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Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity

Zia Nisani [a,*], Danilo S. Boskovic [a,b], Stephen G. Dunbar [a], Wayne Kelln [c], William K. Hayes [a]

[a] Department of Earth and Biological Sciences, School of Science & Technology, Loma Linda University, Loma Linda, CA 92350, USA
[b] Division of Biochemistry, Department of Basic Sciences, School of Medicine, Loma Linda University, Loma Linda, CA 92350, USA
[c] Department of Basic Sciences, School of Medicine, Loma Linda University, Loma Linda, CA 92350, USA

ABSTRACT

We investigated the biochemical profile of regenerated venom of the scorpion *Parabuthus transvaalicus* in relation to its metabolic cost and toxicity. Using a closed-system respirometer, we compared oxygen consumption between milked and unmilked scorpions to determine the metabolic costs associated with the first 192 h of subsequent venom synthesis. Milked scorpions had a substantially (21%) higher mean metabolic rate than unmilked scorpions, with the largest increases in oxygen consumption occurring at approximately 120 h, 162 h, and 186 h post-milking. Lethality tests in crickets indicated that toxicity of the regenerated venom returned to normal levels within 4 d after milking. However, the chemical profile of the regenerated venom, as evaluated by FPLC and MALDI-TOF mass spectrometry, suggested that regeneration of different venom components was asynchronous. Some peptides regenerated quickly, particularly those associated with the scorpion's "prevenom," whereas others required much or all of this time period for regeneration. This asynchrony could explain the different spikes detected in oxygen consumption of milked scorpions as various peptides and other venom components were resynthesized. These observations confirm the relatively high metabolic cost of venom regeneration and suggest that greater venom complexity can be associated with higher costs of venom production.

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1. Introduction

Scorpions use their venom to immobilize prey items and defend themselves against aggressors. Their venom is a cocktail of water, salts, small molecules, peptides, and proteins (Zlotkin et al., 1978; Yahel-Niv and Zlotkin, 1979; Simard and Watt, 1990). The venom composition of many scorpion species has been characterized, leading to the observation that certain constituent peptides have the greatest biological effects on particular target organisms. Scorpion venom toxicity has been shown to be specific for invertebrates, vertebrates, or both (Possani et al., 1999; Inceoglu et al., 2001). Among the peptides present in venom, short-chain neurotoxins (SCNs) act on potassium and chloride channels, whereas long-chain neurotoxins (LCNs) primarily act on sodium channels (Possani et al., 1999; De La Vega and Possani, 2004, 2005; Du Plessis et al., 2008).

Recent work (Nisani et al., 2007) revealed that venom regeneration in scorpions is an expensive metabolic investment, providing further insight into reasons why scorpions appear to be judicious in their stinger and venom use (Rein, 1993; Edmunds and Sibly, 2010; Nisani and Hayes, 2011). Biosynthesis of proteins and peptides seems to be slower than regeneration of the total venom. Emptied *Parabuthus transvaalicus* scorpion glands were able to regenerate venom volume in 72 h, whereas protein

concentration remained diluted within this time frame (Nisani et al., 2007). Boeve et al. (1995) documented a similar pattern of venom regeneration in the spider *Cupiennius salei*, for which the protein concentration of newly regenerated venom was significantly lower, and the concentration of free amino acids significantly higher, than that obtained from the initial milking. In scorpions, maximum levels of toxin mRNA appeared in the venom gland of *Androctonus mauretanicus* at 24 h following emptying (Alami et al., 2001). Venom composition of the scorpion *Tityus serrulatus* varied substantially during venom regeneration (20 days post-feeding, and 1 and 8 d after milking), with smaller proteins appearing to regenerate more slowly than larger ones (Pimenta et al., 2003).

Venom gland morphology in scorpions has a generalized scheme across taxa, with main differences occurring in the presence or absence of folds in the secretory epithelium (Pawlowsky, 1924; Mazurkiewicz and Bertke, 1972). The lumen of the venom gland likely serves as an extracellular storage site for venom. Abundant membrane-bound vesicles within the lumen segregate the different secretory products that are presumably mixed during injection (Mazurkiewicz and Bertke, 1972). Kovoor (1973) demonstrated that the venom gland of *Buthotus judacius* consists of a series of three lobes that differ in morphology and histochemistry. Some lobes within the gland contained only acidic mucosubstances, whereas others contained mainly protein, or acidic and protein products combined.

Zlotkin and Shulov (1969) reported that the appearance of venom collected from a series of successive stings from *Leiurus quinquestriatus* changed from a transparent, to an opalescent, and finally to a milky viscous secretion, with the opalescent fraction having the highest total and specific toxicity (Yahel-Niv and Zlotkin, 1979). Studies conducted on *P. transvaalicus* found that this species possesses two types of secretion: "prevenom" and "venom" (Inceoglu et al., 2003). Prevenom primarily contains a high concentration of K^+ salt and several peptides that elicit significant pain and toxicity, whereas venom is mostly proteinaceous with physiological levels of K^+ salt. More recent work by Nisani and Hayes (2011) suggests a continuum between prevenom and venom, with scorpions delivering a variable number of defensive stings in succession having clear (prevenom), opalescent (mixed), or milky (venom) secretions. Scorpions may, for example, issue several stings with prevenom under low threat, yet deliver venom with every sting under high threat. The presence of such a continuum from prevenom to venom has been reported in a number of scorpion species (Yahel-Niv and Zlotkin, 1979; Gopalakrishnakone et al., 1995; Abdel-Rahman et al., 2009; Fox et al., 2009). Inceoglu et al. (2003) hypothesized that prevenom would be replenished more rapidly than venom that requires more extensive protein synthesis and folding.

