Yamanouye et al.

116   1997; Luna et al. 2009), tissue-specific TF ESE-3 upregulated group II PLA$_2$s genes in the Habu

117   (*Protobothrops flavoviridis*) (Nakamura et al. 2014), and CREs for binding GRHL- and NFI-family TFs

118   were enriched within SVMP, SVSP, and group II PLA$_2$ gene promoters in the Prairie and Tiger rattlesnakes

119   (*Crotalus viridis* and *C. tigris*, respectively) (Schield et al. 2019; Margres et al. 2021; Perry et al. 2022), in

120   addition to co-opted TFs from the Unfolded Protein Response (UPR) pathway (Perry et al. 2022). Current

121   evidence suggests that for the viperids *C. viridis* and *C. tigris,* toxin gene expression is regulated by

122   chromatin structure, TFs, and gene methylation levels (Schield et al. 2019; Margres et al. 2021). Here, we

123   compare active (milked) and resting (unmilked) venom glands of *P. textilis* to provide the first evidence of

124   multiple toxin gene regulatory networks in an elapid snake.

125   *Pseudonaja textilis* is the second most lethal terrestrial venomous snake in the world, based upon

126   murine models of toxicity (Broad et al. 1979), and is responsible for the majority of snakebite deaths in

127   eastern Australia (Sutherland 1992; White 2009). Venom gland transcriptomics and venom proteomics have

128   been used to profile *P. textilis* venom composition (Birrell et al. 2006; Viala et al. 2015; Reeks et al. 2016),

129   and studies have documented geographic, seasonal, ontogenetic, and individual venom variation (Williams

130   and White 1992; Flight et al. 2006; Skejić and Hodgson 2013; Skejić et al. 2015; Jackson et al. 2016;

131   McCleary et al. 2016; van Thiel et al. 2023). Using high-throughput transcriptomics, we evaluated *P. textilis*

132   mRNAs and microRNAs (miRNAs) in a Milked Venom Gland (MVG) and Unmilked Venom Gland

133   (UVG) from an individual snake, eliminating contributing factors involved in venom variation, to identify

134   mechanisms regulating toxin expression. We identified higher-level venom synthesis activation pathways

135   common to both *P. textilis* and viperid venom glands*,* but differences in *cis*- and *trans*-acting regulation of

136   toxin expression. Further, posttranscriptional miRNA regulation was not conserved between venom glands

137   from the two snake families. Therefore, distinct gene regulatory networks produce elapid and viperid venom

138   phenotypes, and thus, our results suggest an independent origin of toxin genes and their associated

139   regulatory mechanisms in elapid and viperid snakes.

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141   **Results**

142   **Toxin genes exhibit high and variable expression in the *Pseudonaja textilis* venom gland**

143   From a *P. textilis* individual, the left venom gland was milked to stimulate venom production while the

144   right venom gland remained unmilked as an 'unstimulated' control. Four days later (96 hours post venom

145 milking, hpvm), both glands were collected, RNA and miRNA isolated and sequenced, and the genome

146 (EBS10Xv2-PRI) used as a reference to profile gene expression between glands. Evaluations of *P. textilis*

147 toxin transcript diversity and expression were also completed with transcripts from *de novo* assembled

148 transcriptomes for each gland (Supplemental Table 1). Fold-changes in transcript expression between the

149 MVG versus UVG were determined with GFOLD (Feng et al. 2012) (Supplemental Table 2). A total of

150 7,062 transcripts were upregulated and 8,038 transcripts downregulated in the MVG.

151   For both the MVG and UVG, toxins were among the most highly expressed transcripts and the

152 same toxin superfamilies made up similar proportions of overall toxin expression (Supplemental Figure

153 1A-B). From a combined *de novo* assembly of each gland (Supplemental Table 1), 28,569 transcripts were

154 expressed (>1 Transcript Per Million, TPM), and 52 full-length toxins from 18 toxin superfamilies were

155 identified. Toxin superfamilies that were expressed, in order of total abundance in the MVG, include

156 fourteen 3FTxs (78% of all toxin transcripts), three group I PLA_2s (13%), four Kunitz-type serine proteinase

157 inhibitors (KUNs, 3%), 13 snake venom C-type lectins (Snaclecs, 2%) and the prothrombin activator

158 pseutarin C (venom coagulation factors V and X, 2%) (Supplemental Figure 1A). Low abundance toxins,

159 making up 1% or less of reads, were two natriuretic peptides (NPs,), one cysteine-rich secretory protein

160 (CRISP), three cystatins (CYSs), two SVMPs, one hyaluronidase (HYAL), one nerve growth factor (NGF),

161 one waprin (WAP), one 5'-nucleotidase (5'NUC), one cobra venom factor (CVF), one L-amino acid

162 oxidase (LAAO), one acetylcholinesterase (AChE) and one vespryn (VES). All transcript isoforms in a

163 superfamily were not expressed equally; for the two superfamilies with the highest expression levels, the

164 fourteen 3FTx isoforms ranged from 236,773 TPM to 10 TPM, and the three PLA_2 isoforms ranged from

165 100,141 TPM to 1,846 TPM.

166   This *de novo* assembled venom gland transcriptome is currently the most comprehensive to date

167 for *P. textilis*, having detected all previous reported toxin transcripts along with full-length transcripts for

168 low abundance CYSs, HYAL, NGF, WAP, 5'NUC, CVF, LAAO, AChE, and VES toxins (Supplemental

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169   Table 3). Three 3FTxs (3FTx_6, 7, and 8), one CRISP (CRISP_1), one KUN (KUN_1), and one PLA_2

170   (PLA2_1) were identical to previously identified sequences (Supplemental Figure 2A-D). PLA_2s and short-

171   chain 3FTxs exhibited the greatest sequence variation (as low as 43% and 48% amino acid sequence

172   identity, respectively) between isoforms. There was an absence of transcripts sequences for textilotoxin, a

173   pentameric PLA_2 complex unique to *P. textilis* venom (Tyler et al. 1987a). Pseutarin C, a toxin composed

174   of two subunits homologous to the mammalian coagulation factor Xa-Va complex (Rao and Kini 2002),

175   were highly conserved, 99% and 95% identical to the published sequences for *P. textilis* venom coagulation

176   factors V and X, respectively (Rao et al. 2003; Rao et al. 2004) (Supplemental Figure 2E-F), and >84%

177   identical to venom coagulation factor homologs in *Oxyuranus microlepidotus* and *O. scutellatus* venom.

178   **Upregulation of toxin gene expression after venom milking is lower in an elapid compared to viperids**

179   To compare changes in toxin gene expression in the MVG of an elapid to that of vipers, we retrieved

180   available RNA-seq libraries from the National Center for Biotechnology Information (NCBI) for MVGs

181   and UVGs of *C. viridis* at 0 and 72 hpvm (Schield et al. 2019), and *C. tigris* at 0, 72 and 96 hpvm (Margres

182   et al. 2021). To have a comparable time course of 0 to 96 hpvm for all species, we milked venom from a *C.*

183   *viridis* individual of the same northeastern Colorado locality as snakes used by Schield *et al.* (2019),

184   collected both venom glands at 96 hpvm, and performed RNA-seq on these tissues. Annotated

185   transcriptomes from genome assemblies (UTA_CroVir_3.0 and ASM1654583v1 for *C. viridis* and *C. tigris*,

186   respectively) and myotoxin transcripts from a *de novo* assembled venom gland transcriptome for *C. viridis*

187   (96 hpvm individual) (Supplemental Table 4) were used as references to determine toxin transcript

188   abundances. Toxin transcripts were identified from transcriptomes from key word searches for each venom

189   protein family, in addition to using previously annotated toxin genes (Supplemental Table 5 and 6 for *C.*

190   *viridis* and *C. tigris*, respectively) (Schield et al. 2019; Margres et al. 2021). We reanalyzed fold-changes

191   in expression of transcripts between MVGs and UVGs at each time point with GFOLD (Feng et al. 2012)

192   (Supplemental Table 7), allowing for comparisons to the *P. textilis* dataset.

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193   Toxin expression in the venom gland of *C. viridis* from northeastern Colorado was predominately

194   myotoxins (75% of total toxin transcripts), SVSPs (11%), PLA$_2$s (7%), SVMPs (6%), and minor toxins (<

195   1%) (Supplemental Table 5). For *C. tigris,* PLA$_2$s were the highest expressed (56% of all toxin transcripts),

196   followed by SVSPs (21%), bradykinin-potentiating peptides (BPPs; 12%), vascular endothelial growth

197   factor (VEGF; 5%), SVMPs (4%), and all other minor toxins (1% or less) (Supplemental Table 6). A

198   myotoxin from *C. viridis* had the highest expression level (520,544 TPM at 96 hpvm) of any toxin from

199   either the two rattlesnake venom glands or from the elapid *P. textilis* (Figure 1). For all species, the highest

200   expressed toxins (>50% of toxin transcripts) were the major components in each venom proteome (Figure

201   1), and are also the primary lethal or tissue damaging toxins in these venoms (Cameron and Tu 1978; Ho

202   and Lee 1981; Su et al. 1983; Tyler et al. 1987b; Calvete et al. 2012; McCleary et al. 2016).
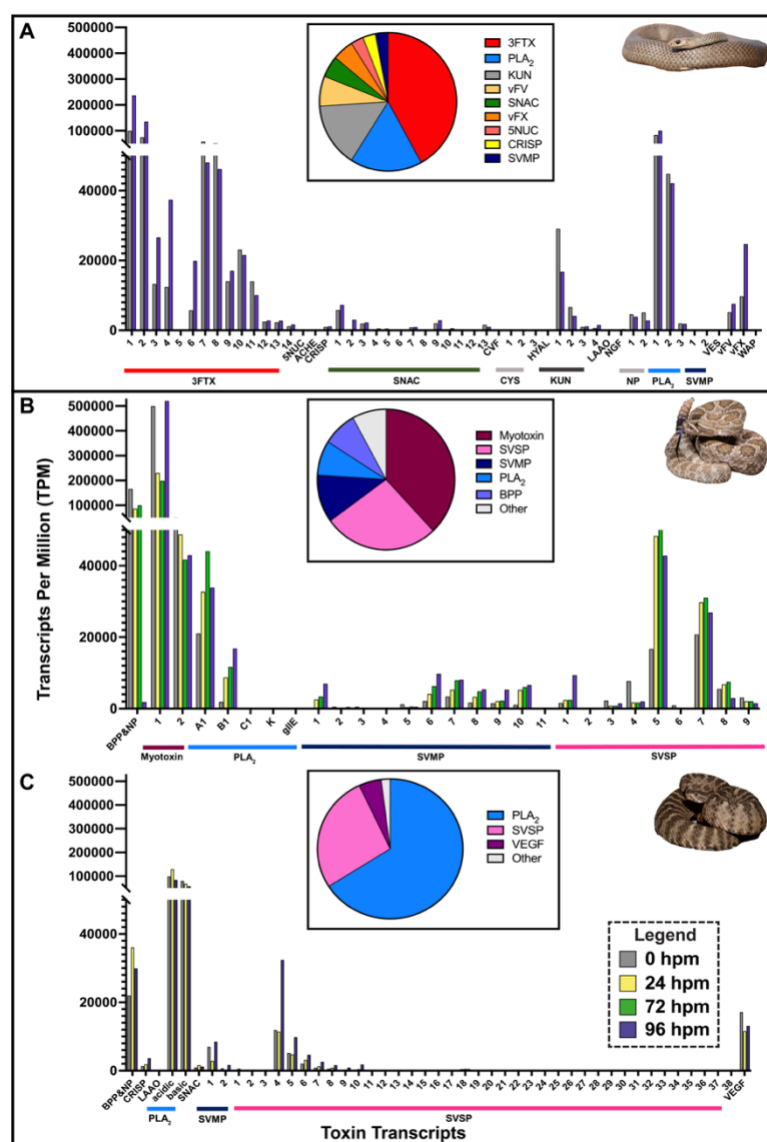

