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Zia Nisani, Stephen G. Dunbar, William K. Hayes

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Cost of Venom Regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae)

Zia Nisani*, Stephen G. Dunbar, and William K. Hayes

Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, CA 92350, USA

*Corresponding Author
znisani07g@llu.edu
Phone: (661) 703-1609
Fax: (909) 558-0259

Abstract

Scorpion venom has many components, but is mainly made up of water, salts, small molecules, peptides, and proteins. One can reasonably assume that the production and storage of this complex secretion is an expensive metabolic investment. However, to date, no study has addressed the costs associated with the regeneration of venom by scorpions. Using a closed-system respirometer, we examined the difference in oxygen consumption between milked and unmilked scorpions to determine the metabolic costs associated with the first 72 h of subsequent venom synthesis. During this time period, milked scorpions had a significantly higher (39%) metabolic rate than unmilked scorpions. The regenerated venom from a second milking had significantly lower (74%) protein concentration, suggesting that venom regeneration was incomplete after 72 h. The protein content in the regenerated venom was not correlated with oxygen consumption. The significant increase in oxygen consumption after milking supports existing hypotheses about the metabolic cost associated with venom regeneration and provides further insight on why scorpions appear to be judicious in their stinger use.

Key words: Metabolic Rate, Oxygen Consumption, Scorpion, Toxins, Secretion, Venom optimization.

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1. Introduction

The toxic properties of scorpion venom have attracted researchers from the clinico-pathological and chemico-pharmacological perspectives. Numerous studies have shown that scorpion venom is a mixture of water, salts, small molecules, peptides and proteins (Zlotkin et al., 1978; Yahel-Niv & Zlotkin, 1979; Simard & Watt, 1990). The venom composition of many scorpion species has been characterized, with peptides having the greatest biological effects on target organisms. Scorpion venom toxicity has been shown to be specific for invertebrates, vertebrates, or both (Possani et al., 1999; Inceoglu et al., 2001).

Production and storage of protein-rich venom is an expensive metabolic investment, especially for organisms that live in extreme environments (Inceoglu et al., 2003; McCue, 2006). Variation in sting use suggests that scorpions regulate venom expenditure (Bub & Bowerman, 1979; Casper, 1985; Rein, 1993). Rein (1993), for example, demonstrated that *Parabuthus*

liosoma and *P. pallidus* used their stinger only if the prey item was difficult to handle. Large larvae of the Yellow Mealworm Beetle, *Tenebrio molitor*, were stung more often than smaller larvae (which were often not stung), presumably because the larger larvae struggled more intensely. Similar patterns of stinger use have been described in other scorpions such as *Hadrurus arizonensis* (Bub & Bowerman, 1979), *Paruroctonus boreus* (Cushing & Matherne, 1980), and *Pandinus imperator* (Casper, 1985).

Although previous investigations with scorpions did not measure venom expenditure, other studies have done so with spiders and snakes (Malli et al., 1999; Hayes et al., 2002; Wigger et al., 2002). For example, Malli et al. (1999) by artificially controlling the struggle intensity of crickets (as prey) and using enzyme-linked immunosorbent assay (ELISA) were able to show that the Wandering Spider, *Cupiennius salei*, delivered more venom into prey items that struggled more intensely. Since *C. salei* controls the amount of venom that it injects, this suggests that the spider regulates the amount of venom expended during predatory bites (Boeve et al., 1995; Malli et al., 1999). These studies support the venom optimization hypothesis, which infers that spiders use their venom as economically as possible (Wigger et al., 2002). Thus, despite our lack of knowledge about how much it costs to make and store venom, evidence from previous studies suggests that venom is an expensive commodity.

To date, only one study has quantified the metabolic expenditure associated with the process of venom regeneration. McCue (2006) showed that North American pitviper snakes completely milked of their venom had a 10% increase in their resting metabolic rate during the first 72 h of venom regeneration. This metabolic increase was an order of magnitude greater than metabolic costs associated with producing an identical mass of body tissue.

The aim of this study was to examine the metabolic cost associated with venom regeneration by measuring the oxygen consumption of *P. transvaalicus* in a closed-system respirometer. We also examined whether the protein content of initially milked venom differed significantly from the venom regenerated after 72 h. Finally, we considered whether there was any correlation between the amount of protein in the regenerated venom and the scorpion's metabolic rate.

