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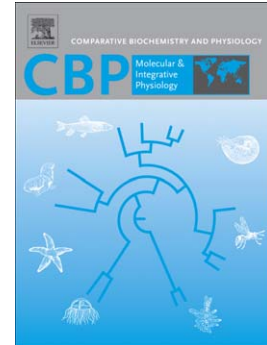
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Cost of Venom Regeneration in *Parabuthus transvaalicus* (Arachnida:

4 **Buthidae)**

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16

18 Abstract

20 Scorpion venom has many components, but is mainly made up of water, salts, small
22 molecules, peptides, and proteins. One can reasonably assume that the production and storage of
24 this complex secretion is an expensive metabolic investment. However, to date, no study has
26 addressed the costs associated with the regeneration of venom by scorpions. Using a closed-
28 system respirometer, we examined the difference in oxygen consumption between milked and
30 unmilked scorpions to determine the metabolic costs associated with the first 72 h of subsequent
32 venom synthesis. During this time period, milked scorpions had a significantly higher (39%)
34 metabolic rate than unmilked scorpions. The regenerated venom from a second milking had
36 significantly lower (74%) protein concentration, suggesting that venom regeneration was
38 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.

36 1. Introduction

38 The toxic properties of scorpion venom have attracted researchers from the clinico-
40 pathological and chemico-pharmacological perspectives. Numerous studies have shown that
42 scorpion venom is a mixture of water, salts, small molecules, peptides and proteins (Zlotkin et
44 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).

46 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).
48 Variation in sting use suggests that scorpions regulate venom expenditure (Bub & Bowerman,
1979; Casper, 1985; Rein, 1993). Rein (1993), for example, demonstrated that *Parabuthus*

50 *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
52 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
54 *Hadrurus arizonensis* (Bub & Bowerman, 1979), *Paruroctonus boreus* (Cushing & Matherne,
1980), and *Pandinus imperator* (Casper, 1985).

56 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
58 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
60 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
62 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
64 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
66 previous studies suggests that venom is an expensive commodity.

To date, only one study has quantified the metabolic expenditure associated with the
68 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
70 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.

72 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
74 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
76 any correlation between the amount of protein in the regenerated venom and the scorpion's
metabolic rate.

78

2. Materials & Methods

80

2.1 Animals

82 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
84 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 $25 \pm 1^\circ\text{C}$ in a 12:12 light-dark cycle and fed one cricket per
86 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
88 no difference in oxygen consumption between male and females used in this study.

90 2.2 Metabolic Chamber and Oxygen Consumption

The experimental chamber was a 5×42 cm (D \times L) transparent PVC pipe (US plastic),
92 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
94 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
96 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
98 monitored for air leaks and was found to be completely sealed.

Oxygen consumption was measured under a 12:12 light-dark cycle at $25 \pm 0.5^\circ\text{C}$ with a
100 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
102 (D \times 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
104 unmilked. The treatment order was random for each scorpion with 21 days separating the two
trials.

106 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
108 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

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

112 For the milked treatment, each scorpion was first weighed and then re-weighed after the
114 initial milking. The scorpion was milked by having it sting a parafilm-covered microcentrifuge
116 tube (1 mL). This was done by securing the telson with forceps and repeatedly pushing the
118 vesicle against the parafilm without removing the aculeus (stinger). We refrained from using
120 electrostimulation since this method may unduly stress the scorpions, so much so that it may
122 cause premature death to the animal (Berea, per. comm.; Z Nisani, unpublished data). Venom
124 released with this technique is likely to represent defensive venom expenditure, more than
126 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 -10°C 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
 $= 35.60 \pm 3.11 \mu\text{L O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$).

128 After 72 h in the metabolic chamber, each scorpion was removed and reweighed. The
130 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.

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

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

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

140

2.3 Venom Measurements

142 We obtained two measures of venom: wet mass and protein mass. Wet mass (nearest
 144 0.01 g) was determined by weighing the scorpion on an analytical balance before and after
 146 venom milking. Protein mass was determined by Coomassie Protein Assay (Pierce Chemical
 148 Co., Rockford, Illinois). The venom standards (0, 5, 10, 15, 20, and 25 $\mu\text{g}\cdot\text{mL}^{-1}$) were prepared
 150 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, Corning 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:

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

154 where P_V is the mass (μg) of protein in venom, m is the slope of the line, A_{570nm} is the absorbance
 156 at 570 nm, and b is the Y-intercept. Protein concentration was measured as $\mu\text{g}\cdot\text{mL}^{-1}$ (assuming
 158 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

162 Because the data met parametric assumptions, a paired t-test was used to compare the
 164 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.
 166 A Pearson correlation was employed to investigate the relationship between metabolic rate and
 the amount of protein in the regenerated venom (Zar, 1999).

168 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).
 170 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

172 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
174 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.

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

178

3. Results

180

3.1 Metabolic Rate of Unmilked and Milked Scorpions

182 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 $\mu\text{L O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, respectively; $t_{10} =$
184 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; $t_{10} = 1.48$, $p = 0.170$;
186 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 ($F_{1,10} =$
188 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 ($F_{5,50} = 0.789$, $p = 0.562$,
190 partial $\eta^2 = 0.07$) indicated that metabolic rates were consistent during the 72 h period.