Figure 1. Toxin gene expression in an elapid (*Pseudonaja textilis*) and two viperids (*Crotalus viridis* and *C. tigris*) over a time course of 96 hours post venom gland milking (hpvm) in comparison to venom proteomes. Time points are shown for (A) *P. textilis* at 0 and 96 hpvm, (B) *C. viridis* at 0, 24, 72, and 96 hpvm, and (C) *C. tigris* at 0, 24, and 96 hpvm. Bar colors represent the time after venom milking. Pie charts (insets in respective panels) correspond to the venom proteomes of *P. textilis* (McCleary et al. 2016), *C. viridis* (Saviola et al. 2015) and *C. tigris* (Calvete et al. 2012). Toxin identifications are as follows: 3FTx = three-finger toxin, 5NUC = 5'-nucleotidase; AChE = acetylcholinesterase;

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219   BPP = Bradykinin-potentiating peptide; CRISP = cysteine-rich secretory protein; CVF = cobra venom

220   factor; CYS = cystatin; HYAL = hyaluronidase; KUN = Kunitz serine proteinase inhibitor; LAAO = L-

221   amino acid oxidase; NGF = nerve growth factor; NP = natriuretic peptide; PLA2 = phospholipase A_2;

222   SNAC = snake C-type lectin; SVMP = snake venom metalloproteinase; SVSP = snake venom serine

223   protease; VEGF = vascular endothelial growth factor; VES = vespryn; vFV = venom factor V (pseutarin C

224   non-catalytic subunit); vFX = venom factor X (pseutarin C catalytic subunit); WAP = waprin. Photo credits:

225   *P. textilis,* Ákos Lumnitzer; *C. viridis*, Wolfgang Wüster; *C. tigris,* Ben Lowe.

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227   For toxin expression in the *P. textilis* MVG, highly abundant toxins (3FTx_1, 2, 3, 4 and PLA2_1)

228   exhibited TPM increases (Figure 1A) with overall fold-changes of two-fold or less in comparison to the

229   UVG. There were small decreases in expression (less than 30%) for 5'NUC, two 3FTxs (3FTx_8 and 10),

230   two PLA_2 (PLA2_2 and 3) and HYAL, and larger decreases (36-66%) for two 3FTxs (3FTx_7 and 11),

231   AChE, two CYSs (CYS_1 and 2), two KUN (KUN_1 and 2), two NPs, four snaclecs (SNAC_5, 8, 11, and

232   13) and WAP. Snaclec transcript isoforms exhibited the greatest variability between the MVG and UVG;

233   SNAC_2 had the overall highest increase (11.4-fold) and SNAC_5 had the greatest decrease (0.31-fold).

234   For toxin expression in the viperid MVG, SVMP_1 in *C. viridis* was the top upregulated transcript

235   by 43-, 58-, and 127-fold at 24, 72, and 96 hpvm, respectively. However, there were not parallel increases

236   in expression for all rattlesnake toxins over the time course. For *C. viridis,* the highest expressed PLA_2

237   (PLA2_A1) peaked 72 hpvm and another PLA_2 (PLA2_B1), the majority of SVMPs (7 out of 11) and

238   myotoxin_1 peaked at 96 hpvm (Figure 1B). SVSP transcripts in *C. tigris* venom glands showed the greatest

239   fold changes over the time course: SVSP_1 (XM_039325703.1) was upregulated 699-fold at 24 hpvm and

240   SVSP_2 (XM_039360544.1) was the top upregulated toxin transcript (45-fold) at 96 hpvm.

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243   **UPR, Notch signaling, and cholesterol homeostasis are enriched in MVGs, and different biological**

244   **process regulated between an elapid and viperids**

245   A Gene Set Enrichment Analysis (GSEA) (Mootha et al. 2003; Subramanian et al. 2005) was conducted

246   using human gene orthologs for all non-toxin genes expressed in MVGs and UVGs. No gene sets were

247   found to be significantly enriched in MVGs for any of the three species, but the lowest familywise error

248   rate (p-value=0.50) was for UPR in *P. textilis*, Notch signaling in *C. viridis* (familywise error rate p-

249   value=0.49) and cholesterol homeostasis in *C. tigris* (familywise error rate p-value=0.49), although these

250   three biological processes were listed in enriched datasets of all three species with the exception of

251   cholesterol homeostasis in *C. viridis* (Supplemental Table 8).

252   Focusing on genes with the greatest fold-change between the MVG and UVG from *P. textilis*, we

253   selected all genes upregulated at least 10-fold (373) and downregulated to less than 0.10-fold (415) in the

254   MVG. A gene ontology and network analysis of the upregulated gene set found the following

255   overrepresented biological processes: chromatin and histone remodeling (GO:006325, GO:0016569, and

256   GO:006338), regulation of transcription (GO:0006355, GO:0006357, and GO:0045944) and phospholipid

257   biosynthesis (including phosphatidylinositol-3-phosphate signaling GO:0036092 and inositol phosphate

258   metabolic process GO:0043647) (Figure 2A,C). Chromatin organization was found to be the only

259   significantly upregulated biological process (p=0.0005; for details, see below). Downregulated genes were

260   significantly enriched for proteins involved in striated muscle contraction and sarcomere assembly (p <

261   0.002; GO:0006936, GO:0014733, GO:0006937, GO:0090257, GO:0055002, and GO:0030239) (Figure

262   2B,C).

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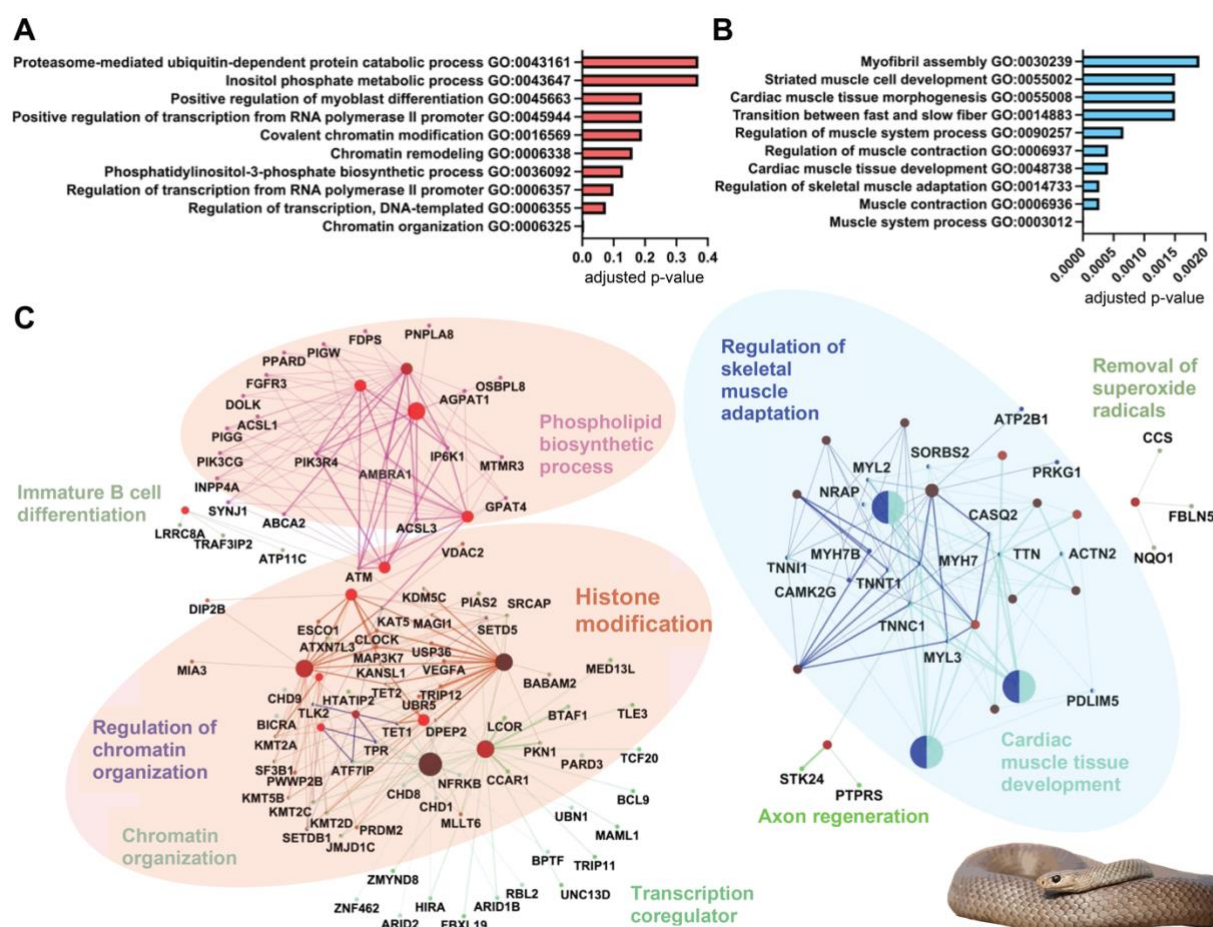**264**

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Figure 2. Enriched biological processes and associated networks for genes up- and downregulated in *Pseudonaja textilis* milked venom gland. The top ten biological processes are shown with their level of significance for genes (A) upregulated at least 10-fold and (B) downregulated to less than 0.10-fold 96 hpvm. Gene ontology analysis was completed using DAVID Bioinformatics Resources (Huang da et al. 2009b; Sherman et al. 2022) and Benjamini-Hochberg adjusted p-values were used for identifying levels of significance. For each up- and downregulated gene set, (C) gene networks and associated biological processes were generated using the ClueGo app plug-in (Bindea et al. 2009) in Cytoscape (Shannon et al. 2003) with *Homo sapiens* orthologs. Light red colored ovals highlight upregulated gene networks with the greatest number of related nodes, and the light-colored blue oval for those downregulated. Photo credit: *P. textilis*, Ákos Lumnitzer.

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280   For the viperids *C. viridis* and *C. tigris*, 16 and 197 genes were upregulated, and 85 and 515 genes

281   downregulated 96 hpvm, respectively, using the same thresholds as used for *P. textilis*. A gene ontology

282   and network analysis of the upregulated gene set found the following overrepresented biological processes:

283   transcription (GO:0045944, GO:0000122, and GO:006354), protein translation and transport

284   (GO:0017148, GO0001822, and GO:0015031), and the UPR (GO:0030968) (Figure 3A, C). Positive

285   regulation of transcription and negative regulation of translation were significant upregulated biological

286   processes (p < 0.05); Figure 3A). Downregulated biological processes were complement activation

287   (GO:0006958 and GO:0006956), immune response (GO:0006954, GO:0045087, and GO:0006955),

288   cellular component organization (GO:0016043, GO:0071840, and GO:0051128) and metabolic processes

289   (GO:0019219, GO:0051173, GO:0009893, and GO:0031325) (Figure 3B); all were significant (p < 0.008).