2. Materials & Methods

2.1 Animals

Adult *Parabuthus transvaalicus* scorpions (1 male and 10 female) were purchased from Glades Herp, Inc. (Bushnell, Florida, USA) and Hatari Invertebrates (Portal, Arizona, USA). The scorpions were housed in clear plastic containers measuring 35 × 16 × 11 cm (L × W × H) with sand substrate. They were kept at 25 ± 1ºC in a 12:12 light-dark cycle and fed one cricket per week. Prior to testing, scorpions were fasted for 7 days. None of the female scorpions were gravid, and all the specimens used were from 5.10 to 8.75 g. Preliminary analyses demonstrated no difference in oxygen consumption between male and females used in this study.

2.2 Metabolic Chamber and Oxygen Consumption

The experimental chamber was a 5 × 42 cm (D × L) transparent PVC pipe (US plastic), with both ends sealed with rubber stoppers. One rubber stopper was drilled to insert a 1.8 cm (D) oxygen probe through it. A small glass vial (2.2 × 6 cm) with two holes (5 mm) drilled into the top was placed inside the tube opposite the probe. The vial contained Ascarite and Drierite to remove CO_2 and water vapor, respectively. The entire chamber was submerged in a 30 L water bath. Two, 2.7 kg bricks kept the chamber underwater, and a heated immersion circulator (VWR, #1112A, Westchester, PA, USA) controlled the temperature. The chamber was monitored for air leaks and was found to be completely sealed.

Oxygen consumption was measured under a 12:12 light-dark cycle at 25 ± 0.5ºC with a TPS 90D dissolved oxygen meter (TPS, Queensland, Australia) in a closed-system respirometer. Prior to testing, the scorpion was placed in a cylindrical plastic chamber measuring 5 × 8.5 cm (D × L) with multiple holes (3 mm) in both ends. The chamber minimized the animal's movement. Each scorpion was tested once under each of two different treatments: milked and unmilked. The treatment order was random for each scorpion with 21 days separating the two trials.

For the unmilked treatment, the scorpion was weighed and placed in the plastic chamber, which in turn was inserted in the experimental chamber at a distance of 8 cm from the oxygen probe. Oxygen consumption was measured for 72 h with readings logged every 30 min. The unmilked scorpions were allowed to acclimate for 30 min before starting the readings. To

minimize possible circadian rhythm effects, all trials were initiated between 0800 and 1100 h, during the light period.

For the milked treatment, each scorpion was first weighed and then re-weighed after the initial milking. The scorpion was milked by having it sting a parafilm-covered microcentrifuge tube (1 mL). This was done by securing the telson with forceps and repeatedly pushing the vesicle against the parafilm without removing the aculeus (stinger). We refrained from using electrostimulation since this method may unduly stress the scorpions, so much so that it may cause premature death to the animal (Berea, per. comm.; Z Nisani, unpublished data). Venom released with this technique is likely to represent defensive venom expenditure, more than predatory stinging. The venom was collected using a sterile microcapillary pipette and transferred into a separate microcentrifuge tube containing 0.5 mL distilled water. The sample was frozen at -10ºC and stored until the analysis could be done. Milked scorpions were treated the same way as unmilked ones, except milked animals were allowed to acclimate for 2 h instead of 30 minutes. This was done to ensure that the scorpion was well rested from the effects of the milking process. Preliminary analysis of two unmilked scorpions agitated by shaking in a small beaker for 30 min showed that oxygen consumption returned to baseline values within 2 h (mean = 35.60 ± 3.11 μL $O_2 \cdot g^{-1} \cdot h^{-1}$).

After 72 h in the metabolic chamber, each scorpion was removed and reweighed. The milked scorpions were milked once again to determine how much venom was regenerated and weighed again after the milking. The venom collected was treated the same way as previously described.

Metabolic rates were calculated after 72 h from oxygen consumption using the following equation from Vleck (1987), with modifications to adjust for the mass of each scorpion and differences in apparatus:

$$MR = V_{O_2} \cdot g^{-1} \cdot t^{-1} \quad (1)$$

where MR is the mass-specific metabolic rate, V_{O_2} is the volume of oxygen consumed, g is the scorpion mass, and t is the time in hours. We also calculated metabolic rates in six, 12 h periods from the 72 h data.

