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Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering

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Venom is a metabolically expensive commodity that animals should use judiciously. The venom-metering hypothesis proposes that venomous animals control, or meter, the quantity of venom they deploy during predatory or defensive situations. We sought to clarify experimentally whether the buthid scorpion *Parabuthus transvaalicus* can regulate defensive venom expenditure based on perceived risk. Scorpions were tested under two threat conditions by inducing them to sting repeatedly a parafilm-covered cup. The high-threat condition involved five sting presentations at 5 s intervals, and the low-threat condition comprised five sting presentations at 5 min intervals. Venom metering appeared to be modulated at three levels: wet versus dry sting, composition of venom injected and volume of venom delivered. Scorpions delivered dry stings more often under the low-threat condition, but in both conditions were more likely to employ wet stings as the threat persisted. Venom appearance changed during successive stings from clear (potassium-rich 'prevenom'), to opalescent, to milky (protein-rich 'venom'), with biochemical analyses (protein assay and MALDI-TOF) confirming the compositional changes. However, progression through this sequence depended on quantity of venom expended, with milky secretion appearing only after the limited quantity of clear secretion was exhausted (i.e. composition and volume covaried). Scorpions injected approximately two-fold more venom per sting during the high-threat compared to the low-threat condition, with milky venom appearing more quickly within the sequence of five stings for the high-threat condition. Thus, these scorpions perceive risk and regulate venom expenditure during stinging according to level of threat, providing further support for the venom-metering hypothesis.

Many animals rely on behavioural trade-offs associated with perceived risk of predation (Lima & Dill 1990; Caro 2005; Lima & Steury 2005; Bednekoff 2007; Ferrari et al. 2009). Studies examining predator risk assessment, or threat sensitivity, have focused on behavioural choices involving foraging, courtship and mating, vigilance, fleeing or hiding, sleep, and defence of self or young. Although risk assessment has been studied most frequently in vertebrates, even invertebrates demonstrate behavioural responses that vary with different levels of threat (e.g. Taylor et al. 2005; Castellanos & Barbosa 2006; Lohrey et al. 2009).

According to the venom-metering (Hayes et al. 2002; Hayes 2008), or venom optimization (Wigger et al. 2002), hypothesis, venomous animals should use their venom as economically as possible. Venom can be viewed as a limited commodity due to storage constraints, metabolic costs of production and ecological costs of depletion (Hayes et al. 2002; McCue 2006; Nisani et al. 2007; Hayes 2008; Nisani 2008). Indeed, many studies have shown that venomous animals regulate their venom expenditure during predatory or defensive situations (Boeve et al. 1995; Malli et al. 1999; Hayes et al. 2002; Stewart & Gilly 2005; Hayes 2008). Studies of snakes suggest that venom metering occurs with different levels of threat. When physically restrained during venom extraction (i.e. the head grasped by a human hand), three viperid and two elapid species injected more venom than during unrestrained strikes at model human limbs (Herbert 1998; Hayes et al. 2002; Rehling 2002). Southern Pacific rattlesnakes, *Crotalus oreganus helleri*, in contrast, expended similar quantities of venom in the two contexts (Rehling 2002).

To date, no study has examined whether scorpions vary venom expenditure during defensive or predatory stinging. Studies of scorpion predatory behaviour demonstrate that small prey, which can be easily handled by the pedipalps, often are not stung, whereas prey that are larger and more difficult to handle are envenomated (Bub & Bowerman 1979; Cushing & Matherne 1980;

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Casper 1985; Rein 1993, 2003). However, whether scorpions can control the volume of venom they deliver during a sting, or vary the volume with intensity of threat, remains unknown.

Although there are no studies demonstrating that scorpions meter the volume of their venom, several show that venom composition may be regulated. When collecting venom from some scorpions, such as *Leiurus quinquestriatus*, appearance of the initial venom tends to be transparent and, over successive stings, the venom becomes opalescent and finally assumes a milky-viscous appearance (Zlotkin & Shulov 1969; Yahel-Niv & Zlotkin 1979). Yahel-Niv & Zlotkin (1979) demonstrated that both composition and toxicity of the secretion varied among consecutive stings. Recent studies indicate that *Parabuthus transvaalicus* similarly secretes a small quantity of transparent venom (termed 'prevenom') with initial stings, followed by a milky 'venom' in subsequent stings (Inceoglu et al. 2003). The prevenom contains a high concentration of potassium (K^+) salt and small peptides, whereas the more toxic venom contains a high concentration of protein. These findings raise the possibility that scorpions might use a different venom composition in different contexts. Inceoglu et al. (2003) proposed that the prevenom is used as an efficient predator deterrent and for immobilizing small prey items, thereby conserving the metabolically expensive venom (Nisani et al. 2007) for more urgent situations.