290   A gene regulatory network analysis of the downregulated gene sets found the largest gene network related

291   to negative mechanisms of regulating nucleobase-containing macromolecules, which included

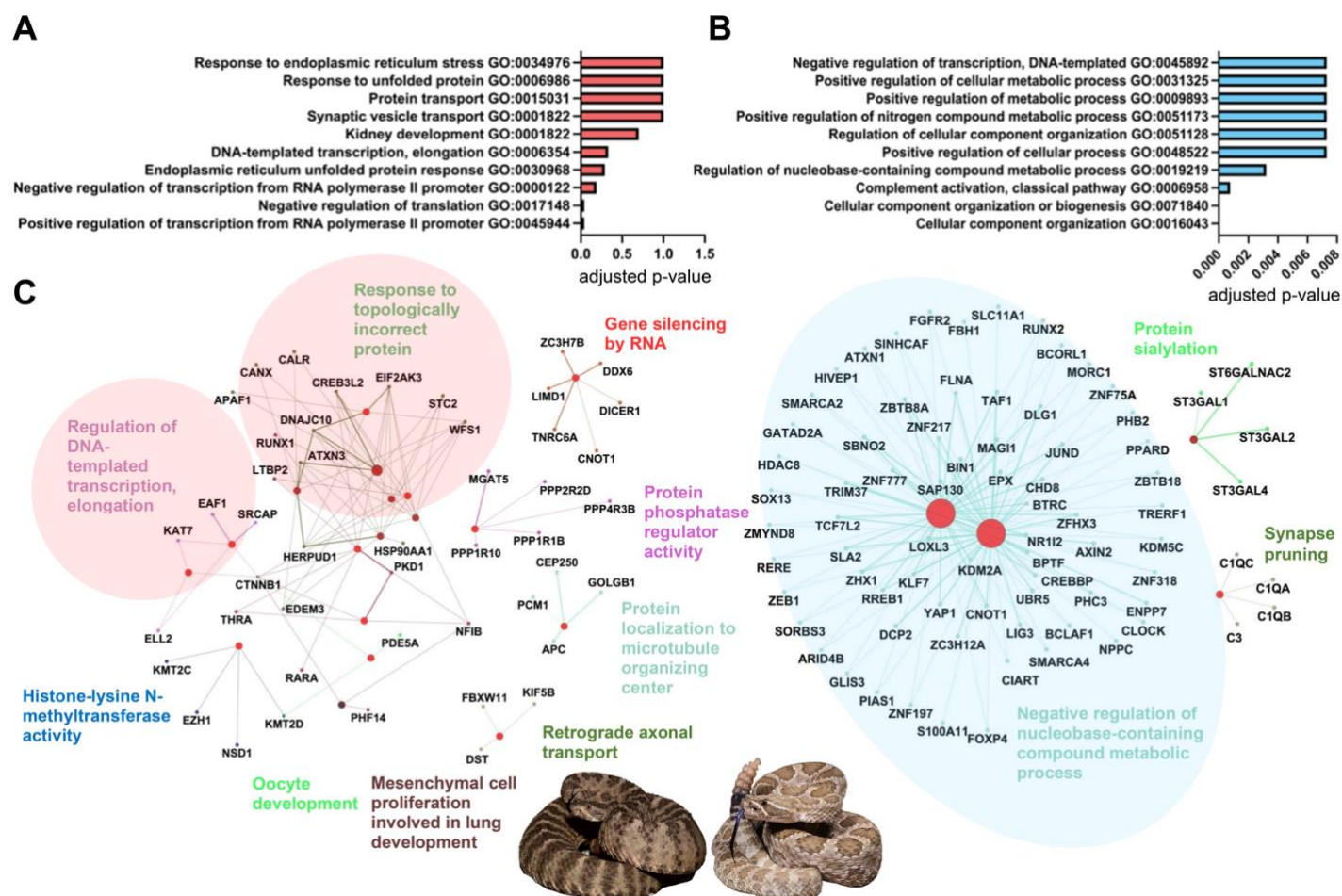292   transcriptional repressors (Figure 3C).

Figure 3. Enriched biological processes and associated networks for genes up- and downregulated after venom milking viperids *Crotalus viridis* and *C. tigris*. The top ten biological processes are shown with their level of significance for genes (A) upregulated at least 10-fold and (B) downregulated to less than 0.10-fold at 96 hpvm. Gene ontology analysis was completed using DAVID Bioinformatics Resources (Huang da et al. 2009b; Sherman et al. 2022) and Benjamini-Hochberg adjusted p-values were used for identifying levels of significance. For each up- and downregulated gene set, (C) gene networks and associated biological processes were generated using the ClueGo app plug-in (Bindea et al. 2009) in Cytoscape (Shannon et al. 2003) with *Homo sapiens* orthologs. Light red colored ovals highlight upregulated gene networks with the greatest number of related nodes, and the light-colored blue oval for those downregulated. Photo credits: *C. tigris,* Ben Lowe*; C. viridis,* Wolfgang Wüster.

13

304 **Chromatin remodelers and transcription factors are differentially regulated after venom miking**

305 **between an elapid and viperids**

306 Given the upregulation of genes involved in chromatin organization and histone modification in the *P.*

307 *textilis* MVG (Figure 2A, C), we identified all chromatin modifiers upregulated at least 40-fold: Snf2 related

308 CREB activator protein (*SRCAP*, 86-fold), jumonji domain containing 1C (*JMJD1C*, 43-fold), lysine

309 methyltransferases 2A (*KMT2A,* 80-fold), KMT2C (*KMT2C,* 45-fold), KMT2D (*KMT2D*, 64-fold), and

310 chromodomain-helicase-DNA-binding protein 8-like (*CHD8,* 52-fold) (Table 1 and Supplemental Table

311 9). *CHD8* was upregulated only 2-fold in the *C. viridis* MVG at 72 hpvm and *SRCAP, KMT2C* and *KMT2D*

312 were upregulated 12-, 24- and 18-fold, respectively, in the *C. tigris* MVG at 96 hpvm. Chromatin modifiers

313 were not seen as highly upregulated in viperid MVGs (Supplemental Table 10).

314 Although chromatin structure regulates the accessibility of gene regulatory elements, TFs play vital

315 roles in the regulation of transcription. The TF with the greatest fold upregulation in the *P. textilis* MVG,

316 and with a transcript variant X2 uniquely expressed in the MVG, was specificity protein 1 (*SP1*, 79-fold)

317 (Table 1 and Supplemental Table 9). In the MVG of the rattlesnakes *C. viridis* and *C. tigris*, *SP1* was only

318 slightly upregulated comparatively, 1.5-, 2- and 4-fold at 24, 72 and 96 hpvm in *C. viridis* and 14- and 5-

319 fold at 24 and 96 hpvm, respectively, in *C. tigris.* In addition, Forkhead box N2 (*FOXN2*) and ligand-

320 dependent corepressor (*LCOR*) were upregulated 41- and 40-fold, respectively, in the *P. textilis* MVG

321 (Supplemental Table 9), but not to this extent in either viperid (Table 1). The TF with the greatest fold-

322 change in the *C. viridis* MVG was cAMP-responsive element binding protein 3-like (*CREB3L3*), which

323 was upregulated 14-, 12-, and 11-fold at 24, 72, and 96 hpvm, respectively. Nuclear factor I isoforms (*NFIA,*

324 *NFIB,* and *NFIX*) were found to be the TFs with the greatest upregulation in *C. tigris*. *NFIA* was upregulated

325 31-fold at 96 hpvm, *NFIB* was upregulated 104-fold and 133-fold, and *NFIX* was upregulated 8- and 24-

326 fold at 24 hpvm and 96 hpvm, respectively, in *C. tigris* (Table 1 and Supplemental Table 10). *NFIA* and

327 *NFIB* were also found upregulated 5-fold and 6-fold, respectively, at 96 hpvm in *C. viridis* (Table 1).

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328 **Table 1. Gene expression and activities of upregulated chromatin remodeler genes and transcription**

329 **factors in elapid and viperid milked venom glands.**

Chromatin remodeler genes	Elapid 96 hpvm	Viperids 96 hpvm	Activity
SRCAP	86-fold	*C. viridis*: 3-fold *C. tigris*: 12-fold	A coactivator for several TFs (CREB, the glucocorticoid receptor and the androgen receptor), and functions by exchanging nucleosome histones to render DNA accessible for transcription (Monroy et al. 2003; Wong et al. 2007; Watanabe et al. 2013).
JMJD1C	43-fold	*C. viridis*: N.C. *C. tigris*: 3-fold	Alters chromatin accessibility by histone demethylation, activating transcription (Viscarra et al. 2020).
KMT2A, KMT2C, and *KMT2D*	80-, 45-, and 64-fold	*C. viridis*: 1-, N.C., and 2-fold *C. tigris*: 2-, 24-, and 18-fold	Methylate lysine 4 of histone 3 (H3), a tag for epigenetic transcriptional activation (Nakamura et al. 2002; Cho et al. 2007; Zhang et al. 2012).
CHD8	52-fold	*C. viridis*: N.C. *C. tigris*: 5-fold	Interacts with H3 di- and tri-methylated at lysine 4, recruiting histone H1 and repressing genes regulated by β-catenin (Wnt signaling pathway) (Thompson et al. 2008; Sims and Wade 2011; Nishiyama et al. 2012).
Transcription factor genes			**Activity**
SP1	79-fold	*C. viridis*: 4-fold *C. tigris*: 5-fold	Binds GC box and related GT/CACC box CREs in promoter, enhancer and locus control regions of housekeeping and tissue-specific genes (Philipsen and Suske 1999).
FOXN2	41-fold	*C. viridis*: N.C. *C. tigris*: 12-fold	Binds purine-rich regions and members of this TF family have been implicated as regulators of embryogenesis, cell cycling, and cell lineage restriction (Tuteja and Kaestner 2007).
LCOR	40-fold	*C. viridis*: N.A. *C. tigris*: 2-fold	Can function as either a transcription activator or repressor and has been linked to polycomb-group target genes, promoting methyltransferase activity (Fernandes et al. 2003; Conway et al. 2018).
CREB3L3	N.C.	*C. viridis*: 11-fold *C. tigris*: N.A.	TF involved in ER stress and activating the unfolded protein response (Zhang et al. 2006).
NFIA, NFIB, and *NFIX*	17-, 5-, and 17-fold	*C. viridis*: 5-, 6-fold, and N.C. *C. tigris*: 31-, 133-, and 24-fold	NFI family of TFs regulates genes across many different cell types, recognizing a palindromic consensus DNA sequence $TGGA/C(N)_5GCCAA$ (de Vries et al. 1987).