The venom of *P. transvaalicus* contains fewer than 100 major peptides, and thus is considered to be relatively simple compared to other scorpion venoms (Possani et al., 1999; c.f., Inceoglu et al., 2001). This venom exhibits high specificity toward both insects and mammals (Inceoglu et al., 2001). Its dual specificity could be attributed to a diet that presumably consists largely of insects (Polis, 1979) and the susceptibility of these large scorpions to mammalian predators (i.e., they offer a high caloric yield as prey).

To date, no studies of the chemical profile of regenerated scorpion venom in relation to metabolic cost and toxicity have been reported in the literature. Thus, the aim of this study was to investigate the biochemical profile and quality of *P. transvaalicus* venom regenerated over time in relation to oxygen consumption and killing effectiveness. Understanding the rates of regeneration for various components can shed light on why scorpions appear to optimize venom expenditure. To accomplish these goals, we analyzed the biochemical profile of regenerating venom using FPLC and MALDI-TOF mass spectrometry. We also recorded oxygen consumption during venom regeneration over an 8-d period, and measured changes in toxicity via bioassay of crickets.

2. Materials and methods

2.1. Animals

We purchased 10 large adult female *P. transvaalicus* scorpions, ranging in weight from 5.10 to 8.75 g, from two suppliers: Glades Herp, Inc. (Bushnell, Florida, USA), and Hatari Invertebrates (Portal, Arizona, USA). Scorpions were housed in clear plastic containers measuring $35 \times 16 \times 11$ cm ($L \times W \times H$) with sand substrate. They were kept at $25 \pm 1\,°C$ under a 12:12 light–dark cycle and fed one cricket per week. Prior to testing, scorpions were fasted for 7 d. None of the female scorpions were gravid.

2.2. Reagents

We used insect Ringer's solution (pH = 7.05) containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). The following buffers were used: buffer A (2% ACN (acetonitrile), 98% H_2O, 0.065% TFA (trifluoroacetic acid)) and buffer B (80% ACN, 20% H_2O, 0.05% TFA).

2.3. Venom collection

We milked scorpions of their venom by having them sting a parafilm-covered microcentrifuge tube (1 ml). This was done by securing the telson with forceps, forcing the aculeus (stinger) to penetrate the parafilm, and then repeatedly pushing the vesicle against the parafilm without withdrawing the aculeus until no further venom was secreted from the aculeus. Thus, we considered the venom gland to be functionally emptied. We refrained from using electro-stimulation, since this method may unduly stress the scorpions to the point of impaired venom production (G. A. Fox and A. Cooper, pers. comm.) and premature death (Z. Nisani, unpubl. data). Venom released with our manual technique presumably represented defensive venom expenditure rather than predatory stinging, and included both prevenom and venom. We measured the quantity of venom expended using a sterile, calibrated microcapillary pipette (10 μl), then transferred the sample into a separate microcentrifuge tube containing 5 ml of distilled water.

This preparation was frozen at $-10\,°C$ and stored for further analysis.

Subsequent to the initial forced milking, scorpions were milked once more at a randomly-selected 2-d ($N = 2$), 4-d ($N = 3$), 6-d ($N = 3$), or 8-d ($N = 2$) interval. The regenerated venom was measured and treated in the same way as initial venom.

2.4. Oxygen consumption

The closed-system respirometer, data collection methods, and computations were described in Nisani et al. (2007). Six scorpions were available for this part of the study, with each tested once in milked and unmilked treatments (i.e., a repeated-measures design); however, three specimens died before or during the experiment. We randomized the treatment order for each scorpion, with 21 d separating the two trials. Oxygen consumption in each treatment was measured for 8 d, with readings logged every 1 h.

2.5. Bioassay

To assess the biological activity of regenerating venom, we injected five groups of 30 crickets (randomly assigned to each group) with venom samples collected on days 0, 2, 4, 6, and 8. Subsequent to the initial forced milking, scorpions were milked once more at a randomly-selected 2-d ($N = 3$), 4-d ($N = 2$), 6-d ($N = 3$), or 8-d ($N = 3$) interval. We delivered 2 μl of dilute venom (1:5 dilution in insect Ringer's solution) intrathoracically between the second and third pairs of legs of each cricket (mean cricket mass $\pm$ SD: 112 ± 12 mg) by means of a 5-μl Hamilton syringe (Hamilton Company, USA). At 24 h following injection, the state of each cricket kept at 25 °C was recorded using the criteria of Boeve et al. (1995). We considered state to be normal (normal crawling and capable of self-righting when placed on dorsum), immobilized (incapable of self-righting, but retaining rapid leg movements), or paralyzed/killed (incapable of self-righting, very slow movements, or motionless). Paralysis was indistinguishable from death. None of the 30 additional control crickets injected with 2 μl of insect Ringer's solution were affected within 24 h.