The genus *Parabuthus* Pocock 1890 is an exclusively Old World scorpion that includes some of the largest buthid scorpions (Prendini 2004). Since these scorpions are concentrated in some of the world's most arid regions, which are sparsely populated by humans, the incidence of scorpion envenomation (scorpionism) is relatively low. Nevertheless, envenomation by these scorpions is of significant medical importance, particularly in western regions of southern Africa (Newlands 1978; Bergman 1997). The primary effects of a sting by *P. transvaalicus* are neuromuscular, with significant parasympathetic nervous system and cardiac involvement (Bergman 1997).

The venom of *P. transvaalicus* is a cocktail 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. Some scorpion toxins have been shown to be specific for invertebrates, some specific to vertebrates, and others to both (Possani et al. 1999; Inceoglu et al. 2001). Among the peptides present in venom, the short-chain neurotoxins (SCNs) are known to act on potassium and chloride channels, whereas the long-chain neurotoxins (LCNs) mostly act on sodium channels (Possani et al. 1999; De La Vega & Possani 2004, 2005; Du Plessis et al. 2008). These different venom components may be unequally represented among consecutive stings, which warrants investigation.

The purpose of the study was to clarify experimentally whether the buthid scorpion *P. transvaalicus* can regulate defensive venom expenditure based on perceived threat. Thus, we examined both the volume and composition of venom delivered across a succession of stings at two levels of threat.

METHODS

Animals

Six adult female scorpions, *P. transvaalicus* (5–10 g), were purchased from Hatari Invertebrates (Portal, Arizona, U.S.A.). Scorpions were housed individually in clear plastic containers measuring 35 × 16 × 11 cm (L × W × H) with sand substrate and a wet sponge. They were kept at 25 ± 1 °C on a 12:12 h light:dark cycle and fed one cricket per week. Prior to testing, scorpions were fasted for 9 days. None of the female scorpions were gravid.

Defensive Stinging

Each scorpion was tested under two threat levels: high threat and low threat. The high-threat condition consisted of five consecutive stings separated by 5 s intervals, whereas the low-threat condition consisted of five consecutive stings separated by 5 min intervals. These scenarios presumably represent persistent (high-threat) and less persistent (low-threat) attacks. Because the level of threat for the first sting was equivalent for the two conditions, differences in venom attributes would be anticipated only for stings later in the sequence.

Scorpions were transferred individually to a 150 ml glass beaker and allowed to acclimate for 10 min. Scorpions were manipulated into the beaker without physically contacting their bodies. Each scorpion was tested twice, once in each condition, with an intertrial interval of 10 days. Half the scorpions were tested in the high-threat condition first, and the others were tested in the low-threat condition first. The entire procedure involving both conditions ('first trial') was then repeated 1 month later ('second trial').

After the scorpion was allowed to acclimate in the beaker, we provoked the scorpion to sting by gently touching its dorsum with the edge of a round, parafilm-covered plastic cup (2 cm high × 4.5 cm diameter). The cup was presented using a pair of forceps, 29 cm long. In all cases, the scorpion generated the stinging action on its own without our grasping either the metasoma or telson (the latter stimulus often provokes a squirt; Nisani 2008). The venom injected into the container was collected and measured (nearest 0.1 μl) using a sterile, calibrated, 5 μl micropipette. We also noted the appearance of the secretion as clear, opalescent, or milky. We considered the clear secretion to be potassium-rich 'prevenom', the opalescent secretion to be transitional, and the milky secretion to be protein-rich 'venom', with relative lethality (per volume) increasing among these three secretions (Inceoglu et al. 2003). This venom collection procedure was repeated for each of the four remaining stings in the sequence of five stings. Venom samples collected in the first trial were pooled among the individual scorpions for each successive sting and for each of the two conditions (thus, 10 samples were retained). These samples were then transferred into a microcentrifuge tube containing 0.5 ml of phosphate-buffered saline (PBS, pH = 7) and frozen at −10 °C until analysis by protein assay. The 10 samples similarly collected in the second trial were transferred into 0.5 ml of buffer (2% acetonitrile, 98% H_2O, 0.065% trifluoroacetic acid), frozen at −80 °C, and stored until analysis via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

