Differences in venom toxicity and antigenicity between females and males *Tityus nororientalis* (Buthidae) scorpions

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ABSTRACT

Venom from male and female specimens of the medically important Venezuelan scorpion *Tityus nororientalis* have been compared. Males showed a significantly higher venom yield (2.39 mg/individual) compared to female scorpions (0.98 mg/individual). Female venom was significantly more toxic than that of males, with a median lethal dose (LD$_{50}$) in C57BL/6 mice of 9.46 µg venom protein/gm body weight [95% confidence interval (8.91-9.94)] whereas LD$_{50}$ for males was 13.36(12.58-14.03) µg/gm. Mass spectral analyses by MALDI-TOF revealed differences in venom composition between males and females. From a clinical standpoint, the time course of toxicity course indicated a tendency, in the case of the female venom, to elicit the earlier occurrence of severe signs such as sialorrhea, dyspnea (bradypnea/apnea) and exophthalmus particularly in the late toxicity phase. Female venom was significantly less efficient than male venom to inhibit the binding of anti-*T. discrepans* antibodies to immobilized *T. discrepans* venom in ELISA assays, suggesting sex-related differences in the bioactive surfaces of *T. nororientalis* toxins. These results indicate that males and females of *T. nororientalis* produce venoms with different composition and activity which may have epidemiological implications.

KEYWORDS: Intersexual variation; scorpion; *Tityus nororientalis*; Venezuela

INTRODUCTION

Envenoming by species belonging to the scorpion genus *Tityus* is a pediatric emergency in several countries in South America (Biondi-Queiroz et al, 1996; Otero et al, 2004; De Sousa et al, 2007; Chippaux and Goyffon, 2008; Lira-da-Silva et al, 2009; Gómez et al, 2010), and also Panamá (Coronado et al, 2008), and the Caribbean (Daisley et al, 1999). Clinical manifestations of scorpionism are dependent on the scorpion species, amount of venom injected, and also the age and venom sensitivity of the victim, which is significantly higher in children (<8 year-old) and the elderly (Borges, 1996; Borges and De Sousa, 2006). Venezuela is one of the South American countries with highest incidence of scorpionism, mainly due to stings by the genus *Tityus* (Borges and De Sousa, 2009; De Sousa and Borges, 2009; Borges et al, 2010a).

Of the seven endemic macroregions of scorpionism identified in Venezuela (De Sousa et al, 2000; Borges and De Sousa, 2006), the Andean and Northeastern regions have
In Sucre state, northeastern Venezuela, total deaths for the period 1996-2000 were 7, with a rate of 1.73 deaths per million inhabitants for the period 1996-2000; national mortality rate for the same period was 0.42 (Borges and De Sousa, 2006). This species was previously described by González-Sponga from Catuaro, Ribero municipality, central Sucre state (González-Sponga, 1996; Rojas-Runjaiac and De Sousa, 2007). As well as other Tityus species in the Venezuelan northeast (González-Sponga, 1996, 2001; Quiroga et al, 2000; Quiroga et al, 2004; De Sousa et al, 2006; De Sousa et al, 2008), T. nororientalis displays a marked sexual dimorphism, with males exhibiting a larger length of metasomal segments compared to females (González-Sponga, 1996).

While intersexual differences in morphology (e.g., size and color) are common in a number of arachnid species, including scorpions, only a few studies have examined such differences in regard to venom yield, composition or bioactivity. Thus far, intersexual differences have been found in theraphosids (Celerier et al, 1993) and araneomorph spider venoms (Herzig et al, 2002; Herzig and Hodgson, 2009). For scorpions, only in the case of Opisthacanthus madagascariensis a male specific venom component has been identified (Yamaji et al, 2004). Intersexual differences in toxicity and antigenicity in scorpion venoms may have clinical, therapeutic, as well as epidemiological implications considering the 2:1 female-to-male ratio in house dwellings, at least in the Venezuelan northeast, for several species of the medically important genus Tityus (De Sousa et al, 2009a). In this regard, the present report is the first to document intersexual variations in venom composition and activity in Tityus species, reporting a reduced venom production and higher lethality for female T. nororientalis specimens compared to male individuals. Differences in toxicity correlate well with proteomic differences evaluated by mass spectrometry and reactivity of female venom towards the commercial antivenom is significantly reduced compared to male individuals.

**MATERIALS AND METHODS**

All animal experiments reported in this article were performed according to protocols approved by the Department of Physiological Sciences, School of Health Sciences, Universidad de Oriente, Anzoátegui Campus (for details see De Sousa et al, 2009b). The ethical procedures recommended by the Fondo Nacional de Ciencia, Tecnología e Innovación (Ministry of Science and Technology, Venezuela) were strictly followed during the research.