2.6. FPLC analysis

Venom samples collected were spun at 10,000 rpm for 5–6 min to remove particulates prior to injections. Due to small volumes, venom samples for day 2 were pooled. We conducted fast protein liquid chromatography (FPLC) on a SOURCE 15RPC ST 4.6/100 reversed phase column using a GE Lifesciences AKTA FPLC. The column had a bed volume of 1.66 ml, and flow rate of 2 ml/min. All venom samples were diluted at 1:40 (5 μl sample in 195 μl buffer A). The column was equilibrated in buffer A, a 100 μl diluted sample was injected onto the column, and eluted with a 20 column volume linear gradient of 0–100% buffer B. The active fractions of the venom standard sample were collected for further analysis by mass spectrometry.

To establish an FPLC profile of the venom, one scorpion was milked 21 d prior to the start of this experiment. The venom sample was stored at -10 °C in 200 μl of buffer A solution.

2.7. MALDI-TOF analysis

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was performed on selected fractions collected from the initial FPLC analysis of venom, using an Autoflex instrument (Bruker Daltonics, Billerica, Massachusetts, USA). Venom samples (1 μl) were loaded on the Polished Steel MALDI plate with 1 μl α-cyano-4-hydroxycinnamic acid (α-CHCA; Aldrich, St. Louis, Missouri, USA) or 3,5-dimethyl-4-hydroxycinnamic acid (sinapinic acid – SA; Aldrich) followed by air drying. The instrument was calibrated using angiotensin II (MW 1047.20 Da), somatostatin 28 (MW 3149.61 Da), insulin (MW 5734.5 Da), myoglobin (MW 8475.70 Da), and cytochrome C $[M + 2H]^{2+}$ (MW 6181.05 Da). All mass spectra were recorded with two reference peptides as internal standards using a two-point calibration. The errors to the masses of the spectra were within the 0.05% range. All spectra were recorded in the 1000–15,000 m/z range using accelerating grid and guide wire potentials of 20,000, 19,000 and 1000 Vs, respectively, and 400 ns delayed extraction setting.

2.8. Data analysis

FPLC profiles of the venom samples (2.5 μl venom diluted to 100 μl in buffer A) were analyzed with respect to peak height as well as peak area for up to 20 distinct peaks (when present). Since the venom volumes as well as the constituents varied with each sample, the peaks were corrected only with respect to the total volume of venom obtained (V_T in μl) and the body weight of the scorpion (W in grams). Standardized peak heights or areas ($P_{i(s)}$) were obtained from their direct measurements (P_i) via the equation: $P_{i(s)} = P_i(V_T/(2.5^*W))$. The peak heights and peak areas then were represented in units of mAU/g and mAU·ml/g, respectively.

We calculated Cohen's d for an effect size (Cohen, 1988) using pooled standard deviation (Hojat and Xu, 2004) to compare the metabolic rates of milked and unmilked scorpions after 72 h, with values of $\sim$0.5 and $\geq$0.8 considered medium and large effects, respectively (Zar, 1996). We employed linear regression analysis to analyze the differences in quantity of venom regenerated over the 8-d period post-initial milking (Zar, 1996), with r^2 computed for effect size and values of $\sim$0.9 considered moderate and $\geq$0.25 large (Cohen, 1988). Finally, we used a Chi-square (χ^2) test to assess the results of the venom toxicity bioassay (Zar, 1996), with Cramer's V or Phi (φ) calculated for effect size and values of $\sim$0.3 deemed moderate and $\geq$0.5 large (Cohen, 1988). Effect sizes, in contrast to statistical significance, are independent of sample size and more readily compared among different data sets and different studies. All analyses were conducted using SPSS version 11.5 (SPSS Inc., Chicago, Illinois, USA), with alpha set at 0.05. Data were screened when

appropriate to assure that parametric assumptions were met.

3. Results

3.1. Oxygen consumption

Milked scorpions had a higher (21%) mean metabolic rate than unmilked scorpions for the duration of the study (means $\pm$ 1 S.E.: milked = 35.64 $\pm$ 1.05 and unmilked = 25.98 $\pm$ 2.02 μl O_2 g^{-1} h^{-1}, $N = 3$ for each mean). Although the sample size was too small for statistical comparison, the effect size was exceptional (Cohen's $d = 3.46$; c.f. Cohen, 1988) and even larger than that of Nisani et al.'s (2007) prior analysis of $N = 11$ milked and unmilked scorpions (Cohen's $d = 1.38$), which was highly significant (t-test: $p < 0.0001$). Some circadian variation in metabolism was evident, particularly during the first 96 h, after which dampening of rhythmicity became apparent (Fig. 1). Several notable peaks in oxygen consumption occurred relative to controls; taking variance into consideration, the largest was at 120 h, followed by smaller peaks at 162 h and 186 h (Fig. 1; variance not shown).