330   N.C. = No Change; N.A. = Not Annotated.

331

332   **_Cis_-regulatory elements (CREs) and _trans_-factor upregulation vary between elapids and viperids**

333   Using toxin gene promoter regions from elapids and viperids, we predicted CREs and evaluated the

334   expression of corresponding _trans_-regulatory factors in MVGs. Promoter activities determined either by

335   reporter gene chloramphenicol acetyl transferase or luciferase assays have identified the importance of

336   CREs within the first 500 base pairs upstream of toxin genes (Jeyaseelan et al. 2000; Kwong et al. 2009).

337   Due to variability in toxin gene expression, we evaluated regions upstream each toxin gene isoform

338   independently to determine CREs potentially contributing to differential expression. For _P. textilis,_ this

339   included 341 bp for the 3FTx pseudonajatoxin b (AY027493) (Figure 4A), 684 bp for short-chain 3FTx

340   (AF204969) (Figure 4B), and 714 bp for a non-conventional 3FTx (Figure 4C). Only one gene is present

341   for pseudonajatoxin b (Gong et al. 2001), and five different short-chain 3FTx genes share the same 684 bp

342   promoter sequence (Gong et al. 2000). Interestingly, among 3FTxs, although many Old World elapids have

343   non-conventional 3FTxs in their venoms with a fifth disulfide bond in the first loop (Nirthanan et al. 2003),

344   _P. textilis_ did not express non-conventional 3FTxs in the venom gland, despite the presence of a non-

345   conventional 3FTx gene in its genome (XP_026561523). We included this toxin gene in the analysis to

346   determine if unique CREs were present for a non-expressed 3FTx.
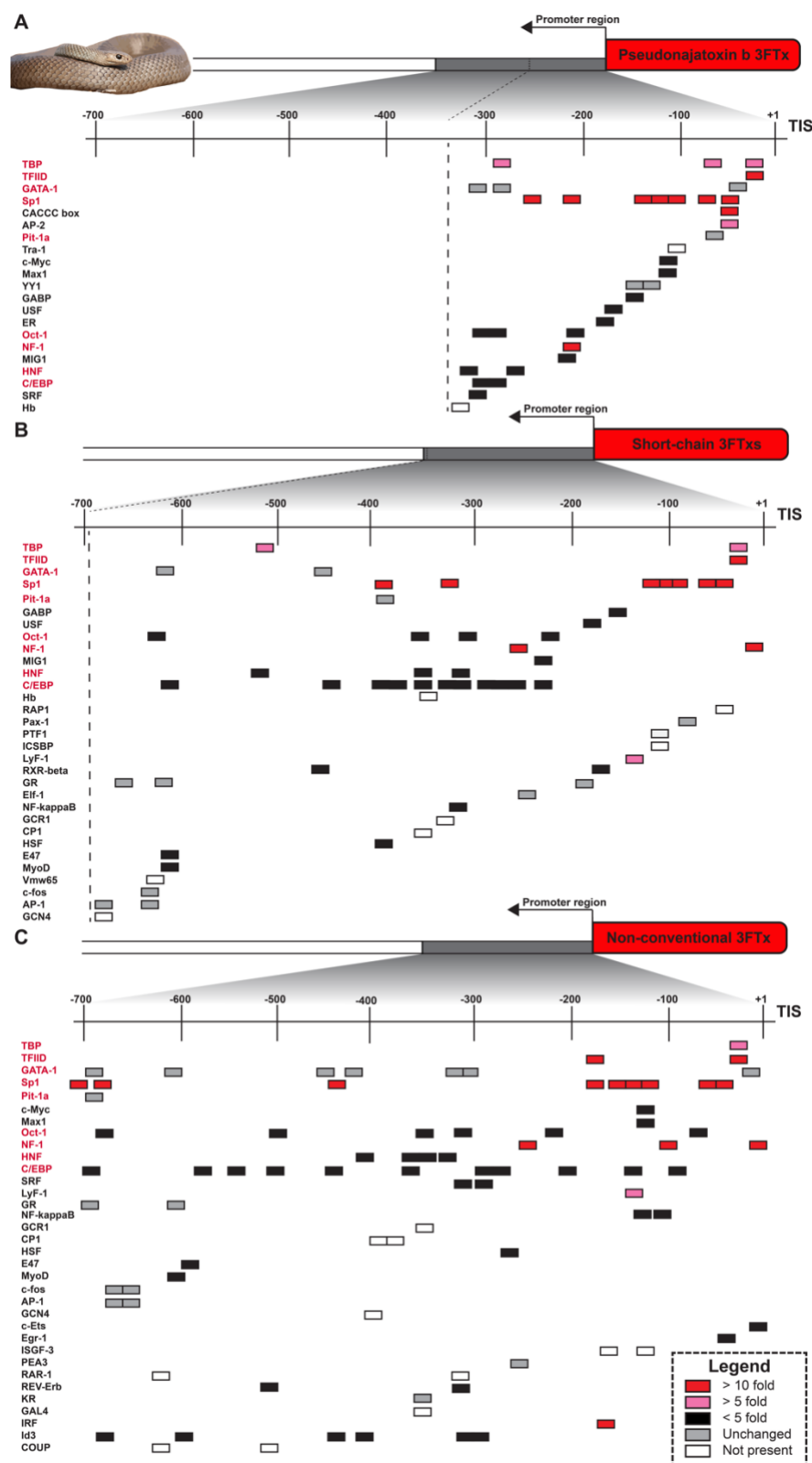
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Figure 4. Predicted *cis*-regulatory elements in the promoter regions of *Pseudonaja textilis* three-finger toxins. Only the first 341 bp upstream from the Transcription Initiation Site (TIS) could be evaluated for (A) three-finger toxin (3FTx) pseudonajatoxin b (AY027493) and 684 bp upstream from the TIS for (B) short-chain 3FTxs (AF204969) due to limited sequence availability and fragmented genome assembly. *Pseudonaja textilis* did not express transcripts encoding non-conventional 3FTxs in the venom gland, despite the presence of a non-conventional 3FTx gene in its genome (XP_026561523). From the genome reference, 713 bp upstream from what would be the TIS for this (C) non-conventional 3FTx was evaluated. Fold-changes in expression levels are shown
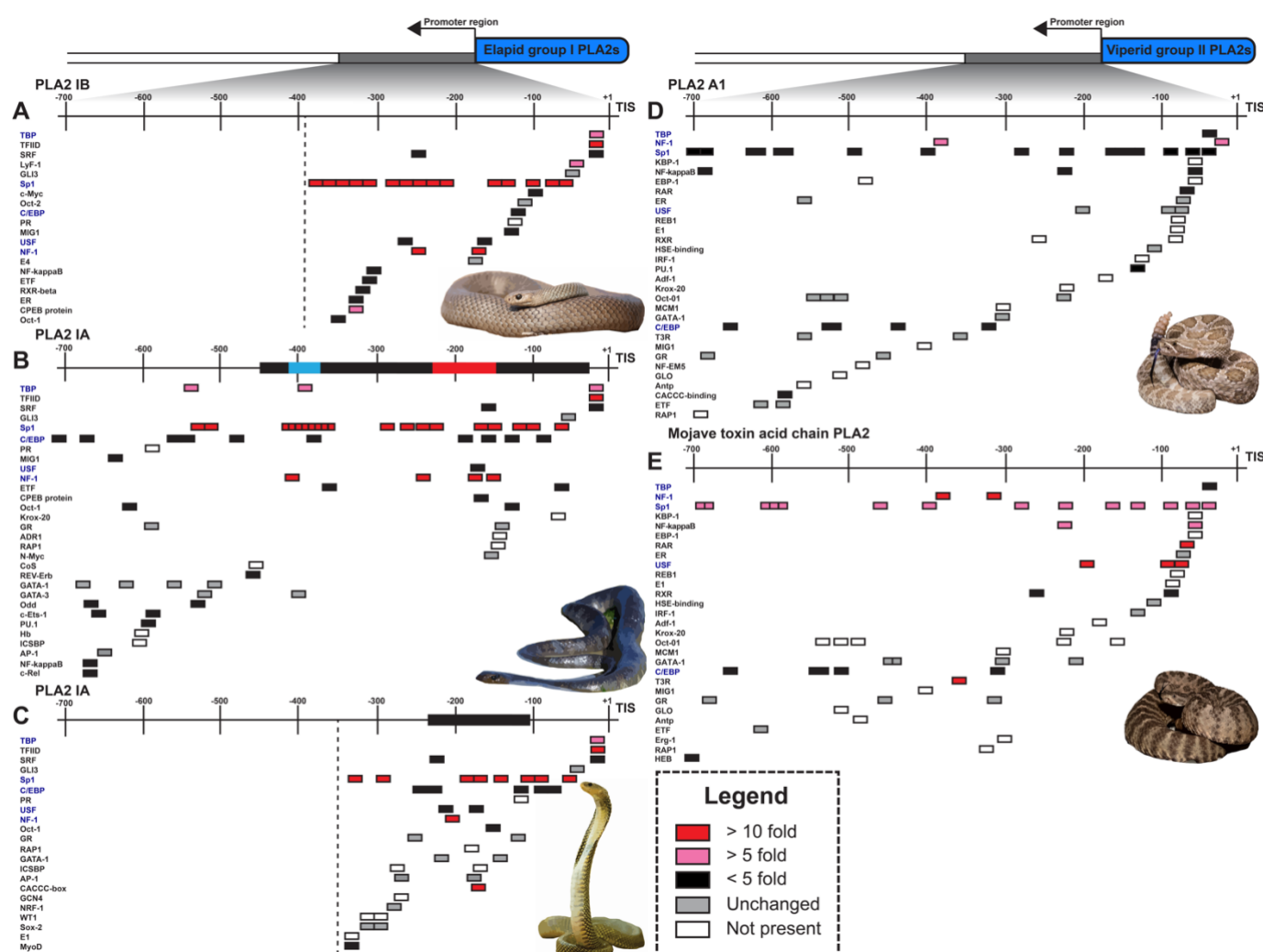
between the *P. textilis* milked venom gland and unmilked venom gland for *trans*-factors known to interact with predicted *cis*-regulatory elements (CREs). CRE predictions were completed with the online server

17

382   AliBaba2.1 using the TRANSFAC 4.0 database. CREs shared across panels are bolded red. Photo credit:

383   *P. textilis,* Ákos Lumnitzer.

384

385   Predicted CREs in promoter regions of the three 3FTx classes varied, likely due to nucleotide

386   sequence diversity, as there was 86-88% conserved sequence in the first 341 bp; however, we did identify

387   shared sites for TATA-box binding proteins (TBP) and TATA-box binding protein associated factors of the

388   RNA polymerase II preinitiation complex (TFIID). Additionally, there were GATA-1, Sp1, Pit-1a, Oct-1,

389   NFI, HNF, and c/EBP binding sites across all promoters, but these varied in number and location. All 3FTx

390   gene promoter sequences did have multiple (six to nine) Sp1 binding sites (Figure 4). Of the predicted TFs

391   binding to 3FTx gene promoter regions, Sp1/CACCC-box, NFI, and interferon regulatory factor (IRF) were

392   found upregulated at least 10-fold in the *P. textilis* MVG. The IRF binding site was only present in the non-

393   conventional 3FTx gene promoter sequence. We did not find any TFs that were downregulated to less than

394   0.10-fold, which was our threshold.

395   CREs upstream of PLA$_2$ genes that are highly expressed in elapids (group I PLA$_2$) and viperids

396   (group II PLA$_2$) were also evaluated (Figure 5). Elapid group I PLA$_2$s are subdivided into group IA and IB,

397   group IB is likely the ancestral PLA$_2$ gene with the presence of the complete pancreatic loop (Fujimi et al.

398   2004). For *P. textilis,* group IB PLA$_2$s are the most abundant and two group IB PLA$_2$ genes have been found

399   in the *P. textilis* genome with identical 385 bp sequence upstream from the transcription initiation site (TIS)

400   (Figure 5A) (Armugam et al. 2004). For *Laticauda semifasciata* and *Naja sputatrix*, group IA PLA$_2$s are

401   the most abundant, and 706 bp and 367 bp upstream from TISs were evaluated, respectively (Figure 5B,C)

402   (Jeyaseelan et al. 2000; Fujimi et al. 2004). Group II PLA$_2$s with the highest expression levels in *C. viridis*

403   and *C. tigris* venom glands were PLA2_A1 and PLA2_acidic (XM_039367474), respectively, and just over

404   700 bp of promoter regions were evaluated for each (Supplemental Table 5 and 6, Figure 5D,E). Regardless

405   of group IA, IB or II, CREs for binding TBP, Sp1, c/EBP, USF, and NFI were present for all PLA$_2$ genes.