**Scorpion collection**

*Tityus nororientalis* specimens were collected at night from its type locality in Catuaro (10°23’59.1”N, 63°30’00.6’’W, 455 meters above sea level), Ribero municipality, Sucre state (González-Sponga, 1996). Scorpions were found under the bark of fallen, decomposing trees, and also in the base of coffee plants (*Coffea arabica*) using ultraviolet lamps (Stachel et al, 1999). Geographical coordinates were determined using a Global Positional System device, model eTrex® H (Garmin). After completion of the experimental procedures, scorpions used during this work were deposited at the Scorpion Collection (CELT), Escuela de Ciencias de la Salud, Universidad de Oriente, where they were given the following catalog numbers: $♀♀$, CELT-1142 to CELT-1155; $♂♂$, CELT-1156 to CELT-1162. *T. discrepans* specimens were collected in San Antonio de Los Altos, Miranda state, Venezuela ($10°23’01”N, 66°56’58”W”) and venom extracted as described above.

**Venom extraction and protein determination**

Venom was milked by electrical stimulation of the telson (the last caudal segment of the scorpion metasoma) according to the method of Quiroga et al (1982) using a neurostimulator Phipps-Bird (Richmond, Virginia, USA). Fourteen adult females and 7 adult males were milked according to this procedure. Venom was kept at -20°C until further use. Protein content was determined by measuring absorbance of venom solutions at 280nm using a 6405 Jenway UV/vis spectrophotometer (Staffordshire, UK) considering one unit of absorbance equivalent to 1 mg/ml (Possani et al, 1977). Previous to mass spectrometry and electrophoretic analyses venom was lyophilized at 50mBar of pressure at -50°C.

**Mice**

17-23 g female mice of the strain C57BL/6 (Bioterium, Venezuelan Institute for Science Research, IVIC) were used throughout. Animals were maintained using natural light cycles, room temperature, feeding and water *ad libitum* as described by De Sousa et al (2009b).

**Signs of acute toxicity and LD$_{50}$ evaluation**

Fifty lethal doses (LD$_{50}$) for female and male *T. nororientalis* venoms were calculated as described by Dixon and Mood (1948) (with the modifications presented by Sevcik, 1987) for a one-hour period of experimentation as presented by De Sousa et al (2009b) for scorpion venom lethality titration. Venom was injected intraperitoneally (ip) with a Hamilton 505µl microsyringe. Doses administered were calculated based on the weight of individual mice. Clinical signs elicited by scorpion venom toxicity were chronologically recorded during the observation period. Briefly, LD$_{50}$ were calculated as follows: The first mouse injected ip received an initial dose, $X_1 = \text{antiLog } 1 = 10\mu g$ venom protein/g of body weight. If the first animal dies, a second mouse will receive a dose $X_2 = \text{antiLog } (X_1) - d$, where $d$ is a constant factor corresponding to 0.05 (De Sousa et al, 2009b). If the first animal survives, a second mouse will receive a dose $X_2 = \text{antiLog } (X_1) + d$. The protocol is continued thereby increasing or reducing the dose depending on the outcome (death or survival) of the mouse previously injected until completing four cycles of increasing/decreasing doses, i.e., $† 0 \uparrow 0 \downarrow 0 \uparrow \Theta$ ($† = \text{dead mouse, } 0 = \text{surviving mouse, } \Theta = \text{end point}$) or $0 \uparrow 0 \downarrow 0 \uparrow 0 \Theta$. The LD$_{50}$ value was determined from the median of the doses starting from the first inflexion point in the lethality curve, which comprise the valid data sequence.

**Statistical analysis**

The median of the doses taken from the first inflexion point in the lethality curves was calculated according to Hodges
and Lehmann (1963) together with the confidence intervals for the LD₅₀ values and for the occurrence times of the acute toxicity signs. Differences between medians were tested using the Kruskal-Wallis analysis of variance (Kruskal and Wallis, 1952) considering a level of significance of p < 0.05 (De Sousa et al, 2009b). For data processing, the program V-8.34 was utilized, which was developed by Dr Carlos Sevcik, Venezuelan Institute for Science Research. To assess the significance of the difference in venom production between female and male T. nororientalis specimens, difference of proportions were calculated with independent sampling (Z value) and considering a p < 0.05. Variability index (VI) was evaluated as VI = [(LD₅₀ top limit - LD₅₀ bottom limit)/Median of LD₅₀] x100.

Mass spectrometry
Mass spectra of positively charged ions from female and male T. nororientalis venoms were analyzed by MALDI-TOF MS in a Biflex III MALDI-TOF MS (Bruker, FRG) basically as described by Borges et al (2006a,b). Briefly, samples for analyses (200-500µg) were lyophilized, resuspended in 100µl of MilliQ water and diluted 10-fold with 0.1% (v/v) trifluoroacetic acid (TFA). One µl of the diluted sample was mixed with 5µl of matrix solution (10mg/ml of 3,5-dimethoxy-4-hydroxycinnamic acid in a 1:1 mixture of acetonitrile and 0.1% (v/v) TFA). One µl from this mixture was spotted on the target plate. Mass spectra of positively charged ions were recorded on the Biflex III instrument operated in the linear mode. The total acceleration voltage and the detector voltage were 19kV and 0.55kV, respectively. A total of 100 to 150 single shots were accumulated for each sample. Molecular masses were calculated from at least three independent analyses.