3.2 Venom Measurements and Metabolic Rate

192 An equal volume of venom was obtained from the initial milking when compared with
194 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
196 than the venom regenerated after 72 h (mean = 2.30 and 0.60 $\mu\text{g} \cdot \mu\text{L}^{-1}$, respectively; $t_{10} = 3.88$, p
= 0.003) (Table 2). No correlation was detected between the amount of protein in the
198 regenerated venom and the metabolic rate measured over the 72 h time period ($r_{11} = 0.133$, $p =$
0.696).

200

4. Discussion

202

We found that *Parabuthus transvaalicus* incurred considerable metabolic cost when
204 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
206 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
208 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
210 toxic components into extracellular compartments (Mackessy, 1991).

Although venom regeneration required a 39% increase in metabolic rate compared to the
212 unmilking 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
214 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
216 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
218 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
220 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
222 encounters. The quantity of venom we extracted (mean = 40 μ L) was higher than values
obtained in other studies (Inceoglu et al., 2003; mean = 22 μ L; scorpion size not indicated).
224 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
226 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
228 underestimate of the actual cost.

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

234 *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
236 at 25° C, mean oxygen consumption was approximately 30 $\mu\text{L O}_2\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ and 50 $\mu\text{l O}_2\cdot\text{g}^{-1}\cdot\text{h}^{-1}$,
respectively, for the two studies. These values are consistent with what we obtained from our
238 unmilked scorpions (36 $\mu\text{L O}_2\cdot\text{g}^{-1}\cdot\text{h}^{-1}$; Table 1). The agreement of these values increases our
confidence in the oxygen consumption measurements obtained in the current study.

240 Understanding the metabolic expense associated with venom regeneration is important in
understanding why scorpions judiciously use their stingers (Rein, 1993). Although venom
242 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
244 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
246 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).
248 Moreover, scorpions having less-toxic, protein-depleted venom might be less efficient in venom
use.

250 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
252 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-
254 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
256 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
258 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
260 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).
262 Therefore, the apparent time minimization could be due to the time required to produce venom
lethal enough to protect the scorpion from predators.

264 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
266 handle. The increased cost associated with venom production is central to the venom
optimization hypothesis. Moreover, the lack of biochemically efficient venom could explain
268 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
270 with the chemical profile of regenerated venom, will further elucidate the costs associated with
venom production and use by these scorpions.

272

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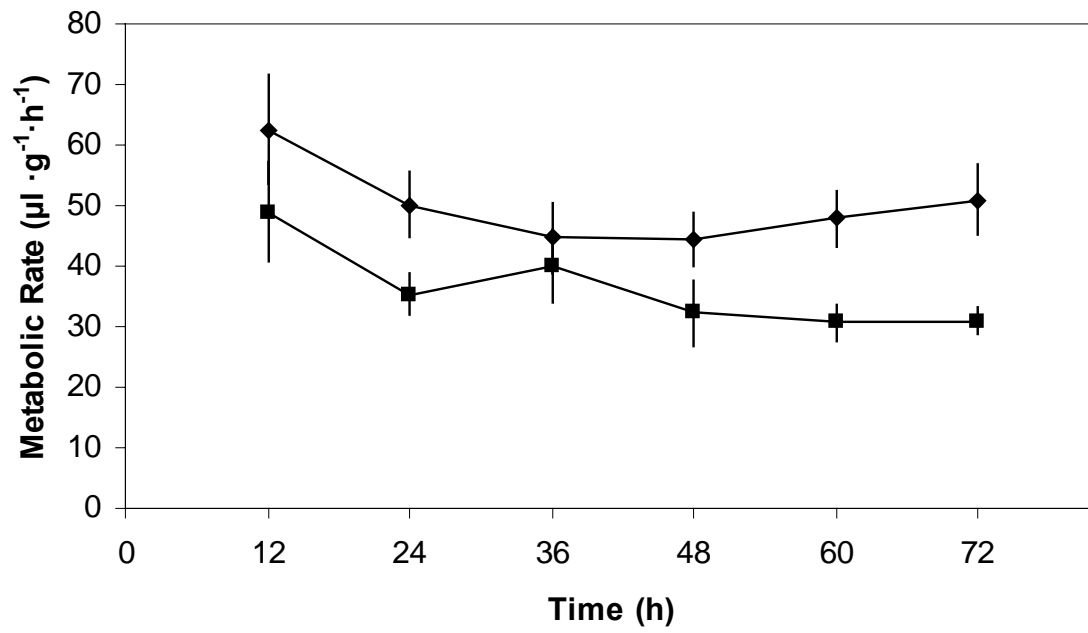
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352

Captions to Figure

354

Fig. 1: The mean (± 1 S.E.) metabolic rate ($\mu\text{L O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) for milked (\blacklozenge) and unmilked (\blacksquare)
356 scorpions for every 12 h post-milking. N = 11 for each treatment.



358

ACCEPTED

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

Group	N	Mass (g)	MR ($\mu\text{L O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$)
Unmilked	11	6.63 \pm 0.32	36.12 \pm 2.88
Milked	11	6.25 \pm 0.21	50.29 \pm 3.30*

362 * $p < 0.0001$

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

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