2.3 Venom Measurements

We obtained two measures of venom: wet mass and protein mass. Wet mass (nearest 0.01 g) was determined by weighing the scorpion on an analytical balance before and after venom milking. Protein mass was determined by Coomassie Protein Assay (Pierce Chemical Co., Rockford, Illinois). The venom standards (0, 5, 10, 15, 20, and 25 $\mu g \cdot mL^{-1}$) were prepared from the lyophilized venom of the Western Diamondback Rattlesnake, *Crotalus atrox* (protein = 90% dry mass; Tu, 1982). Venom standards and scorpion venom samples were assayed in triplicate on a 96-well flat-bottom microplate (Costar® 3595, Coring Inc., New York). Samples were analyzed using the protocol provided by Pierce using a μQuant microplate reader (Bio-Tek Instruments, Inc.) at 570 nm absorbance. The amount of protein was calculated using the following regression equation:

$$P_V = m \cdot A_{570nm} + b \quad (2)$$

where P_V is the mass (μg) of protein in venom, m is the slope of the line, A_{570nm} is the absorbance at 570 nm, and b is the Y-intercept. Protein concentration was measured as $\mu g \cdot mL^{-1}$ (assuming specific gravity = 1.0, such that 1 mg wet mass = 1 μl volume). Venom measurements were obtained twice from each animal, including the initial venom extraction and the subsequent milking 72 h later.

2.4 Data Analysis

Because the data met parametric assumptions, a paired t-test was used to compare the metabolic rate of milked and unmilked scorpions after 72 h (Zar, 1999). The same analysis was utilized to test for differences in scorpion mass for each treatment group and to compare protein concentration in initially milked venom and the subsequent venom sample collected after 72 h. A Pearson correlation was employed to investigate the relationship between metabolic rate and the amount of protein in the regenerated venom (Zar, 1999).

We used a 2 × 6 repeated-measures ANOVA to investigate the effects of treatment (milked vs. unmilked) and time (the six successive, 12 h periods) on metabolic rate (Zar, 1999). For this analysis, we used rank-transformed data to meet parametric assumptions, with treatment being a between-subjects factor and time being a within-subjects factor. Effect sizes were

obtained as partial η^2 values, indicating the approximate proportion of variance in the dependent variable explained by an independent variable or interaction (Cohen, 1988). Because the partial η^2 values provided by Statistical Package for the Social Sciences (SPSS) summed to >1, we adjusted these values by dividing each by the sum of all partial η^2 values for the effects tested.

All analyses were conducted using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA), with alpha set at 0.05.

3. Results

3.1 Metabolic Rate of Unmilked and Milked Scorpions

In Table 1, we show that milked scorpions had a significantly (39%) higher mean metabolic rate than unmilked scorpions (mean = 50.29 and 36.12 μL $O_2 \cdot g^{-1} \cdot h^{-1}$, respectively; t_{10} = 7.0, $p < 0.0001$). In spite of milking, no significant difference was observed in the mass of milked and unmilked scorpions (mean = 6.25 and 6.63 g, respectively; $t_{10} = 1.48$, $p = 0.170$; Table 1). The ANOVA revealed that the milked scorpions had higher metabolic rates throughout the 72 h time period (Fig. 1), with the main effect of treatment being highly significant ($F_{1,10}$ = 38.569, $p < 0.001$, partial $\eta^2 = 0.77$). However, the main effect of time ($F_{5,50} = 1.857$, $p = 0.119$, partial $\eta^2 = 0.16$) and lack of an interaction between time and treatment ($F_{5,50} = 0.789$ $p = 0.562$, partial $\eta^2 = 0.07$) indicated that metabolic rates were consistent during the 72 h period.

3.2 Venom Measurements and Metabolic Rate

An equal volume of venom was obtained from the initial milking when compared with the milking after 72 h (mean = 39.69 and 37.23 μL, respectively; $t_{10} = 0.24$, $p = 0.815$). However, the venom from the initial milking had approximately four-fold higher protein content than the venom regenerated after 72 h (mean = 2.30 and 0.60 $\mu g \cdot \mu L^{-1}$, respectively; $t_{10} = 3.88$, $p = 0.003$) (Table 2). No correlation was detected between the amount of protein in the regenerated venom and the metabolic rate measured over the 72 h time period ($r_{11} = 0.133$, $p = 0.696$).