Protein Assay

Protein mass of the pooled venom samples for each sequential sting of the first trial was determined by Coomassie Protein Assay (Pierce Chemical Co., Rockford, IL, U.S.A.). Venom standards (0, 5, 10, 15, 20 and 25 μg/ml) were prepared from the lyophilized venom of the western diamond-backed 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., Coring, NY, U.S.A.). Samples were analysed using the protocol provided by Pierce using a μQuant microplate reader (Bio-Tek Instruments Inc., Winooski, VT, U.S.A.) at 570 nm absorbance. Venom standards were within a linear range and the amount of protein was calculated using the following regression equation:

$$P_V = m \times A_{570\text{nm}} + b \quad (1)$$

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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.

MALDI-TOF Analysis

We subjected the venom samples, pooled among the six scorpions for each successive sting within high- and low-threat conditions of the second trial, to MALDI-TOF using an Autoflex instrument (Bruker Daltonics, Billerica, MA, U.S.A.). The samples were first dried using a speed-vacuum and then redissolved in 1 μl of buffer. The venom samples (1 μl) were loaded on the polished steel MALDI plate with 1 μl of α-cyano-4-hydroxycinnamic acid (α-CHCA, Aldrich, St Louis, MO, U.S.A.) and air dried. The instrument was calibrated using angiotensin II (molecular weight = 1047.20 Da), somatostatin 28 (3149.61 Da), insulin (5734 Da), myoglobin (8475.70 Da) and cytochrome c $(M + 2H)^{2+}$ (6181.05 Da). All mass spectra were recorded, with two reference peptides as internal standards, using a two-point calibration. Errors to the masses of the spectra were within the 0.05% range. All spectra were recorded in the m/z range of 1000–15 000 using an accelerating grid and guide wire potentials of 20 000, 19 000 and 1000 V, respectively, and 400 ns delayed extraction setting. Because identifying peaks and their intensities is complicated by high frequency of noise and lack of preferred methods of distinguishing noise from true signal, there is no consensus on which properties of the spectra are truly relevant in inferring peptide abundance (Randolph et al. 2005); thus, interpretation of peptides present within venom samples was limited to presence or absence of the five major peptides identified in *P. transvaalicus* venom by Inceoglu et al. (2003).

Statistical Analyses

We used a 2 × 5 repeated measures ANOVA (Zar 1999) to investigate the effects of threat level (high threat versus low threat) and sting sequence (the five successive stings) on relative lethality of venom expended. Individual stings were ranked by appearance (1 = dry or no venom; 2 = clear venom; 3 = opalescent venom; 4 = milky venom), which corresponded to increasing level of lethality. Rather than treat the two trials as a separate variable (i.e. 'replication'), values from each scorpion in the two different trials were averaged for each of the corresponding stings (when analysed separately, the two trials yielded identical conclusions). Data were inspected to ensure that they met parametric assumptions.

We used two ANOVA models to examine how threat and sting sequence influenced the quantities of venom expended. The volumes of venom measured in the two trials were averaged for corresponding stings. The first 2 × 5 (threat × sting sequence) repeated measures ANOVA considered total venom expended and included all five successive stings, regardless of whether they were 'dry' (no venom expended) or 'wet' (venom expended). For this analysis, we used rank-transformed data to meet parametric assumptions. The second ANOVA considered only the first three wet stings; hence, this 2 × 3 (threat × sequence) repeated measures ANOVA removed the confounding effect of dry stings and allowed us to assess whether scorpions metered their venom volume more subtly than simply choosing to deliver a wet versus a dry sting. For this analysis, no data transformation was required. By using only wet stings, the between-sting interval sometimes increased from 5 to 10 s in the high-threat condition, and from 5 to 10–15 min in the low-threat condition. Nevertheless, the clear distinction between the two threat levels (seconds versus minutes between consecutive stings) remained for the second and third stings (again, the first stings were equivalent for the two conditions).