3.2. Venom volume regeneration and bioassay

After milking and emptying the glands, the volume of venom regenerated relative to the initial venom yield clearly increased over the subsequent 8-d period (linear regression: percent venom volume regenerated = 0.046 [days] + 0.595; $t = 2.47$, $r^2 = 0.361$, $p = 0.039$, $N = 10$; note the large effect size; Fig. 2). Venom regeneration appeared to be complete by day 8 (reaching asymptote). From the bioassay, venom toxicity differed among the five venom samples collected over the 8-d period ($\chi^2 = 86.45$, df = 8, $p < 0.001$, Cramer's $V = 0.54$; Fig. 3). Compared to the initial milking on day 0, toxicity was clearly reduced on day 2 ($\chi^2 = 36.61$, df = 2, $p < 0.001$, $\varphi = 0.78$), with the majority of crickets injected (76%) showing no effects of envenomation. However, toxicity returned to initial levels by day 4 and remained equally toxic thereafter (pairwise Chi-square p-values relative to day 0 were all >0.77).
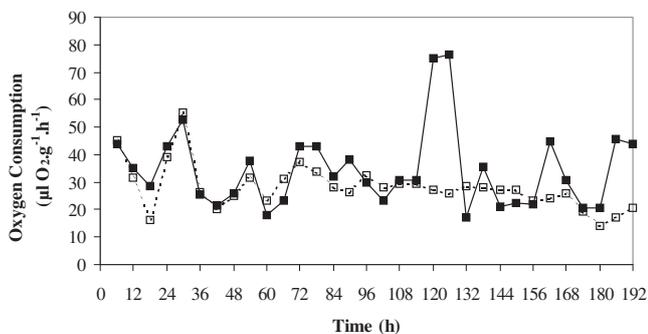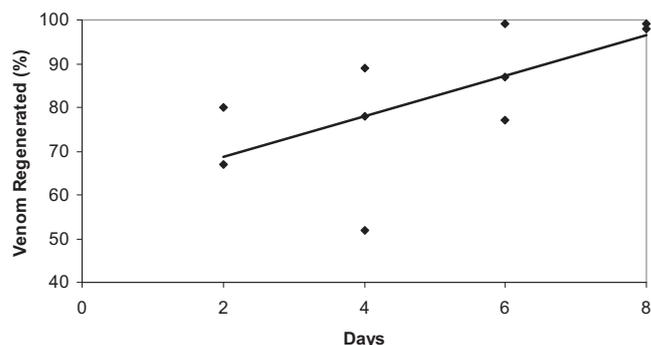

Fig. 2. Percentage of venom volume regenerated by *P. transvaalicus* scorpions over an 8-d period in relation to the initial amount of venom milked ($y = 0.0462x + 0.595$). $N = 2$–3 for each day.

3.3. FPLC analysis

Results of the FPLC are shown in Fig. 4 and summarized in Table 1. Five of 20 fractions collected (Fig. 4) appeared to possess biologically active peptides, as determined previously from *P. transvaalicus* venom (Inceoglu et al., 2003) and identified by MALDI-TOF (see next section). Using FPLC, we tracked these fractions from venoms obtained during different regeneration days, and standardized them based on volume of total venom and scorpion mass (Fig. 5). Fractions 6 (parabutoxins) and 17 (25 kDa peptide group) appeared to be completely regenerated by day 4 (Fig. 5A and E), and fraction 12 (alpha toxin, kurtoxin) by day 6 (Fig. 5C). Fractions 11 (seven identified toxins; Table 1) and 14 (parakinins) possibly required the full 8-d period for regeneration (Fig. 5B and D). Peak regeneration for the 25 kDa protein group (fraction 17) preceded the peaks for fractions 11, 12, and 14, and their maxima in turn coincided with reduced levels of fraction 17.

3.4. MALDI-TOF analysis

The mass spectrum of *P. transvaalicus* venom standard (initial milking) is shown in Fig. 6. The peaks clustered in two main groups separated by an m/z range in which no
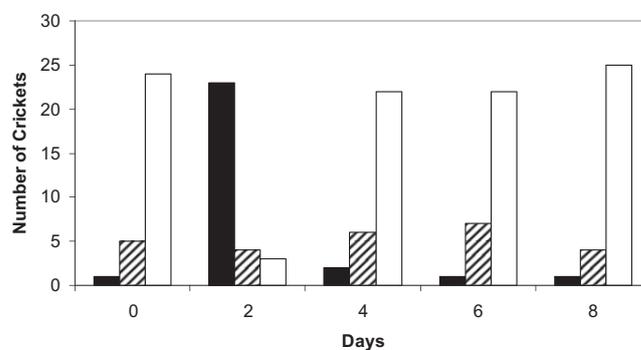

Fig. 1. Mean metabolic rate (μl O_2 g^{-1} h^{-1}) for milked ($\blacksquare$) and unmilked ($\square$) *Parabuthus transvaalicus* scorpions for every 6 h post-milking. $N = 3$ for each group.