406   Multiple Sp1 binding sites (eight to 19) were observed clustered together within all PLA$_2$ upstream gene

407   regions. For the *P. textilis* group IB PLA$_2$, TFs with CREs present and upregulated at least 10-fold in the

18

408   MVG were Sp1/CACCC-box and NFI, the same two TFs as seen for 3FTxs. For group II PLA$_2$s in the

409   viperids, TFs with CREs and upregulated over 10-fold were NFI, retinoic acid receptor (RAR), upstream

410   stimulatory factor 1 (USF1), and thyroid hormone (3,5,3'-triiodothyronine) receptor (T3R) (Figure 5D,E).

411   Interestingly, although Sp1 was slightly upregulated (5-fold) in *C. tigris* 96 hpvm, this was not observed

412   for *C. viridis*, but NFI was upregulated at least 5-fold in both viperids. TFs that were downregulated less

413   than 0.10-fold did not have any predicted CREs.

Figure 5. Predicted *cis*-regulatory elements in promoter regions of highly expressed group I and group II

phospholipase A$_2$s. Promoters regions upstream from Transcription Initiation Sites (TISs) are shown: 385

bp for (A) group IB PLA$_2$ (AY027495) from *Pseudonaja textilis*, 706 bp for (B) group IA PLA$_2$

(AB111958) from *Laticauda semifasciata*, 367 bp for (C) group IA PLA$_2$ (AF101235) from *Naja sputatrix*,

418   702 bp for (D) group II PLA$_2$_A1 from *Crotalus viridis,* and 705 bp for (E) group II PLA$_2$_acidic

419   (XM_039367474) from *C. tigris.* Fold-changes in expression levels are shown between milked and

420   unmilked glands for *trans*-factors known to interact with the predicted *cis*-regulatory elements (CREs).

421   Identified regulatory regions are bolded in promoter regions for AB111958 (Fujimi et al. 2004) and

422   AF101235 (Jeyaseelan et al. 2000). This includes a region from -232 to -162 in AB111958 that was found

423   to be responsible for an increase in promoter activity (bolded red) and a suppressor region from -410 to -

424   382 (bolded blue) (Fujimi et al. 2004). CRE predictions were completed with the online server AliBaba2.1

425   using the TRANSFAC 4.0 database. CREs shared across panels are bolded blue. Photo credits: *P. textilis*,

426   Ákos Lumnitzer; *L. semifasciata*, Patrick Davis; *N. sputatrix,* Matej Dolinay; *C. viridis*, Wolfgang Wüster;

427   *C. tigris*, Ben Lowe.

428

429   Additionally, we evaluated upstream regions that have been experimentally shown to regulate toxin

430   gene promoter activity. Fujimi et al. (2004) found a 411 bp insertion sequence (-444 to -34) present in the

431   highly expressed group IA PLA$_2$s that was absent in the lowly expressed group IB for *L. semifasciata*.

432   Luciferase activity assays from construct variations of this insertion identified a region from -232 to -162

433   that triggered elevated expression and a suppressor region from -410 to -382 (Fujimi et al. 2004). Jeyaseelan

434   et al.(2000) used chloramphenicol acetyl transferase reporter gene assays and DNase 1 footprinting

435   approaches with promoter constructs from a *N. sputatrix* group I PLA$_2$ gene to identify a region from -116

436   to -233 that contained crucial CREs (Jeyaseelan et al. 2000). We found CREs with binding sites for Sp1

437   and NFI in all of these identified regulatory regions (Figure 5B, C). Previously, we identified a 271 bp

438   insertion (-308 to -37) upstream of the gene for pseutarin C catalytic subunit (venom coagulation factor X)

439   that differed from the endogenous coagulation factor X gene. We termed this segment *VERSE* (*Ve*nom

440   *R*ecruitment/*S*witch *E*lement) (Reza et al. 2007). Within the *VERSE* core promoter there are two regions

441   that upregulate the pseutarin C catalytic subunit (Up1 and Up2) and one that suppresses expression (Sup1)

442   (Kwong et al. 2009). Here, we re-analyzed the TFs binding to these regulatory regions and found CREs for

20

443   Sp1 in Up2 and c/EBPdelta in Sup1 (Supplemental Figure 3). c/EBPdelta was downregulated 0.82-fold in

444   the *P. textilis* MVG.

445   **Venom gland miRNA profiles and targets are distinct between elapid and viperid snakes, and after**

446   **venom milking**

447   MiRNAs are known to post-transcriptionally regulate over 60% of mammalian genes (Friedman et al.

448   2009). We sequenced small RNA-seq libraries from the *P. textilis* MVG and UVG, and MVGs from *C.*

449   *viridis* (96 hpvm), to examine miRNA expression and regulation in snake venom glands. miRNAs were

450   identified by lengths of 18-23 bp, alignment to genomes, and the presence of a transcribed hairpin structure,

451   predicted by miRDeep2 (Friedländer et al. 2012). A total of 366 miRNAs (308 non-redundant mature

452   sequences) in the MVG and 375 miRNAs (299 non-redundant mature sequences) in the UVG were

453   identified in *P. textilis* (Supplemental Table 11), and 501 miRNAs (420 non-redundant mature sequences)

454   were identified in the MVGs from *C. viridis* (Supplemental Table 12). Approximately 50% of miRNAs

455   from the *P. textilis* MVG and UVG were common (Supplemental Figure 4A), but only 18% of miRNAs

456   found in *C. viridis* were also present in *P. textilis* (Supplemental Figure 4B). The most abundant miRNAs

457   in *P. textilis* venom glands were found to be *miR-148a-3p* with 359,436 Counts Per Million reads (CPM)

458   in the MVG and *miR-10c* with 210,834 CPM in the UVG (Supplemental Figure 5A, B). *miR-375* had been

459   found to be the most abundant miRNA in the venom gland of the king cobra (*Ophiophagus hannah*) (Vonk

460   et al. 2013) (Supplemental Figure 5C), but this was not the case for *P. textilis* where it was ranked 13th in

461   the MVG and 7th in the UVG. Interestingly, *miR-375* was not present in the venom gland of *C. viridis*. For

462   *C. viridis, miR-21-5p* was the most abundant (157,080 CPM), followed by *miR-148a-3p* (140,288 CPM)

463   (Supplemental Figure 5D), both of which were present but varied in expression between the elapid and

464   viperid venom glands (Table 2).

465   miRNAs post-transcriptionally repress mRNAs by base pair complementary binding (Bartel 2004),

466   usually within the 3' untranslated region (3' UTR) of target mRNAs, but interactions can also be within the

467   5' UTR or coding sequences (O'Brien et al. 2018). Predicted miRNA:mRNA interactions are largely

468   controlled by seed complementarity and duplex free energy (Doench and Sharp 2004). To explore potential

21

469   miRNA regulation of toxin transcripts (Durban et al. 2013), toxin transcript targets were predicted for the

470   top ten most abundant miRNAs. This was done with the miRanda position-weighted local alignment

471   algorithm using a criteria of -19 kcal/mol or less free energy pairing between miRNA:mRNA (Enright et

472   al. 2003; John et al. 2004), as used previously to identify toxin transcripts targeted by venom gland miRNAs

473   (Durban et al. 2013; Durban et al. 2018). In the *P. textilis* MVG, miRNAs targeted transcripts for seven C-

474   type lectin-like toxins (SNAC_2, 3, 5, 7, 8, 9, and 12), all three PLA$_2$s, and the pseutarin C catalytic subunit.

475   In the *P. textilis* UVG, the same toxin transcripts were targeted with an additional six short-chain 3FTxs

476   (3FTx_7, 8, 10, 11, 12 and 13) and the pseutarin C non-catalytic subunit (Table 2). In the *C. viridis* venom

477   gland, two snake venom metalloprotease transcripts (SVMP_4 and 9) were targeted (Supplemental Table

478   13), although not all toxin transcripts were evaluated (only those listed in Supplemental Table 5). *miR-215-*

479   *5p,* which targeted the SVMP_4 transcript, was found uniquely expressed in the *C. viridis* venom gland

480   (Table 2 and Supplemental Figure 5D).

481

482   **Table 2. miRNA differences between elapid and viperid milked venom glands.**

miRNA	Elapid 96 hpvm	Viperid 96 hpvm	Activity
miR-375	Present	Absent	Multiple transcript targets predicted that relate to ubiquitin regulation, stress response, collagen biosynthesis, translation, endocytosis and trafficking (Supplemental Table 14) (The UniProt 2021).
miR-148a-3p	359,436 CPM	140,288 CPM	Predicted to target C-type lectin-like toxin transcript in *P. textilis* (SNAC_5), no predicted targets in viperids (Supplemental Table 13 and Supplemental Table 15).
miR-21-5p	22,809 CPM	157,080 CPM	No predicted mRNA targets in the elapid but predicted to target the N-alpha-acetyltransferase 25 auxiliary subunit in viperid (Supplemental Table 15).
miR-215-5p	Absent	Present	Predicted to target a SVMP transcript (SVMP_4) in *C. viridis* MVGs (Supplemental Table 13).

22

Top ten miRNAs	Toxin transcripts targeted: Snaclecs, group I PLA$_2$s, 3FTxs and pseutarin C.	Toxin transcripts targeted: SVMPs	Different toxin transcript targets were predicted from elapid and viperid venom glands (Supplemental Table 13).
miRNAs over 100 CPM	mRNA transcripts targeted: Intracellular transport, catabolic processes, metabolic processes, organelle organization, and ER to Golgi mediated transport.	mRNA transcripts targeted: Regulation of mRNA processing, catabolic processes, negative regulation of cytoskeleton organization, steroid hormone receptor signal, stress granule assembly, transport, and ER stress.	Transcripts for proteins involved in different biological processes were targeted in elapid and viperid MVGs (Figure 6).
Pte-miR-1	Present in the *P. textilis* MVG	Absent	Predicted to target 510 mRNAs expressed in the *P. textilis* MVG. These included transcripts for proteins involved in ER to Golgi vesicle transport, ubiquitin-dependent ERAD pathway and proteasome-mediated ubiquitin-dependent protein catabolic process (Supplemental Figure 6).