To evaluate whether stings yielding clear secretion (prevenom) differed in volume from those yielding either opalescent or milky secretion, we assumed all wet stings were independent (dry stings were excluded) and subjected wet sting volumes to a 2 × 2 ANCOVA, with threat and appearance (clear versus opalescent or milky) treated as between-subjects factors and wet sting sequence (up to five stings) treated as a cofactor. Although this test involved pseudoreplication, we were able to compare the volumes of the different-appearing secretions while controlling for threat level and sting sequence. Data were rank-transformed for this analysis.

We used a Spearman rank correlation to evaluate the relative complexity of venom delivered across the sequence of five stings. For this analysis, we pooled samples for the two threat levels and then summed the number of recognized peptides from *P. transvaalicus* venom (up to five; Inceoglu et al. 2003) that were detected by MALDI-TOF in each of the five consecutive stings.

All analyses were conducted using SPSS version 11.5 (SPSS Inc., Chicago, IL, U.S.A.), with alpha set at 0.05. For each ANOVA model, 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 SPSS sometimes summed to greater than 1, we adjusted these values by dividing each by the sum of all partial η^2 values for the effects tested.

RESULTS

Venom Appearance

In Table 1, we show the appearance of venom obtained from successive stings under the two threat conditions for both trials. In a typical sequence of five stings, the first venom to appear was clear, followed by opalescent and then milky venom. No reverse transitions occurred through the sequence; that is, once opalescent venom appeared, no subsequent stings involved clear venom, and once milky venom appeared, no subsequent stings involved either clear or opalescent venom. In some sequences, opalescent venom appeared with the first sting, and in two sequences milky venom appeared in all wet stings. Dry stings, representing 12.5% of the 120 stings measured, usually occurred early in the sequence, more so for high-threat (all five dry stings were the first sting) than low-threat (first sting: $N = 3$; second sting: $N = 3$; third–fifth stings: $N = 4$). Among wet stings in high threat, clear venom appeared in an average of 0.67 stings (range 0–2), opalescent 0.50 (range 0–2) and milky 3.42 (range 2–4). Among wet stings in low threat, clear venom appeared an average of 1.25 stings (range 0–4), opalescent 1.08 (range 0–3) and milky 1.83 (range 0–3). A 2 × 5 (threat × sting

Table 1
Appearance* of venom obtained from five successive stings under low- and high-threat conditions by *Parabuthus transvaalicus* scorpions

Scorpion	Trial	High-threat stings					Low-threat stings				
		1	2	3	4	5	1	2	3	4	5
1	1	C	M	M	M	M	D	C	O	M	M
	2	C	M	M	M	M	O	D	D	O	O
2	1	D	O	M	M	M	D	C	O	M	M
	2	O	M	M	M	M	O	M	M	D	M
3	1	O	M	M	M	M	C	C	O	O	M
	2	D	M	M	M	M	C	O	O	M	M
4	1	O	O	M	M	M	C	C	M	M	M
	2	D	M	M	M	M	C	C	M	M	M
5	1	D	C	C	M	M	C	D	C	C	C
	2	C	O	M	M	M	C	O	M	D	D
6	1	D	C	C	M	M	C	D	O	M	M
	2	C	M	M	M	M	D	O	M	M	M

The study was replicated; hence, two trials are indicated for each scorpion.
* D = dry sting; C = clear venom; O = opalescent venom; M = milky venom.

sequence) ANOVA confirmed that both threat ($F_{1,5} = 12.14$, $P = 0.018$, adjusted partial $\eta^2 = 0.39$) and sequence ($F_{4,20} = 30.38$, $P < 0.001$, adjusted partial $\eta^2 = 0.47$) significantly influenced the appearance of venom, with the most lethal stings (milky venom) being delivered more often for high threat, and for both conditions later in the sting sequence. There was no interaction between threat and sequence ($F_{4,20} = 1.74$, $P = 0.18$, adjusted partial $\eta^2 = 0.14$).

Venom Expenditure

When all five successive stings were considered, the 2×5 (threat $\times$ sting sequence) ANOVA revealed that both threat ($F_{1,20} = 16.82$, $P = 0.009$, adjusted partial $\eta^2 = 0.47$) and sequence ($F_{4,20} = 8.64$, $P < 0.001$, adjusted partial $\eta^2 = 0.38$) significantly influenced venom expenditure (Fig. 1). Scorpions expended 2.2-fold more venom per sting in the high-threat condition (mean ± SE: 1.38 ± 0.15 μl, $N = 30$; for each individual, values for the two trials were averaged for each sting in the sequence, thereby halving sample size) compared to the low-threat condition (0.62 ± 0.07 μl, $N = 30$), and more in subsequent stings compared to the first sting (quadratic effect of sequence: $F_{1,5} = 192.03$, $P < 0.001$). No interaction between threat and sting sequence was detected ($F_{2,20} = 1.71$, $P = 0.19$, adjusted partial $\eta^2 = 0.15$).