4. Discussion

We found that *Parabuthus transvaalicus* incurred considerable metabolic cost when replenishing its venom. Scorpion venom is a complex mixture containing mucus, inorganic salts, low-molecular weight organic molecules, and many different small proteins, with the latter being neurotoxins (Muller, 1993; Debont et al., 1998). Studies of other venomous animals, such as snakes, suggest that the relatively high metabolic cost may reflect both the indirect costs of catabolizing and mobilizing endogenous materials and the direct costs of secretion up-regulation (c.f., Secor et al., 1994), synthesis of complex components (Bdolah, 1979), and secretion of the toxic components into extracellular compartments (Mackessy, 1991).

Although venom regeneration required a 39% increase in metabolic rate compared to the unmilked condition, our measurements likely underestimated the actual cost of venom synthesis by scorpions. The protein concentration of venom was not fully restored 72 h after milking, the metabolic rate did not return to baseline within 72 h, and no correlation was detected between metabolic cost and protein content of the regenerated venom. However, we concede that the cost for venom regeneration might be less than what we measured for scorpions that deploy much smaller quantities of venom. Still, we recognize that venom regeneration is a process that possibly includes the production of indole compounds, neutral and acidic mucosubstances, and that the synthesis and movement of these molecules is likely to have associated metabolic costs beyond protein production (Tu, 1977; Halse et al., 1980; Farley, 1999). At present, we do not know how much of the total venom available is expended during typical predatory or defensive encounters. The quantity of venom we extracted (mean = 40 μL) was higher than values obtained in other studies (Inceoglu et al., 2003; mean = 22 μL; scorpion size not indicated). Although we assume our milking procedure fully depleted the venom reserve, we may not have done so for several or all scorpions. In the only other study to address the cost of venom synthesis, McCue (2006) similarly measured the metabolic rates of North American pitvipers during the first 72 h of venom regeneration. He likewise concluded that the 10% increase was an underestimate of the actual cost.

While we acknowledge that both milking and pre-chamber handling of scorpions is stressful, our data suggest that metabolic rates of both milked and unmilked scorpions returns to steady state with 24 h and despite this, milked scorpions continued to have a higher metabolic rate than unmilked scorpions. Oxygen consumption rates measured for the unmilked *Parabuthus transvaalicus* scorpions in our study corresponded to reported values in the literature for other

Parabuthus species. Robertson et al. (1982) and Bridges et al. (1997) measured oxygen consumption rates of *P. villosus* at several temperatures. From their results, we extrapolated that at 25º C, mean oxygen consumption was approximately 30 µL $O_2 \cdot g^{-1} \cdot h^{-1}$ and 50 µl $O_2 \cdot g^{-1} \cdot h^{-1}$, respectively, for the two studies. These values are consistent with what we obtained from our unmilked scorpions (36 µL $O_2 \cdot g^{-1} \cdot h^{-1}$; Table 1). The agreement of these values increases our confidence in the oxygen consumption measurements obtained in the current study.

Understanding the metabolic expense associated with venom regeneration is important in understanding why scorpions judicially use their stingers (Rein, 1993). Although venom optimization has not been directly measured in scorpions as it has in spiders (Malli et al., 1999; Wigger et al. 2002), restrictive stinger use in scorpions suggests that scorpions optimize venom expenditure. The restrictive sting use in scorpions is likely advantageous from an energetic point of view (Rein, 1993), as discussed above, but may also be advantageous from an ecological perspective. Scorpions that expend excessive venom, for example, may be left with insufficient reserves to secure additional food or to adequately defend themselves (c.f., Hayes et al., 2002). Moreoever, scorpions having less-toxic, protein-depleted venom might be less efficient in venom use.

Boeve et al. (1995) demonstrated that the newly-regenerated venom of the spider, *Cupiennius salei*, not only had lower protein concentrations compared to older venom (initial milking), but also showed less acute symptoms when injected into crickets. The need for biochemically efficient venom could explain the lack of surface activity reported in post-ingestive scorpions. In field enclosures, desert grassland scorpions, *Paruroctonus utahensis*, returned to the surface an average of 20.3 days following meal consumption, a period of time far exceeding that required to digest their meals (Bradley, 1982). Since the digestive pause was not shown to be a possible explanation for this long, post-feeding interruption of surface activity, it may be reasonable to suggest that this surface time minimization might be a response to predation risk (Bradley, 1982). The danger of cannibalism, along with predation, plays an important role in controlling scorpion activity patterns (Polis, 1980). The biosynthesis of protein in venom seems to be slower than regeneration of total venom volume (Boeve et al., 1995). Therefore, the apparent time minimization could be due to the time required to produce venom lethal enough to protect the scorpion from predators.