483

484   Next, we predicted mRNAs targeted by abundant miRNAs (over 100 CPM) using all transcript

485   annotations from the *P. textilis* and *C. viridis* genomes to identify the most likely regulated mRNAs and

486   pathways. To reduce false positives, we used a strict criteria of -30 kcal/mol or less free energy pairing

487   between miRNA:mRNA and only evaluated transcripts that were co-expressed (at least over 10 TPM) in

488   the venom glands. A total of 750, 264, and 244 transcripts in the *P. textilis* MVG, *P. textilis* UVG and *C.*

489   *viridis* MVGs, respectively, met these criteria with predicted miRNA binding sites (Supplemental Table 14

490   and 15). Gene regulatory network analyses of miRNA targets identified a greater number of biological

491   processes targeted for downregulation in MVGs, especially for *P. textilis* (Figure 6). Biological processes

492   targeted in *P. textilis* MVG included intracellular transport (GO:0006886 and GO:0006888) and several

493   metabolic and catabolic related processes (GO:0043170, GO:1901565, GO:1901564, among others)

494   (Figure 6A). With the exception of ER to Golgi vesicle transport, different biological processes were

495   targeted for downregulation in the *P. textilis* UVG (Figure 6B). Targeted processes in *C. viridis* MVGs

23

496   shared interestingly little overlap to those of *P. textilis* and included regulation of mRNA processing

497   (GO:0006397), response to endoplasmic reticulum stress (GO:0034976), and ribonucleoside diphosphate

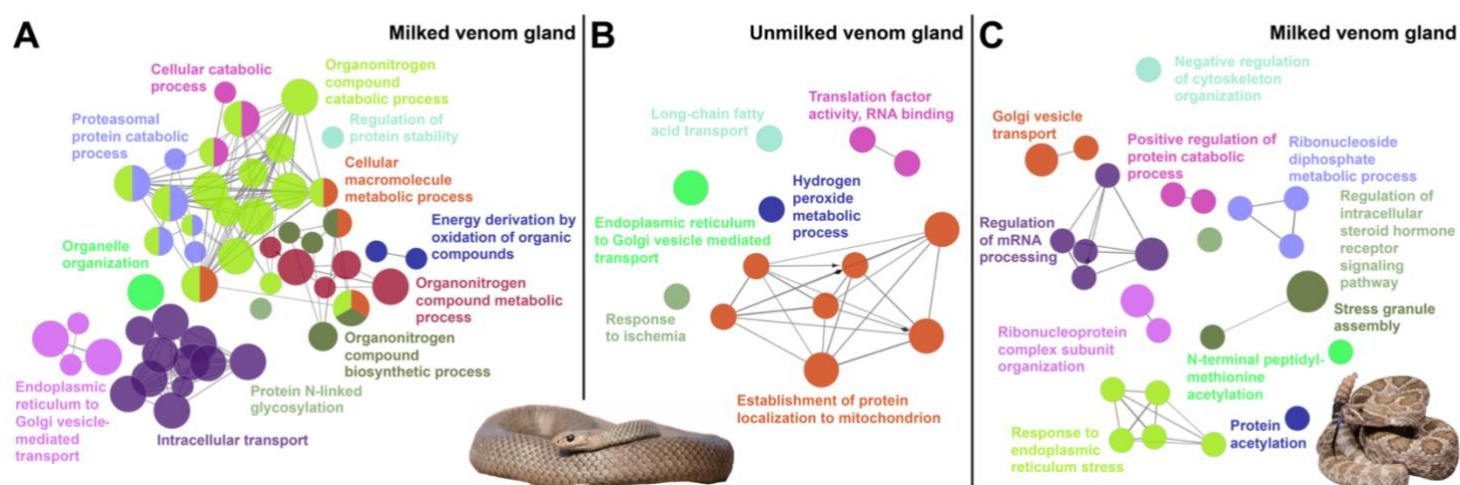498   metabolic process (GO:0009185), among others (Figure 6C and Table 2).

499   **Figure 6.** Gene networks and enriched biological processes for transcripts that are targeted by miRNAs

500   expressed in snake venom glands. Transcripts from *Pseudonaja textilis* and *Crotalus viridis* genome

501   annotations were used to predict mRNA targets for abundant miRNAs (over 100 Counts Per Million), in

502   the (A) milked venom gland from *P. textilis,* (B) unmilked venom gland from *P. textilis,* and (C) milked

503   venom gland from *C. viridis.* Gene networks were generated using *Homo sapiens* ortholog accessions of

504   all targeted transcripts and the ClueGo app plug-in (Bindea et al. 2009) in Cytoscape (Shannon et al. 2003)

505   to visual significant associated biological processes. Photo credit: *P. textilis,* Ákos Lumnitzer; *C. viridis,*

506   Wolfgang Wüster.

507

508   We observed transcripts with multiple miRNA binding sites, as well as miRNAs that targeted

509   multiple transcripts. One novel miRNA (3'-cgCCGCCGCCGTCGCCGCc-5'), uniquely expressed in the

510   *P. textilis* MVG, had 510 targets. This miRNA did not share identity to any currently in the miRBase

511   database (Griffiths-Jones et al. 2006), and due to the potential importance of this miRNA, we have named

512   it *Pte-miR-1*. It is located on the *P. textilis* genome scaffold NW_020769308.1, 14,222,810 bp to 14,222,853

513   bp in the sense direction. Transcripts targeted by *Pte-miR-1* were for proteins involved in the following

514   biological processes: ER to Golgi vesicle transport (GO:0006888), ubiquitin-dependent ERAD pathway

515   (GO:0030433) and proteasome-mediated ubiquitin-dependent protein catabolic process (GO:0043161) (p-

516   value=0.008) (Supplemental Figure 6).

517

518   **Discussion**

519   Snakes of the families Elapidae and Viperidae are responsible for medically relevant snakebite envenoming

520   worldwide, and venom variation between and within these families impacts the management of snakebite

521   victims (Gutiérrez et al. 2017; Casewell et al. 2020). Investigations into how toxin genes, which most

522   commonly arise from gene duplications, are variably expressed have been few due to the paucity of non-

523   model genomes, including those for venomous snake species, and this question has been almost entirely

524   unexplored for elapids. Using high throughput RNA-sequencing of MVGs and UVGs from the elapid *P.*

525   *textilis* and viperids *C. viridis* and *C. tigris*, as well as comparative genomic approaches evaluating CREs

526   in promoter regions of highly expressed toxin genes, we identified distinct toxin gene regulatory networks

527   between these two venomous snake families.

528   3FTxs, group I PLA$_2$s, KUNs, Snaclecs, and pseutarin C were the most abundant toxin families

529   expressed in *P. textilis* venom glands (Figure 1A). Geographic variation in venom composition is found

530   between South Australian (SA) and Queensland (QLD) *P. textilis* populations; SA snakes have an

531   abundance of postsynaptic neurotoxins (3FTxs) and QLD snakes have greater amounts of textilotoxin and

532   pseutarin C in their venoms (Skejić et al. 2015). We previously evaluated the venom proteomes of 12 *P.*

533   *textilis* individuals from SA and found none contained all textilotoxin subunits, three individuals even

534   entirely lacked textilotoxin subunits in their venoms (McCleary et al., 2016), but it was uncertain if this was

535   due to method sensitivity limitations. Our high-throughput transcriptomic results from a *P. textilis* SA

536   individual demonstrates a complete lack of gene expression of all four textilotoxin PLA$_2$ subunits, and

537   pseutarin C expression was only 2% of total toxin transcripts, whereas 3FTxs were highly expressed,

538   exhibiting greater than 70% total toxin transcripts. These data demonstrate that *P. textilis* venom variation

539   is partly due to differences in toxin gene transcription between populations. Evaluation of *P. textilis*

25

540   genomes from different geographic regions would provide insight into resolving whether PLA$_2$ genes for

541   textilotoxin subunits are present but lacking transcription, or if these genes have been lost altogether in

542   certain populations. This could contribute to the noted 'brown snake paradox', where although textilotoxin

543   is a potent neurotoxin in *P. textilis* venom, *P. textilis* envenoming more frequency causes coagulopathy

544   disturbances and rarely neurotoxicity (Barber et al. 2012).

545   Toxin expression in the viperid venom glands consisted primarily of myotoxins, SVSPs, group II

546   PLA$_2$s, and SVMPs for *C. viridis,* and group II PLA$_2$s, SVSPs, BPPs, and VEGF for *C. tigris* (Figure 1B,

547   C). From 0 to 96 hpvm, there was asynchrony in toxin synthesis, corroborating observations for the

548   Palestine Viper (*Daboia palaestinae*) (Oron et al. 1978). However, different individual snakes were used

549   at each time point for the viperid venom glands and intraspecific variation is likely. For this reason, we used

550   a single *P. textilis* individual to investigate elapid toxin gene expression dynamics after venom milking.

551   Using MVGs and UVGs, we compared toxin gene expression between an elapid and viperids.

552   Highly expressed *P. textilis* toxins exhibited fold-changes that were two-fold or less after venom milking.

553   There were similar increases of 2.5 – 3.5-fold for 3FTxs and group I PLA$_2$ genes 96 hpvm the venom gland

554   of another elapid, *N. sputatrix* (Lachumanan et al. 1999). Increases over 40-fold for toxin gene expression

555   were observed for the viperids *C. viridis* and *C. tigris*, with a SVSP gene peaking as high as 699-fold in *C.*

556   *tigris* at 24 hpvm. Similar fold changes in SVSPs and SVMPs (over 50-fold and almost 20-fold,

557   respectively) have been documented for the Puff Adder (*Bitis arietans),* although these levels of expression

558   were determined from mRNA in venom, not venom glands (Currier et al. 2012). The lower fold-changes

559   in toxin gene expression in the elapid MVG is due to the high levels of toxin expression in the UVG,

560   suggesting elapid toxin genes might be more constitutively expressed. Greater fold changes in viperid toxin

561   expression after venom milking is also likely because of the extensive physiological changes (cell

562   elongation and expansion of ER) that take place in secretory cells of viperid MVGs (Oron and Bdolah 1973;

563   Mackessy 1991), resulting in increased toxin synthesis capabilities. These physiological cell changes in

564   viperid MVGs are largely absent in elapid MVGs (Kochva et al. 1982; Lachumanan et al. 1999). This also

565   explains the absence of downregulated cellular component biogenesis and metabolic processes in the *P.*

566   *textilis* MVG that was present in the viperid MVGs (Figure 2B and Figure 3B). Genes involved in striated

567   muscle contraction were downregulated in the *P. textilis* MVG, aligning with the observation of the

568   downregulation of troponin that was seen after venom milking of another elapid, the Many-banded Krait

569   (*Bungarus multicinctus*) (Yin et al. 2020).

570   Genes related to the UPR, Notch signaling, and cholesterol homeostasis were enriched in the MVGs

571   of both *P. textilis* and the two viperids. Increases in toxin synthesis, secretion, and posttranslational folding

572   likely trigger upregulation of UPR and similar ER pathways to mediate ER stress and ensure protein quality

573   control. Perry et al. (2020) identified the UPR pathway as a feedback regulatory mechanism increasing

574   venom production in *C. viridis* (Perry et al. 2020), and UPR pathway components were present in the

575   conserved metavenom network of venom glands from the viper *Protobothrops mucrosquamatus* (Barua

576   and Mikheyev 2021), as well as conserved across venom glands in Metazoa (Zancolli et al. 2022). Cell-

577   surface receptor Notch signaling is important in cell division and development (Bray 2006), and Notch

578   signaling has been found to be critical for salivary gland cell growth and differentiation (Dang et al. 2009).

579   In human salivary glands, β−adrenergic receptor activation upregulates Notch-mediated cell proliferation

580   and differentiation of acinar cells (Wang et al. 2021), and this could be mediated by β-adrenergic receptor

581   activation in the initiation of toxin synthesis in venom glands (Luna et al. 2009). This additionally supports

582   conserved higher-level regulatory networks between venom glands and salivary glands (Barua and

583   Mikheyev 2021). Cholesterol homeostasis enrichment is likely due to increases in vesicle-mediated

584   transport and exocytosis in MVGs. Lipids that include cholesterol, phosphatidylinositol 4,5-bisphosphate

585   and sphingolipids cluster as plasma membrane microdomains, concentrating and regulating SNARE

586   proteins to create active exocytotic sites (Salaün et al. 2005). These data suggest that there is conservation

587   in high-level cellular pathways regulating venom production for both elapids and viperids.