Fig. 3. Status of cricket prey after injection of 2 μl of diluted (1:5 insect Ringer's solution) *P. transvaalicus* venom from milkings at day 0 (baseline) and at 2 ($N = 3$ scorpions), 4 ($N = 2$), 6 ($N = 3$), and 8 ($N = 3$) days following initial milking. Prey status: no effect (black bar), immobilized (hatched bar), or paralyzed/killed (white bar) within 24 h ($N = 30$ crickets injected each day). Killing effectiveness returned by day 4 after milking.
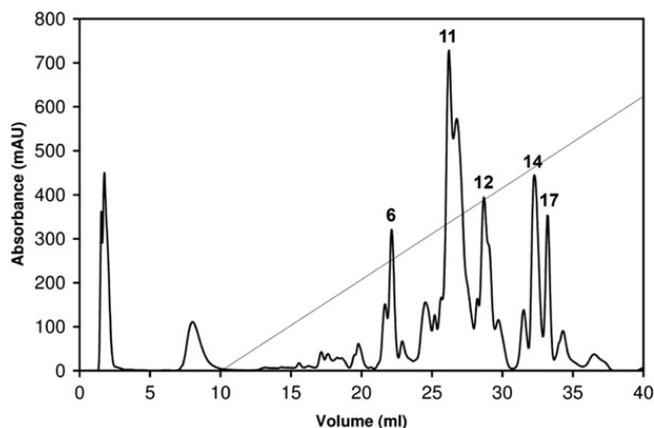
Fig. 4. FPLC profile of *P. transvaalicus* venom standard (initial milking) eluted with continuous gradient of buffer B starting at 10 ml. The biologically active fractions are numbered, and described in Table 1.

peptides occurred. The two main clusters were observed around the 6 kDa and 7 kDa regions, consistent with prior work and suggesting proper taxonomic assignment of our specimens (Dyason et al., 2002).

Fig. 7 shows the mass spectra of the five fractions that were biologically active. Fraction 6 (parabutoxins) showed peaks in the 4 kDa range; fraction 11 (seven identified toxins; Table 1) had two clusters similar to venom standard in the 6 and 7 kDa range; fraction 12 (alpha toxin, kurtoxin) showed peptides only in the 7 kDa range; and fractions 14 (parakinins) and 17 (25 kDa peptide group) had peaks in the smallest (900 Da) and largest (25 kDa) ranges, respectively. These molecular weights are summarized in Table 1, as matched to the FPLC fractions, time to regeneration, and previously-identified toxins.

4. Discussion

Results of this study confirm the relatively high energetic cost of venom replenishment in scorpions. The 21% increase in metabolic rate during an 8-d post-milking period was somewhat less than the 39% increase observed during a 3-d period (Nisani et al., 2007), but was still substantial. Additional metabolic costs may accrue beyond the time of measurement. The findings further suggest that the synthesis of *P. transvaalicus* venom

peptides after initiation of a venom regeneration cycle is asynchronous, as reflected in the different toxins being synthesized at varying rates, and in the apparent fluctuation of energy demands for regenerating the various venom components. Asynchrony in scorpion venom regeneration was previously reported by Pimenta et al. (2003). Some snakes similarly exhibit asynchrony in the regeneration of venom components, whereas others apparently do not (Oron et al., 1978; Paine et al., 1992; Pintor et al., 2010b). Reported differences among taxa could be from dissimilarities in venom composition, venom gland structure and size, and methods used.

The process of venom biosynthesis in *P. transvaalicus* appears to be slower than venom volume regeneration. When volume was approximately 68% replenished at day 2, the newly regenerated venom was not as toxic as the initial venom. However, lethality of the regenerated venom, in terms of killing effectiveness in crickets, appeared to be fully restored by day 4, when volume was 78% replenished (Fig. 2) and protein content was approximately 26% of the original milking (Nisani et al., 2007). Restoration of toxicity in this scorpion appeared to be much more rapid than in the spider *C. salei* (Boeve et al., 1995). When newly regenerated spider venom was compared with older venom, the rate of protein synthesis lagged behind the volume of venom regenerated, as the new venom did not regain its initial toxicity, based on a cricket assay similar to ours, for more than two weeks (Boeve et al., 1995). Toxicity assays involving mammals (e.g., mice) may yield substantially different results from those based on invertebrates (Inceoglu et al., 2003), so the comparison applies only to venom efficacy against invertebrates.

Scorpion toxins have the ability to effectively target sodium, potassium, and chloride channels (Possani et al., 1999; De La Vega and Possani, 2004, 2005; Du Plessis et al., 2008). The different peptides responsible for targeting these different channels are relatively well studied and classified. Inceoglu et al. (2003) developed a chemical profile of the major toxins in *P. transvaalicus*, and documented differences in composition between the prevenom and venom. In the current study, we were able to track the synthesis of major peptides (Table 1) during regeneration of the entire venom gland contents (Fig. 5). Some of the prevenom constituents, especially the parabutoxins (fraction 6), the 25 kDa group (fraction 17), and possibly the

Table 1
Summary of FPLC fractions, MALDI-TOF determination of size class, distribution in whole venom, and approximate time for regeneration following emptying of *Parabuthus transvaalicus* scorpion venom glands.