When we compared only the first three wet stings, the 2×3 ANOVA showed that the effect of threat ($F_{1,5} = 4.45$, $P = 0.089$, partial $\eta^2 = 0.47$) was not significant; however, the effect size was substantial, especially when compared to significant effects in other models, suggesting that the scorpions injected more venom (1.9-fold) per sting during high-threat (1.40 ± 0.18 μl, $N = 18$) compared to low-threat (0.75 ± 0.10 μl, $N = 18$) conditions. The effect of sting sequence was comparatively small ($F_{2,10} = 1.06$, $P = 0.38$, partial $\eta^2 = 0.18$), suggesting that differences between venom expended among successive stings in the previous ANOVA model (for all five stings) were largely the result of dry stings. There was no interaction between threat and sequence ($F_{2,10} = 1.36$, $P = 0.30$, partial $\eta^2 = 0.21$).

When we treated all stings as independent, the ANCOVA model confirmed that threat ($F_{1,100} = 4.82$, $P = 0.030$, partial $\eta^2 = 0.05$) and venom appearance ($F_{1,100} = 21.90$, $P < 0.001$, partial $\eta^2 = 0.18$) significantly influenced venom volume, and that sting sequence ($F_{1,100} = 0.87$, $P = 0.35$, partial $\eta^2 = 0.01$) did not. There were no significant interactions. Because of pseudoreplication (inflated degrees of freedom), the P values should be interpreted with caution; however, the effect sizes indicate that venom appearance explained 3.6-fold more variance than threat level, and 18-fold more variance than sting sequence. Stings yielding opalescent or milky secretion (1.33 ± 0.11 μl, $N = 82$) averaged 2.8-fold more volume than stings yielding clear secretion (0.47 ± 0.08 μl, $N = 23$). Thus, the visible detection and number of stings yielding prevenom depended to a large extent on whether initial stings were of small volume.

Although scorpions expended similar amounts of venom on average in the first, second and third stings, the volume of venom expended among successive wet stings by individual scorpions (not averaged for the two trials) varied substantially, with a 1.7- to 25-fold difference (high-threat: 8.0 ± 1.9; low-threat: 6.2 ± 1.2) between the lowest and highest values. The coefficients of variation (SD $\times$ 100/mean) were 74.9 ($N = 55$) for all wet high-threat stings, 83.6 ($N = 50$) for all wet low-threat stings, and 86.9 ($N = 105$) for all wet stings pooled.

Protein Assay

Because venom samples were pooled from all six scorpions to assay total protein, the data were not amenable to statistical analysis. For the first sting, the dry mass of protein expended was relatively small but similar for the high-threat (24.63 μg) and low-threat (28.44 μg) conditions (Fig. 2). Under high threat, the scorpions expended the greatest quantity of protein with the second sting, and protein expulsion declined for subsequent stings, suggesting venom depletion (Fig. 2). Under low threat, the scorpions injected similar or increasing quantities of protein with stings two through five (Fig. 2). Thus, the pattern of variation suggests that a significant interaction existed, with scorpions expending protein-rich venom earlier in the sting sequence under high threat and later in the sting sequence under low threat.

MALDI-TOF Analysis

Peptide composition of the venom appeared to be similar for the two threat conditions, but varied considerably among the sequence of five stings (Table 2). Five potential peptides were identified based on previously published studies (Inceoglu et al. 2003). The first sting, usually consisting of clear or opalescent venom, had only one or two of these identified peptides present (Table 2). Venom from subsequent stings became increasingly more complex (Spearman rank correlation: $r_S = 0.95$, $N = 5$, $P = 0.014$), with four or five of these identified peptides present in the last two stings (Table 2).