588   In contrast, we observed differences in chromosomal, TF, and CRE regulation between elapid- and

589   viperid-specific venom production. Genes involved in chromatin organization/regulation, histone

590   modification and transcription were upregulated in the *P. textilis* MVG (Figure 2A, C). This included

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591   chromatin-remodeler *SRCAP* and histone lysine methyltransferases *KMT2A, KMT2C* and *KMT2D*. KMT2C

592   and KMT2D are known to function together as super-enhancers (Lai et al. 2017), and could be a potential

593   mechanism to increase transcription related to venom production. Although *SRCAP, KMT2C* and *KMT2D*

594   were also upregulated in *C. tigris* after venom milking, there were less genes overall associated with

595   chromatin organization/regulation upregulated for viperid MVGs (Figure 3C). This could be due to the

596   differences in chromosome locations of major toxins between elapids and viperids, macrochromosomes in

597   elapids (Suryamohan et al. 2020; Zhang et al. 2022) and microchromosomes in viperid snakes (Shibata et

598   al. 2018; Schield et al. 2019; Margres et al. 2021).

599   The transcription factor Sp1 was upregulated 79-fold in the *P. textilis* MVG, and Sp1 binding sites

600   have been identified in promoter regions of *P. textilis* toxin genes for 3FTxs (Gong et al. 2001), group I

601   PLA_2s (Armugam et al. 2004) and the pseutarin C catalytic subunit (Reza et al. 2007), as well as toxin genes

602   from other elapids (Supplemental Table 16). We also identified clusters of Sp1 CREs in toxin gene

603   promoters belonging to different venom protein families and isoforms (Figure 4, 5), and although Sp1

604   binding sites were also present in viperid toxins, Sp1 was not upregulated to the same extent as observed

605   for the elapid. Multiple Sp1 binding sites are known to enhance transcription (Pascal and Tjian 1991) and

606   could be a regulatory mechanism to elevate the expression of toxins. Sp1 binding sites are highly enriched

607   in promoter-proximal regions and instead of initiating the opening of chromatin structure, Sp1 activates

608   transcription through interacting with general transcription machinery and p300, a core member of the

609   enhancer complex (Ibañez-Tallon et al. 2002; Grossman et al. 2018). Thus, Sp1 has been associated with

610   constitutive transcription (Ibañez-Tallon et al. 2002).

611   The shared Sp1 CREs of elapid 3FTx and group I PLA_2 genes could allow for coordinated co-

612   expression of these two venom protein families, which are both abundant toxins in the venom of many

613   elapid snakes (Tasoulis and Isbister 2017), and for a potential pre-adaptive regulatory mechanism that could

614   have contributed to the evolution of defensive venom in spitting cobras (Kazandjian et al. 2021). Notably,

615   Sp1 sites have been identified in regulatory promoter regions for 3FTx and group I PLA_2 genes in the

616   spitting cobra *N. sputatrix* (Jeyaseelan et al. 2000; Ma et al. 2001). In *P. textilis,* shared Sp1 regulation of

617 3FTx, group I PLA$_2$, and pseutarin C catalytic subunit genes could provide collective polygenic

618 upregulation after venom milking. TATA boxes and associated factors were also identified shared across

619 3FTx, group I PLA$_2$, and pseutarin C catalytic subunit genes. The TATA box regulates approximately 24%

620 of genes in humans, and these genes are more tightly regulated by biotic or stress stimuli in comparison to

621 TATA-less genes (Yang et al. 2007; Bae et al. 2015). However, given that the two group I PLA$_2$ genes in

622 *P. textilis* share identical 385 bp sequence upstream the TIS, but we found three group I PLA$_2$ transcripts

623 expressed at variable abundances (100,141 TPM to 1,846 TPM), and therefore additional regulatory

624 mechanisms (e.g. chromatin structure, CREs farther upstream, number of CREs, enhancer sequences

625 present at other sites, or RNA editing) likely contribute to the differential relative abundances of these

626 toxins.

627   We identified potential *cis*-regulatory suppressors of *P. textilis* toxin genes: the non-conventional

628 3FTx found in the *P. textilis* genome had an IRF binding site not found to be present in expressed 3FTx

629 homologs, and a c/EBP binding site was identified within the suppressor region of the pseutarin C catalytic

630 subunit gene. Further, c/EBP was downregulated 0.82-fold in the *P. textilis* MVG. Gamma interferon

631 response elements (γ−IRE) contributed to cell-specific silencing of group I PLA$_2$ genes in *N. sputatrix*

632 (Jeyaseelan et al. 2000), and c/EBP has been previously identified as a toxin suppressor CRE, as deletion

633 of this site resulted in a 2-fold increase in promoter activity for a cytotoxic 3FTx in *N. sputatrix* (Ma et al.

634 2001).

635   Although NFI CREs are present in both elapid and viperid toxin gene promoters, NFI-family TFs

636 appear to likely be of greater importance in the expression of viperid toxin genes in comparison to the elapid

637 *P. textilis* as NFI genes were highly upregulated in both viperids. NFIA, NFIB, and NFIX, are RNA

638 polymerase II core promoter binding TFs with binding sites present and accessible in promoter regions of

639 multiple viperid toxin genes (SVMPs, SVSPs, and group II PLA$_2$s) (Schield et al. 2019; Margres et al.

640 2021), potentially also coordinating the expression of these multiple gene families. NFI-family genes are

641 ubiquitously expressed in different tissues but are known to regulate tissue-specific expression, including

642 mammalian glands (Murtagh et al. 2003), through interactions with other TFs, members of the transcription

643   initiation complex, and epigenetic regulators (Gronostajski 2000; O'Connor et al. 2016). NFI family binding

644   sites are highly correlated with the center of nucleosome depletion regions, suggesting that their binding

645   directly shapes local chromatin structures and can function as pioneer factors (Grossman et al. 2018; Adam

646   et al. 2020). Pioneer factors are the first factors to engage target sites in chromatin and recruit histone

647   modifying proteins, similar to many other identified viperid toxin gene TFs such as AP-1, CREB3, and

648   FOX family TFs (Zaret and Carroll 2011; Perry et al. 2022). In addition, we found multiple CREs and

649   associated *trans*-factors that were upregulated in viperids unique to group II PLA$_2$s (RAR, USF1, and T3R),

650   as well as varying upregulation of these factors between the two rattlesnake species, highlighting potential

651   regulatory differences that could contribution to venom variation between and within the two snake

652   families.

653   Post-transcriptional regulation of venom toxins by miRNAs has also been proposed (Durban et al.

654   2013). We observed that highly expressed miRNAs in the *P. textilis* MVG and UVG shared toxin transcript

655   targets, with additional 3FTxs and pseutarin C non-catalytic subunit transcripts targeted in the UVG. Given

656   that these targeted toxin transcripts are all major components of *P. textilis* venom, and we evaluated only

657   top 10 most abundant miRNAs, there is a high likelihood that miRNAs could be regulating the translation

658   of these toxin transcripts, including the downregulation of toxin translation in UVGs. Post-transcriptional

659   miRNA regulation of venom toxins has been hypothesized to be responsible for ontogenetic venom

660   variation in snakes (Durban et al. 2013; Durban et al. 2017; Durban et al. 2018), and our finding that there

661   are miRNAs targeting both pseutarin C subunit transcripts could contribute to the ontogenetic shift in

662   abundance of pseutarin C, as neonate *P. textilis* venoms lack this toxin complex and have venoms that fail

663   to induce clot formation in plasma and whole blood (Jackson et al. 2016; Cipriani et al. 2017). Venom gland

664   miRNAs from other *P. textilis* age classes will need to be evaluated to test this hypothesis. For *C. viridis,*

665   we only identified two SVMP transcripts targeted, therefore less miRNA regulation of toxin transcripts was

666   seen in the viperid venom gland. Viperid venom gland miRNAs that target SVMP transcripts have also

667   been observed for the Mexican rattlesnakes *C. simus, C. tzabcan* and *C. culminatus* (Durban et al. 2013;

30

668 Durban et al. 2017), suggesting this could be a common post-transcriptionally regulated toxin gene family

669 in viperids.

670 Unique miRNA expression signatures were present in the *P. textilis* and *C. viridis* MVGs. This

671 included the presence of *Pte-miR-1* in the *P. textilis* MVG with no known miRNA homology, the absence

672 of *miR-375,* a highly abundant elapid miRNA, from the venom gland of *C. viridis,* and the unique presence

673 of *miR-215-5p* in the *C. viridis* venom gland that was predicted to target SVMP toxin transcripts. Although

674 there was overall a greater number of identified miRNAs in the viperid MVGs, miRNAs in the *P. textilis*

675 MVG were more abundant and target predictions for miRNAs over 100 CPM demonstrated a greater extent

676 of miRNA regulation in comparison to the viperid (Figure 6). For both elapid and viperid MVGs, miRNAs

677 targeted transcripts for proteins involved in intracellular transport, but a greater extent of metabolic

678 processes were targeted in the elapid MVG, and a greater number of processes related to mRNA processing

679 and response to ER stress were targeted in the viperid MVGs.

680 As we can now produce snake venom gland organoids (Post et al. 2020; Puschhof et al. 2021), it is

681 of even greater relevance to understand the epigenetic and genetic processes that regulate the expression of

682 toxin genes. These insights would be useful to optimize *in vitro* toxin expression, with applications across

683 fields in biotechnology and therapeutics (e.g., drug development from toxins and antivenom production),

684 and would reduce the need for live venomous snakes to be used in research. The toxin gene regulatory

685 mechanisms we have identified that potentially contribute to venom variation between elapid and viperid

686 snakes will require additional evidence from larger snake venom gland and genome datasets. Our dataset

687 is limited by only having one MVG and UVG for *P. textilis*, but this was done to avoid intraspecific

688 variation in toxin expression, and the logistical and ethical considerations of sacrificing multiple animals.

689 Additional sequencing approaches would be insightful, Assaying for Transposase-Accessible Chromatin

690 followed by sequencing (ATAC-seq) would help to determine which toxin genes are in chromatin

691 accessible regions, and Chromatin Immunoprecipitation followed by sequencing (ChIP-seq) for targeted

692 histone markers (lysine 4 methylation of histone 3 associated with methyltransferases KMT2A, KMT2C,

693 and KMT2D) and TFs (Sp1 and NFI-family TFs) would better determine their relationships to highly

31

694   expressed toxin genes. Snake-specific TF antibodies are unfortunately not commercially available at this

695   time, but venom gland organoids may offer an alternative approach where CRISPR/Cas9 technology could

696   be used to Epitope Tag endogenous TFs for ChIP-seq (CETCh-seq) (Savic et al. 2015). Additionally,

697   promoter activities and miRNA targets require experimental validation, and future venom gland organoid

698   experiments could also facilitate such investigations by providing tissue-specific cell cultures for this work.