Fraction	Size class (kDa)	Distribution[a]	Time (days)	Peptides[a]
6	4	Prevenom, venom	4	Parabutoxins[b] (and KTxs[f])
11	6–7	Venom	8+	Birtoxin[c], ikitoxin[c], dortoxin[c], bestoxin[c], altitoxin[c], alpha toxin[d], kurtoxin[d]
12	7	Venom	6	Alpha toxin[d], kurtoxin[d]
14	0.8–0.9	Prevenom, venom	4–8	Parakinins[e]
17	25	Prevenom, venom	4	25 kDa group

[a] Inceoglu et al., 2003; also Chuang et al., 1998 (for kurtoxin), and Universal Protein Resource Knowledgebase (UniProtKB; http://www.uniprot.org/).
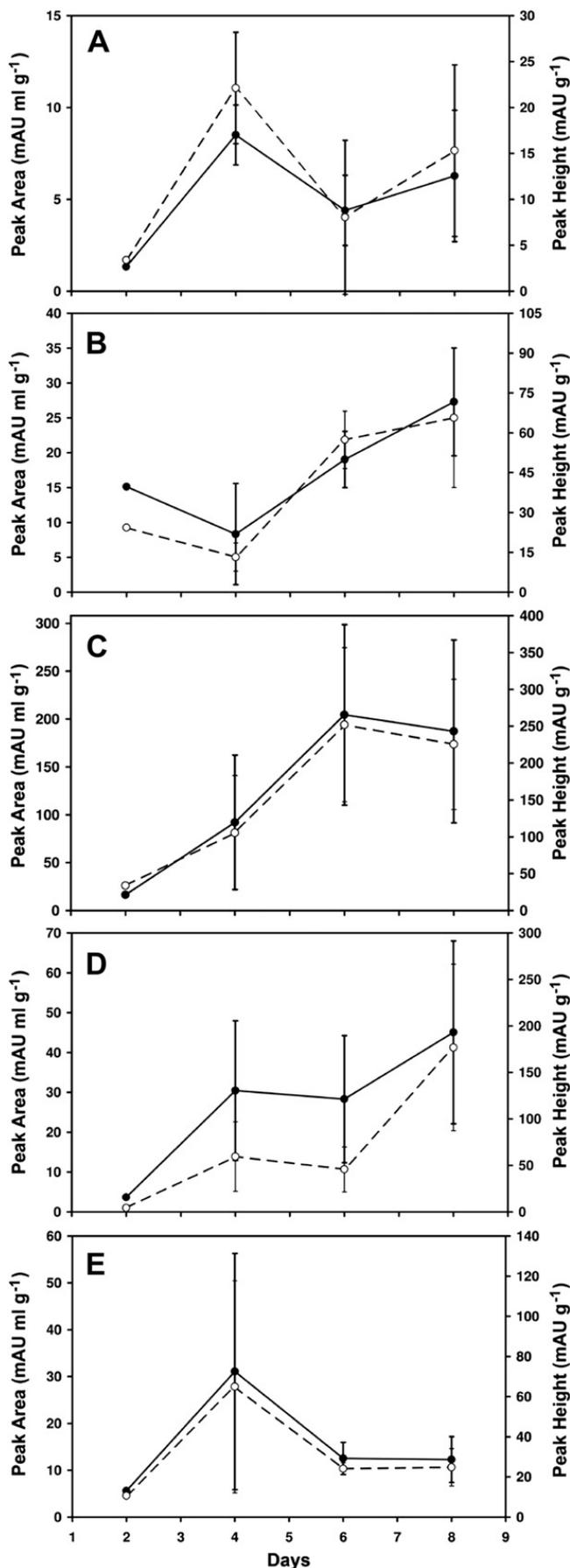[b] Short-chain scorpion toxin superfamily: 37 amino acids, 3 disulfide bridges.
[c] Long-chain scorpion toxin superfamily, beta subfamily: 58 amino acids, 3 disulfide bridges.
[d] Long-chain scorpion toxin superfamily, alpha subfamily: 62–63 amino acids, 4 disulfide bridges.
[e] 8 amino acids (Hammock and Inceoglu, 2007).
[f] K$^+$ channel blockers.

parakinins (fraction 14), appeared to be resynthesized rapidly, whereas other components represented only in venom (the long-chain neurotoxins of fractions 11 and 12) took longer to reappear. The parabutoxins were largely replenished by day 4, by which time toxicity of the venom toward crickets had returned to baseline. Parabutoxins, which belong to the K$^+$ channel blockers (KTxs), are structurally simpler than the long-chain neurotoxins, being shorter in length and having fewer disulfide bridges (Table 1). Accordingly, they are probably synthesized more rapidly. Parabutoxins occur with parakinins and K$^+$ in the prevenom of these scorpions, and prevenom is very effective in paralyzing insect prey and inflicting pain in mammals (Inceoglu et al., 2003). The long-chain neurotoxins render the venom especially potent (in terms of venom volume), and represent at least 20% of the protein content (Inceoglu et al., 2005). Their slower synthesis presumably contributed to the high peak of energetic cost on day 5. The 25 kDa group of proteins, present in both prevenom and venom, appeared to peak on day 4 and waned thereafter, suggesting it includes some precursors of or enzymes involved with the processing of other peptides (e.g., isomerases, proteases).