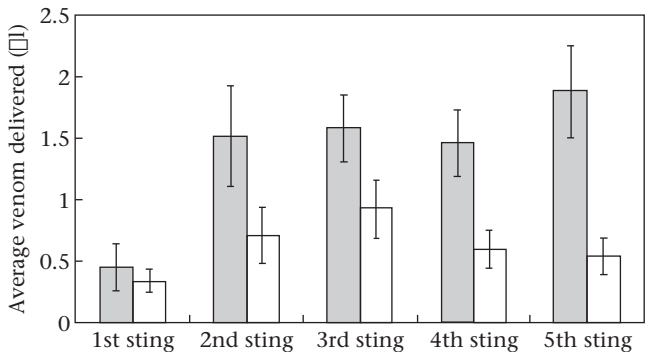

Figure 1. Mean ± SE volume of venom delivered during successive stings by *Parabuthus transvaalicus* scorpions under high-threat (grey bars) and low-threat (clear bars) conditions. $N = 6$ individuals for each mean, with each individual tested twice and values averaged.

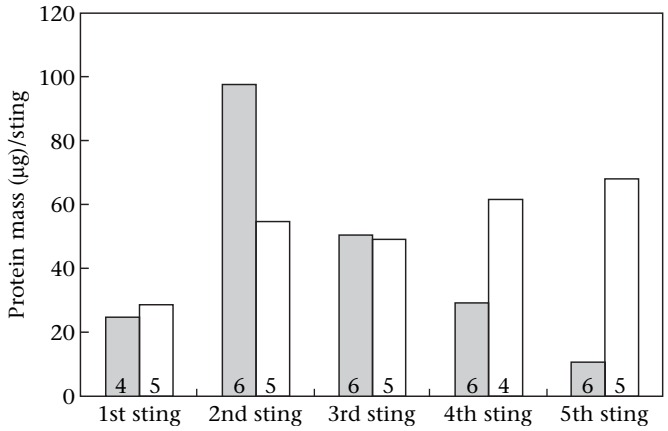

Figure 2. Dry mass of venom protein obtained from successive stings by *Parabuthus transvaalicus* scorpions under high-threat (grey bars) and low-threat (clear bars) conditions. Each bar represents venom pooled for six scorpions. The number at the base represents total number of wet stings from the six individual scorpions in trial 1.

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Table 2
Comparison of *m/z* values of venom composition among different *Parabuthus transvaalicus* stings under two threat conditions (high and low) analysed by MALDI-TOF mass spectrometry

Stinging sequence									
1st sting		2nd sting		3rd sting		4th sting		5th sting	
High	Low	High	Low	High	Low	High	Low	High	Low
	4084.11[a]					**4081.41[a]**			**4083.16[a]**
									4291.41
			4748.21		4748.59			4748.40	
5048.92	5050.58	5049.13	5048.53	5048.92	5049.45	5048.72	5049.27	5048.17	
5256.17		5257.38	5256.92	5257.33	5257.22	5256.71	5257.46	5257.11	
						6543.09[b]	**6544.30[b]**	**6543.33[b]**	**6545.21[b]**
		6604.21[c]	**6604.06[c]**	**6604.18[c]**	**6604.12[c]**	**6603.88[c]**	**6604.53[c]**	**6603.80[c]**	**6604.82[c]**
		6645.26[d]	**6644.97[d]**	**6645.11[d]**	**6645.33[d]**	**6644.99[d]**	**6645.57[d]**	**6644.97[d]**	**6646.02[d]**
						6811.13			
7219.37[e]	**7221.38[e]**	**7220.14[e]**	**7219.42[e]**	**7220.14[e]**	**7220.05[e]**	**7220.27[e]**	**7220.40[e]**	**7219.72[e]**	**7221.25[e]**
			7222.08		7222.15				
7277.73	7279.55				7278.07		7278.99		7279.38
7298.65	7300.67		7298.91				7299.99		7300.23
		7335.10	7334.38	7335.04	7334.83	7335.28	7335.77	7334.69	
7390.39	7391.93	7391.27	7390.55	7391.14	7390.93	7391.01	7391.72	7391. 05	7392.07
									7428.05
	7445.92						7445.88		
									7505.55
7514.76	7516.75	7515.60	7514.98	7515.63	7515.29	7515.44	7516.20	7515.50	

Bold values and associated superscripts indicate major peptides known from the venom: [a]parabutoxins, [b]birtoxin, [c]bestoxin, [d]dortoxin, [e]alpha toxin (Inceoglu et al. 2003); unidentified *m/z* values could be noise, unidentified peptides, or isomers of known peptides.