699

700   **Conclusions**

701   Snake venom glands are tractable models to investigate gene regulation, as toxin gene expression and

702   synthesis are upregulated in MVGs, and we can use manual venom milking to experimentally initiate these

703   processes. Elapid and viperid venom glands are thought to share a common evolutionary origin, supported

704   by anatomical and developmental evidence (Vonk et al. 2008), however, these two families have differing

705   venom delivery systems, notably fang and venom gland morphology (Kerkkamp et al. 2015), and major

706   differences in venom composition (Supplemental Table 17). Although there are shared toxin gene families

707   and phylogenetic analysis of toxins suggests venom evolved once at the base of the advanced snake

708   radiation (Fry and Wüster 2004), there are distinct toxin genes present in these two snake families and these

709   toxin genes have differing chromosomal locations. Here, we used high-throughput RNA-seq to profile gene

710   expression and miRNAs between MVGs and UVGs in these two snake families, in addition to performing

711   comparative genomic analyses to identify *cis-* and *trans*-acting regulation of venom production in

712   an elapid in comparison to previous viperid datasets (*C. viridis* and *C. tigris*). We identified CREs

713   that are common across multiple toxin genes between these two snake families, but differences in potential

714   key chromatin modifiers, TFs, and miRNAs regulating elapid and viperid toxin expression and synthesis

715   (Supplemental Table 17). Therefore, elapid and viperid venom delivery systems, and their toxin genes and

716   associated regulatory mechanisms, likely evolved independently.

717

718

719 **Materials and Methods**

720 **Venom gland collection and RNA-sequencing**

721 An adult *P. textilis* was collected from the Barossa Valley region, South Australia and maintained at Venom

722 Supplies Pty Ltd. (Adelaide, SA, Australia). Venom was milked from the left gland, and the right gland

723 was left untouched. Four days following venom milking, the snake was humanely euthanized, both glands

724 dissected, placed in RNAlater (Thermo Fisher), and then shipped to the National University of Singapore.

725 RNA was then extracted from the venom glands using Qiagen's RNeasy Mini kit, following the

726 manufacturer's protocol. For mRNA libraries, 1 μg of total RNA was used as input into Illumina's TruSeq

727 RNA Sample Preparation v2 protocol. The small RNA library preps from the *P. textilis* MVG and UVG

728 were completed using Illumina's TruSeq Small RNA Sample Preparation protocol with 1 μg of total RNA

729 as input. The finished small RNA library was loaded onto a 6% PAGE gel (Invitrogen) and a band of ~170-

730 320 bp was excised from the gel. The size-selected library was then extracted from the PAGE gel and

731 recovered by ethanol precipitation. Quantitation of libraries was performed using Invitrogen's Picogreen

732 assay and the average library size determined by running the libraries on a Bioanalyzer DNA 1000 chip

733 (Agilent). Library concentration was normalized to 2 nM and concentrations validated by qPCR on a ViiA-

734 7 real-time thermocycler (Applied Biosystems), using qPCR primers recommended in Illumina's qPCR

735 protocol and Illumina's PhiX control library used as a standard. Libraries were then pooled at equal volumes

736 and the two library types sequenced on separate lanes of an Illumina HiSeq2500 rapid run at a final

737 concentration of 11 pM, a read-length of 101 bp paired-end for the mRNA library and 51 bp single-end for

738 the small RNA library.

739 An adult *C. viridis* rattlesnake of the same northeastern Colorado locality as used by Schield et al.

740 (2019) for prior *C. viridis* genome sequencing was collected and venom milked. Four days following venom

741 milking, the snake was humanely euthanized and both glands dissected. RNA isolation was completed for

742 both glands combined, following the TRIzol reagent (Thermo Fisher Scientific) manufacturer's protocol,

743 with an additional overnight −20 °C incubation in 300 μL 100% ethanol with 40 μL 3 M sodium acetate,

744 and resuspension in nuclease-free H$_2$O following incubation. Two different Illumina sequencing libraries

33

745   were prepared, one selecting mRNA and a second selecting small RNA. The same mRNA library

746   preparation protocol as described above for *P. textilis* was also followed for *C. viridis and* paired-end

747   sequencing completed to a read length of 150 bp. The small RNA library preparation was done with 1 µg

748   of total RNA used as input into the NEBNext Small RNA Library Prep Set for Illumina (New England

749   BioLabs) and an AMPure XP Bead (Beckman Coulter) selection step for a 110-160 bp size range. These

750   small RNA libraries were sequenced to 75 bp, single-end.

751   **Venom gland *de novo* transcriptome assembly, toxin annotation and expression**

752   Sequenced reads were assessed with the Java program FastQC (Babraham Institute Bioinformatics, UK) to

753   confirm that all adapters and low quality reads (<Q20) were removed before assembly. To obtain a

754   comprehensive *de novo* venom gland transcriptome assembly for *P. textilis*, separate assemblies for each

755   gland were completed, and three assemblers used: 1) ABySS (release v1.5.0) (Birol et al. 2009; Simpson et

756   al. 2009) with paired-end default parameters and k-mer sizes 30 to 66, increased in increments of 4, and

757   merged with TransABySS (v1.5.1) (Robertson et al. 2010), 2) Trinity (release v2014-07-17) (Grabherr et

758   al. 2011) with genome-guided assembly default parameters using Bowtie2 (v2.2.6) (Langmead and

759   Salzberg 2012) aligned reads to the *P. textilis* genome (assembly EBS10Xv2-PRI), and 3) Extender (Rokyta

760   et al. 2012) with 10,000 starting seeds, where seeds were reads first merged with PEAR (Paired-End read

761   mergeR; v0.9.6 using default parameters) (Zhang et al. 2014) and seed extensions required 100 nucleotide

762   overlaps and quality scores of at least 30. For *C. viridis,* a *de novo* venom gland transcriptome assembly

763   was completed using the same approached detailed above for *P. textilis,* except excluding ABySS and with

764   an additional Trinity assembly using *de novo* parameters. For the *de novo* assembled transcriptomes for

765   both species, contigs less than 150 nucleotides and redundancies between assemblies were removed with

766   CD-HIT (v4.6.6) (Li and Godzik 2006; Fu et al. 2012). Coding contigs were then identified with

767   EvidentialGene (downloaded May 2018) (Gilbert 2013). Abundances of the coding contig set were

768   determined with RSEM (RNA-seq by Expectation Maximization, v1.3.0) (Li and Dewey 2011), using the

769   aligner Bowtie2 (v2.2.6) (Langmead and Salzberg 2012). Contigs less than 1 TPM (Transcript Per Million)

770   were filtered out, and the remaining contigs annotated with Diamond (Buchfink et al. 2014) and BLASTx

34

771   (E-value 10^{-05} cut-off) searches against the *P. textilis* genome-predicted protein set and the National Center

772   for Biotechnology Information (NCBI) non-redundant protein database. OrfPredictor (v3) (Min et al. 2005)

773   was used for final coding and protein sequence prediction with BLASTx input to aid in proper transcript

774   translation. Venomix (Macrander et al. 2018) was used to help to identify all toxin transcripts in addition

775   to toxins being manually evaluated to determine if venom proteins were full-length, shared sequence

776   identity to currently known toxins, and contained a conserved signal peptide sequence within each venom

777   protein family.

778   **Venom gland gene expression and *cis*-regulatory element predictions**

779   From *P. textilis* genome annotations (assembly EBS10Xv2-PRI), the global transcriptome (34,614

780   predicted transcripts) was used as a reference for aligning reads originating from the *P. textilis* MVG and

781   UVG. For rattlesnake MVGs and UVGs, the following NCBI data sets were used: SRR11524062 (*C. tigris*

782   UVG), SRR11524063 (*C. tigris* UVG), SRR11524059 (*C. tigris* 24 hpvm), SRR11524060 (*C. tigris* 24

783   hpvm), SRR11524050 (*C. tigris* 96 hpvm), SRR11524051 (*C. tigris* 96 hpvm), SRR7401989 (*C. viridis*

784   UVG), SRR7402004 (*C. viridis* 24 hpvm), and SRR7402005 (*C. viridis* 72 hpvm). Reads from these data

785   sets were aligned to annotated transcriptomes from genome assemblies (UTA_CroVir_3.0 and

786   ASM1654583v1 for *C. viridis* and *C. tigris*, respectively), in addition to myotoxin transcripts from the *de*

787   *novo* assembled venom gland transcriptome for *C. viridis*. Transcript abundances were determined with

788   Bowtie2 (v2.2.6) (Langmead and Salzberg 2012) read alignments and RSEM (Li and Dewey 2011). RSEM

789   output of expected counts, transcript length and TPM were used as input into GFOLD (Feng et al. 2012) to

790   identify transcript fold-changes between the conditions. A Gene Set Enrichment Analysis (GSEA) (Mootha

791   et al. 2003; Subramanian et al. 2005) was performed using RSEM estimated transcript abundances as input.

792   Transcripts were searched against the UniProt *Homo sapiens* protein database (The UniProt 2021) with

793   Diamond BLASTx (v0.8.34) (Buchfink et al. 2014) to identify orthologs. UniProt accessions were then

794   entered into DAVID Bioinformatics Resources 6.8 (Huang da et al. 2009a) to identify functional

795   annotations and pathways. In addition, gene networks were constructed using the ClueGo app plug-in

796   (Bindea et al. 2009) in Cytoscape (Shannon et al. 2003). *Cis*-regulatory element predictions were completed

797 upstream toxin gene transcription start sites, up to 700 bp if available, using the online server AliBaba2.1

798 (http://gene-regulation.com/pub/programs/alibaba2/) with the TRANSFAC 4.0 database embedded within

799 the webserver. GFOLD determined fold-changes in MVGs for *trans*-factors associated with CREs were

800 then evaluated.

801 **Venom gland microRNA expression and target prediction**

802 TrueSeq small RNA library adapters were trimmed with the fastx_clipper tool, provided in the FASTX-

803 Toolkit (Hannon lab Cold Spring Harbor Laboratory). Trimmomatic (Bolger et al. 2014) was used to filter

804 out low quality (< Q20) reads, evaluated with a sliding window of 4 nucleotides. To filter out rRNA, reads

805 were then aligned with Bowtie2 (v2.2.6) to known snake rRNA sequences. Non-rRNA reads were used as

806 input into miRDeep2 (v2.0.1.2) (Friedländer et al. 2012) to identify species-specific miRNAs from the *P.*

807 *textilis* genome (GCF900518735.1, assembly EBS10Xv2-PRI) and *C. viridis* (UTA_CroVir_3.0).

808 Expression levels of miRNAs in venom glands were estimated by normalization to Counts Per Million

809 (CPMs; CPM = mature miRNA reads / total mapped miRNA reads * 10^6). Target prediction was performed

810 with the position-weighted local alignment miRanda (v3.3) algorithm (Enright et al. 2003; John et al. 2004).

811 A free energy value of at least -19 kcal/mol was used as thresholds for toxin transcript target identification,

812 using only contigs coding for full length venom proteins from the *de novo* assembled transcriptome from

813 *P. textilis* and toxin transcripts from the annotated in *C. viridis* genome, in addition to the *de novo* assembled

814 myotoxin transcript. A stricter free energy value of at least -30 kcal/mol was used for target identification

815 from genome annotated transcriptome datasets. Non-toxin transcripts targeted were searched against the

816 UniProt *Homo sapiens* protein database (The UniProt 2021) with Diamond BLASTx (v0.8.34) (Buchfink

817 et al. 2014) to identify orthologs, and accessions were then entered into DAVID Bioinformatics Resources

818 6.8 (Huang da et al. 2009a) to identify functional annotations and pathways. In addition, gene networks

819 were constructed using the ClueGo app plug-in (Bindea et al. 2009) in Cytoscape (Shannon et al. 2003).

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823 **Data access**

824 All next-generation sequencing data was submitted to NCBI under BioProject ID PRJNA931953

825 (BioSample SAMN33139130 for *P. textilis* and SAMN33139131 for *C. viridis*).

826

827 **Competing interest statement**

828 The authors declare no competing interests.

829 **Acknowledgements**

832

833 **References**

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