Our findings contrasted with those of Pimenta et al. (2003), who reported differences in venom composition of the scorpion *T. serrulatus* among days 1 and 8 after milking and 20 days post-feeding. Their results (Fig. 1 of their paper) suggest that the smaller (m/z = 2643, 2725, 2748, 4507) peptides among the major peptide peaks took longer to regenerate than the larger (m/z = 6883, 6990) peptides. This seems counter-intuitive, but the contrast could have resulted from differences in milking procedures. We were very deliberate in obtaining multiple venom expulsions to ensure that we emptied the venom gland as much as possible. Pimenta et al. (2003) may have failed to fully empty the venom gland, and therefore much of the protein-rich venom with presumably larger proteins remained in the gland, resulting in venom profiles showing depletion and regeneration largely of the presumably smaller prevenom peptides.

Our conclusions are limited by the small sample sizes of our study, as we were unable to acquire additional specimens. Some analyses were based on large effect sizes, which lend credence to our conclusions, whereas others were not amenable to statistical analysis. Some of the differences, particularly in the venom regeneration profiles, could reflect individual variation. Among scorpions, individual variation in venom composition certainly exists (Kalapothakis and Chávez-Olórtegui, 1997; Pimenta et al., 2003; Ozkan and Ciftci, 2010), including those of genus *Parabuthus* (Debont et al., 1998; Dyason et al., 2002). Thus, we encourage further study to confirm and extend our

Fig. 5. Standardized peak heights (dashed line) and areas (solid line) of *P. transvaalicus* venom profile on FPLC during an 8-d period of venom regeneration following milking. (A) Fraction 6 (parabutoxins); (B) Fraction 11 (birtoxin, ikitoxin, dortoxin, bestoxin, altitoxin, alpha toxin, kurtoxin); (C) Fraction 12 (alpha toxin, kurtoxin); (D) Fraction 14 (parakinins); (E) Fraction 17 (25 kDa group). $N = 2$ (at 2 and 8 d, but pooled for day 2 due to low volume) and $N = 3$ (at 4 and 6 d) for each mean ± 1 SD.
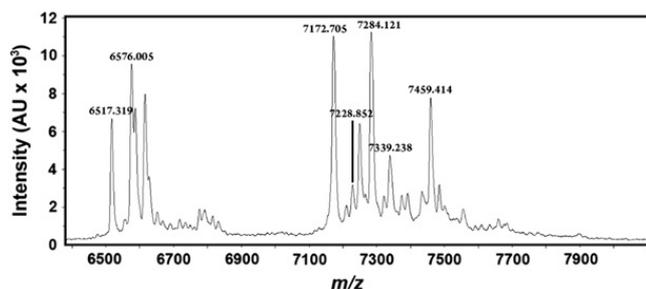
Fig. 6. MALDI-TOF mass spectrum of *P. transvaalicus* venom standard (initial milking).
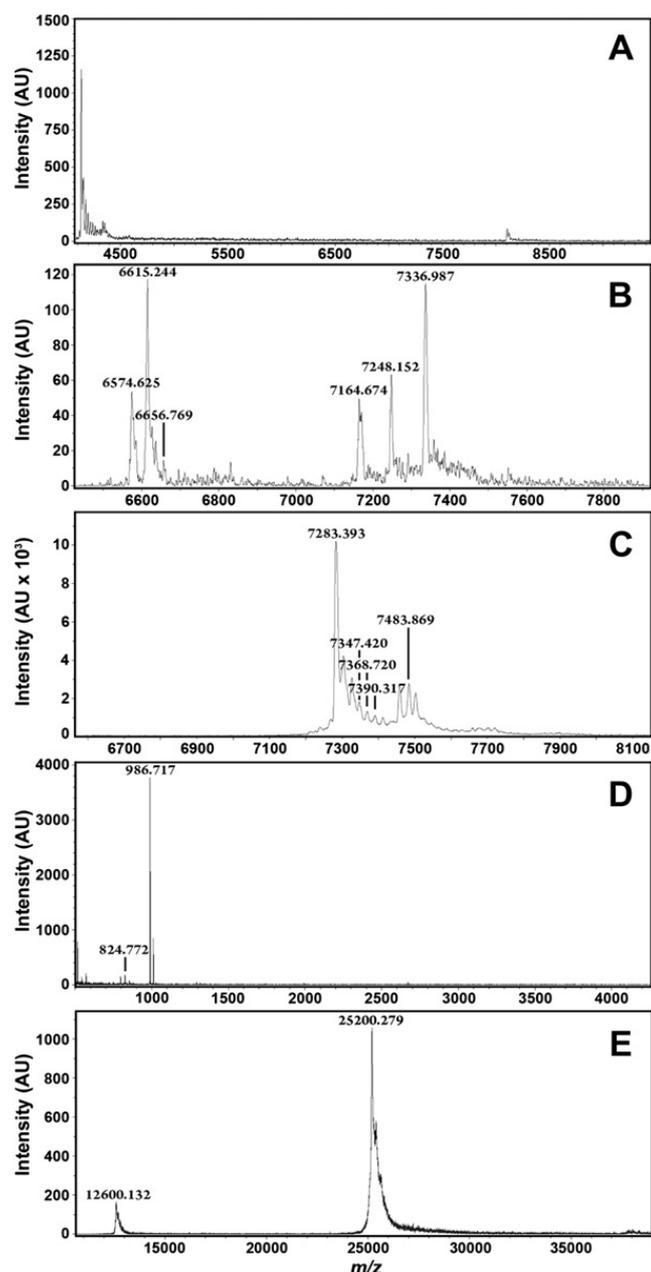