DISCUSSION

Our results suggest that *P. transvaalicus* scorpions regulate venom expenditure at three levels. First, these scorpions can choose between delivering a dry or wet sting. Second, should they deliver a wet sting, they can control, or meter, the volume of venom expended, delivering more under high-threat and less under low-threat conditions. Third, because their stored venom is heterogeneous, they also vary the composition of the venom injected, delivering either potassium-rich prevenom or protein-rich venom. However, the composition of venom injected covaries with venom volume, such that regulation of volume leads to regulation of composition.

Although 'dry bites' have long been recognized for snakes (Hayes et al. 2002), the prevalence of dry defensive stings has not been documented previously for scorpions. Our results suggest that dry stings can be frequent within a defensive context, and suggest judicious use of venom. Dry stings most often occurred early in the succession of stings, suggesting they did not result from venom depletion. Because the target properties were always the same for consecutive stings, the decision not to envenomate was presumably unrelated to tactile cues.

Evidence supporting the conclusion that scorpions inject more venom under higher threat was statistically significant only when all stings, including dry stings, were analysed ($P = 0.009$). However, because we recognize the need to distinguish between decisions involving venom release (dry versus wet stings) and quantity of venom released, we reanalysed the data using only wet stings. In doing so, the difference was not statistically significant ($P = 0.089$), but the effect size was substantial, with threat level explaining approximately 47% of the variance in venom expenditure, a value identical to the first, highly significant comparison including dry stings (47%). Cohen (1988) indicated that an eta-squared value explaining 14% or more of the variance represented a 'large' effect. However, effect size gains relevance only within an appropriate context, which in this case would be comparison to other effects within the same ANOVA, or other ANOVAs using similar data. Clearly, the difference in significance between the two ANOVA models (all stings versus wet stings only) was merely a consequence of sample size and degrees of freedom, as the strength of the relationship was identical. Thus, this study is the first to document venom metering by any scorpion. Our interpretation is supported by the high level of variation in venom expended among consecutive stings by the same scorpion, confirming that the bolus of venom ejected is variable rather than uniform. The coefficients of variation in venom expenditure were similar to those reported by venomous snakes that also meter their venom (Herbert 1998; Hayes 2008).

Inceoglu et al. (2003) compared the relative lethality and functional roles of *P. transvaalicus* prevenom and venom. They concluded that prevenom, which constitutes roughly 5% of the total venom reservoir, was extruded on the first sting and that venom was extruded during subsequent stings. However, our results suggest a continuum in venom composition, with clear, opalescent and milky venom being readily distinguished. Moreover, prevenom sometimes appeared for more than one defensive sting, and sometimes was omitted altogether. The number of stings yielding prevenom depended to a large extent on the volume of initial stings. If the initial sting was of small volume, it and the subsequent sting were more likely to be composed of prevenom. More importantly, the sequence of venom categories expulsed varied with threat level. In the high-threat condition, scorpions more quickly escalated their delivery of milky venom, doing so earlier within the sequence of stings compared to the low-threat condition. Although variation in venom composition has been documented for successive stings by scorpions (Yahel-Niv & Zlotkin 1979; Inceoglu et al. 2003) and for successive spits by cobras (*Naja pallida*; Cascardi et al. 1999), no other venomous animal has been shown to regulate venom composition in different contexts (e.g. levels of threat). *Parabuthus* changes its venom composition depending on threat, but does so indirectly by metering the volume of venom during stings.

Collectively, these findings support the venom-metering (Hayes et al. 2002; Hayes 2008), or venom optimization (Wigger et al. 2002), hypothesis. This hypothesis proposes that venomous animals use their venom judiciously, and make cognitive decisions about how much venom to inject. Venom can be an expensive commodity (McCue 2006; Nisani et al. 2007; Nisani 2008; see also

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Pintor et al. 2010), and many venomous animals have been shown to be judicious in their venom expenditure (e.g. Boeve et al. 1995; Malli et al. 1999; Hayes et al. 2002; Hayes 2008). There are several reasons why scorpions should be judicious when deploying their venom reserves. First, venom regeneration in scorpions has measurable metabolic costs (Nisani et al. 2007; Nisani 2008). For example, a *P. transvaalicus* scorpion that has had its venom gland emptied will experience a 39% increase in its resting metabolic rate (Nisani et al. 2007). Second, scorpions may be disadvantaged by a depleted supply of venom: a scorpion with insufficient venom may be unable to capture additional prey or defend itself against attack until its supply of venom has been at least partially restored. When *P. transvaalicus* venom glands are completely emptied, it usually takes 3 days for the venom volume to return to pre-extraction level, but another 5 days are needed for the venom to regenerate most of its essential components (i.e. major venom peptides; Nisani 2008).