In summary, the high metabolic cost associated with venom regeneration could explain, at least partially, why scorpions seem to use their stinger only when prey items are difficult to handle. The increased cost associated with venom production is central to the venom optimization hypothesis. Moreover, the lack of biochemically efficient venom could explain why, after feeding, scorpions will seek shelter to minimize contact with predators or conspecifics that could result in cannibalism. Future studies looking at long-term venom regeneration, along with the chemical profile of regenerated venom, will further elucidate the costs associated with venom production and use by these scorpions.

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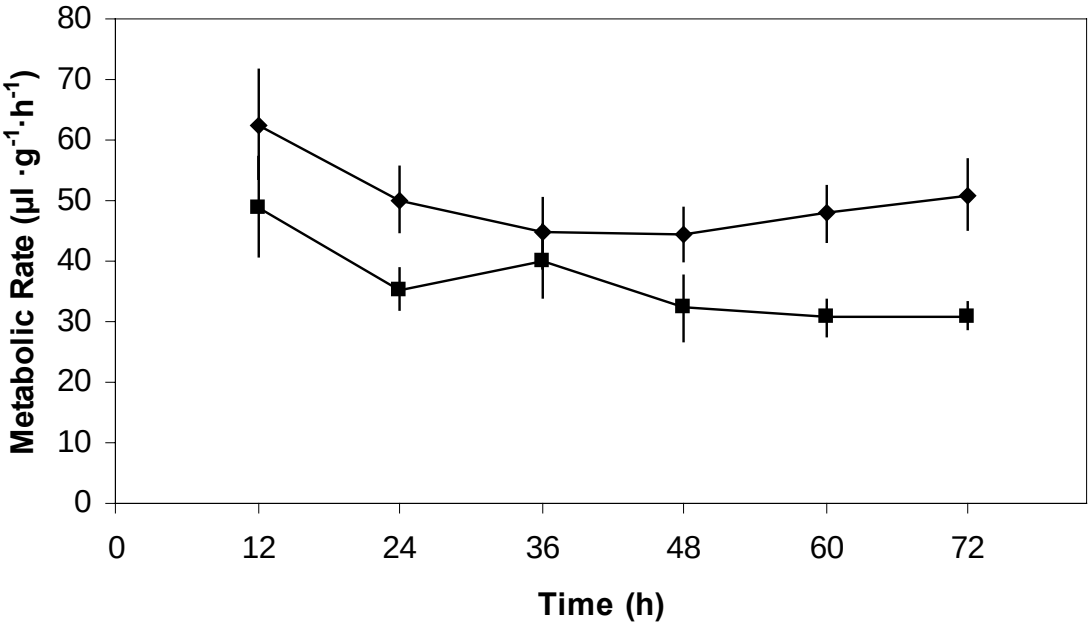
Captions to Figure

Fig. 1: The mean (± 1 S.E.) metabolic rate (μL $O_2 \cdot g^{-1} \cdot h^{-1}$) for milked (♦) and unmilked (■) scorpions for every 12 h post-milking. N = 11 for each treatment.



358

Table 1: Comparison of mean (± 1 S.E.) scorpion mass and metabolic rate (MR) for milked versus unmilked *Parabuthus transvaalicus*.

Group	N	Mass (g)	MR ($\mu L\ O_2 \cdot g^{-1} \cdot h^{-1}$)
Unmilked	11	6.63 ± 0.32	36.12 ± 2.88
Milked	11	6.25 ± 0.21	50.29 ± 3.30*

*$p < 0.0001$

Table 2: Comparison of mean (± 1 S.E.) volume of venom and protein concentration in initially milked venom and venom regenerated after 72 h.

Sample	Volume of venom (µL)	Protein in venom (µg)	Protein Concentration ($\mu g \cdot \mu L^{-1}$)
Initial Milking	39.69 ± 9.23	69.87 ± 8.84	2.30 ± 0.32
Second Milking	37.23 ± 11.62	18.49 ± 7.65	0.60 ± 0.21