Fig. 7. MALDI-TOF mass spectra of selected *P. transvaalicus* venom fractions isolated using FPLC. (A–E) Fractions correspond to those of Fig. 5: (A) Fraction 6 (parabutoxins); (B) Fraction 11 (birtoxin, ikitoxin, dortoxin, bestoxin, altitoxin, alpha toxin, kurtoxin); (C) Fraction 12 (alpha toxin, kurtoxin); (D) Fraction 14 (parakinins); (E) Fraction 17 (25 kDa group).

findings on how venom complexity relates to the rate and energetics of venom replenishment.

The ecological and physiological implications of asynchronous regeneration of venom components are several-fold. One benefit of having prevenom is that it allows these scorpions to conserve metabolically expensive venom that is high in protein, using it only as higher levels of stimulation require (Nisani and Hayes, 2011). Venom regeneration can be a metabolically expensive process (McCue, 2006; Nisani et al., 2007; but see Pintor et al., 2010a); thus, it is reasonable to hypothesize that these scorpions possess adaptive means for judicious venom deployment, i.e., venom metering (Hayes, 2008) or venom optimization (Wigger et al., 2002). The capacity to rapidly regenerate the relatively simple parabutoxins provides these scorpions with efficacious prevenom to capture prey and deter mammalian predators (Inceoglu et al., 2003), thereby avoiding the ecological costs associated with depletion of larger quantities of venom (Malli, 1999; Hayes et al., 2002; Hayes, 2008). Indeed, *P. transvaalicus* can deliver variable quantities of venom in a single sting depending on level of threat (Nisani and Hayes, 2011), and appears to squirt defensively a larger volume of venom than is often expended upon stinging (Nisani, 2008). Some prey may even be procured without the need for venom. Large scorpions, such as *P. transvaalicus*, generally depend on their pinchers in capturing and subduing their prey, but only use the stinger if the prey is difficult to handle (Cushing and Matherne, 1980; Casper, 1985; Rein, 1993; Edmunds and Sibly, 2010). Unfortunately, we do not know well enough the functional roles of other proteins that require lengthier periods of time to regenerate. However, their slower replenishment might be expected to influence the behavior of scorpions for a period of time after an episode of significant venom expenditure, as observed in predator decisions by spiders (Wullschleger and Nentwig, 2002; Wigger et al., 2002).

Not all of the metabolic costs detected in this study can be attributed to protein synthesis. Studies of venomous snakes (McCue, 2006; Pintor et al., 2010a,b) suggest that the metabolic costs of venom regeneration may include both the indirect costs of catabolizing and mobilizing endogenous materials, and the direct costs of secretion up-regulation (Pintor et al., 2010a), synthesis of complex components (Bdolah, 1979), and secretion of toxic components into extracellular compartments (Mackessy, 1991). Furthermore, the granular material within the epithelial cells of scorpion venom glands disappears after venom ejection, and can take some time to appear normal again (Kovoor, 1973; Farley, 1999). Thus, there are multiple costs that could potentially contribute to the fluctuations in metabolic rate that we have documented for scorpions regenerating their venom.

By examining a scorpion that produces a toxic secretion of varying composition (i.e., prevenom and venom; Inceoglu et al., 2003), and which uses different venom components depending on behavioral context (e.g., varying levels of threat and stinging versus squirting; Nisani, 2008; Nisani and Hayes, 2011), this study has demonstrated not only the high metabolic cost of venom production, but also the correspondence between venom complexity and

energy demand. Clearly, this scorpion species can feed and defend itself with the relatively simple prevenom, which apparently can be regenerated quickly and with relatively low metabolic cost. However, the more complex and potent portions of the venom of this species require additional time to regenerate, and demand greater energetic costs than for prevenom alone. Thus, our results support the view that greater venom complexity, at least in terms of venom versus prevenom, corresponds to higher energetic costs of replacement.

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Ethical statement

This paper represents a series of experiments carried out under the standard procedures of scientific ethics, including the care of experimental animals. All authors have read the manuscript and agree to its publication in Toxicon and agree that it has followed the rules of ethics presented in the Elsevier's Ethical Guidelines for Journal Publication.

Conflicts of interest statement

The authors declare that there is no conflict of interest.

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