In addition to the scorpion's need for conserving a valuable commodity, the optimal amount of venom injected may vary with the context of use. Within a predatory context, scorpions do not ordinarily sting small prey that could be easily handled by the pedipalps. Two East African scorpions (*Parabuthus leiosoma* and *Parabuthus pallidus*), for example, were shown to be selective in their sting use, deploying venom only for large or difficult-to-handle prey items (Rein 1993). Other researchers reported similar findings in additional scorpion species (Cushing & Matherne 1980; Casper 1985; Edmunds & Sibly 2010). However, none of these studies measured the amount of venom injected by the scorpions and only assumed that restrictive sting use was advantageous. Within a defensive context, the amount of venom delivered may vary with the identity of attacker or the level of perceived threat. This study addressed the latter issue and has shown that *P. transvaalicus* meters the amount of venom injected depending on threat. Some buthids successfully defend themselves by striking the potential predator with a powerful blow, wherein the force may be sufficient in startling the predator and allowing the scorpion to escape (Newlands 1969). Scorpions in general, and buthids in particular, demonstrate a strong preference for retreat when threatened (Newlands 1969), which is another indicator of venom conservation. Some predators appear to be highly resistant to the venom's toxicity (Rowe & Rowe 2008; Holderied et al., In press), but may still be vulnerable to the venom's pain-inducing properties (Rowe & Rowe 2006).

Bergman (1997) reported that human envenomation by *P. transvaalicus* had a mortality rate of 0.3%, with deaths occurring mainly in children under the age of 10 years and adults over 50. The prevenom portion of these scorpions has been shown to contain a high combination of K^+ salt and some peptides that block rectifying K^+ channels and elicit significant pain and toxicity due to massive depolarization in a mammalian model (Inceoglu et al. 2003). Because the more lethal venom is ejected after the first or several stings, the most severe envenomations of humans probably result from multiple stings, although susceptibility of the patient to envenomation is certainly important (Hayes & Mackessy 2010). As the results of this study show, higher amounts of protein are injected when the threat is persistent enough to solicit multiple stings.

Yahel-Niv & Zlotkin (1979) proposed two possible mechanisms to explain the different profiles of venom from the same scorpion. They proposed that the clear venom released in an initial sting occupies the lumen of the gland, and is subjected to inactivation-degradation and/or reabsorption processes. Alternatively, they suggested that the very presence of different types of secretion is a manifestation of a natural sequential mode of selective venom secretion of different components. Venom gland morphology in scorpions has a generalized scheme, with the main differences occurring in the presence or absence of folds in the secretory epithelium, if present (Pawlowsky 1924; Mazurkiewicz & Bertke 1972). The lumen of the venom gland probably serves as an extracellular storage site for the venom. The abundant numbers of membrane-bound vesicles within the lumen segregate the different secretory products that are presumably mixed during injection (Mazurkiewicz & Bertke 1972). Kovoor (1973) demonstrated that the venom gland of the scorpion *Buthotus judacius* consists of a series of three lobes that differ in their morphology and histochemistry. Some of the lobes contain only acidic mucosubstances, while others contain acidic and protein products combined, or mainly protein. Our results suggest that venom storage is heterogeneous; that is, peptides are not evenly distributed within the duct or lumen of the venom gland of *P. transvaalicus*, as the clear prevenom always comes before the milky venom and the order is never reversed (Table 1). Whether different venom products are regionally secreted and stored within the gland without mixing, or secretion is homogenous but involves inactivation-degradation and/or reabsorption for venom residing in the lumen or a portion of it, remains to be determined.

In conclusion, this study provides evidence that scorpions regulate venom expenditure based on the level of perceived threat. The capacity to make decisions regarding usage (dry versus wet sting), quantity, and, indirectly, the composition (prevenom or venom) of venom injected provides further support for the venom-metering hypothesis.

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