Inhibition of anti-Tityus discrepans antivenom binding
An ELISA assay was used to determine the ability of T. nororientalis venoms (female and male) to inhibit binding of horse anti-T. discrepans antivenom antibodies to T. discrepans venom adsorbed onto microtitration plates according to the method of King et al (1985). Competing venom samples were serially diluted starting from 10µg/ml and incubated for 1hr at 37°C with the antivenom (Biotecfar, Universidad Central de Venezuela, Lot No. 049) at a dilution corresponding to 50% of the maximum antibody binding to immobilized T. discrepans venom. The latter venom was a mixture of equal proportions of female and male T. discrepans venoms. One hundred µl of the competing venom:antivenom mixtures were added onto the plates previously coated with T. discrepans venom (100ng/well) and incubated for 1hr at 37°C. Plates were then washed and horse radish peroxidase-labeled anti-horse IgG antibodies (Sigma) added as described by Borges et al (2008). The antigen:antibodies complexes were recorded by measuring color formation after the peroxide-driven reaction at 492nm. Data was plotted in a semilogarithmic scale and used to determine the percentage of inhibition of antivenom binding to immobilized T. discrepans venom as a result of preincubation with T. nororientalis female and male venoms.

Acetic acid-urea gel electrophoresis
Electrophoresis of protein samples in acid-urea gels was carried out according to Wang et al (1997) in 12.5% (w/v) polyacrylamide gels, using 5% (v/v) acetic acid as running buffer and pyronine Y as tracking dye.

RESULTS

Venom production
Figure 1 shows venom production by female and male T. nororientalis obtained by electrical stimulation. A significantly lesser amount of venom (Z = 2.42; p <0.001) in terms of protein and volume was produced by females (n = 14) compared to males (n = 7). In total females produced 13.77 mg of venom whereas males produced 16.73 mg. The venom yield per individual was 0.98 mg in 2.343 µl for females, and 2.39 mg and 6.252l for males.
Venom lethality and signs of toxicity

Figure 2 compares the lethality of female and male venoms through the determination of their 1-h LD$_{50}$ values in C57BL/6 mice. Female venom LD$_{50}$ was 9.46(8.91-9.46)µg/gm, significantly (Kruskal-Wallis = 18.95; $p$ = 0.00001) lower than the male venom LD$_{50}$, 13.36(12.58-13.36)µg/gm. Variability indexes were 5.8% for both LD$_{50}$ determinations.

Table 1 presents the mortality frequency obtained in the valid data sequences for LD$_{50}$ determination for female and male *T. nororientalis* venoms in mice. For females, the dose corresponding to the highest rate was 10.00µg/gm (n = 4, 80.0%). At 8.91µg/gm the frequency was low (n = 1, 20.0%) and with 7.94µg/gm no deaths were recorded. For males, the dose corresponding to the highest rate was 14.13µg/gm (n = 5, 83.3%). At 12.58µg/gm the frequency was low (n = 1, 16.7%) whereas at 11.22µg/gm no deaths were recorded.

Table 2 compares the signs of toxicity occurring after injection of female and male venoms into mice. With the female venom, 24 signs of toxicity were recorded. Hyperactivity, tachypnea, sialorrhea, piloerection, and deglutatory movements were observed in all mice corresponding to the valid data sequence. 80-90% of the mice developed mouth and nose cleaning, ataxic movements, Straub sign, convulsions, abundant sialorrhea, and dyspnea characterized by forced abdominal breathing. 72.7% of the injected mice presented bradypnea/apnea, ocular secretion, pasty defecation, abdominal distension, and generalized tremors, whereas 63.6% developed exophthalmus and hypotonic rear limbs. Spastic paralysis was observed in 45.6% of the injected animals. Signs associated with high mortality were (in n/n dead mice and n/n surviving mice): bradypnea/apnea (5/5 and 3/6), pasty defecation (5/5 and 3/6), exophthalmus (5/5 and 2/6), hypotonic rear limbs (5/5 and 2/6) and spastic paralysis (5/5 and 0/6).

Similarly, the male venom elicited occurrence of 24 signs of toxicity with frequencies equivalent to those developed by the female venom. Signs associated with high mortality were as follows (mice with the sign/total mice, dead and surviving mice respectively): bradypnea/apnea (6/6 and

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<th>Table 1. Mortality frequency in C57BL/6 mice injected with female or male <em>Tityus nororientalis</em> venom.</th>
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Occurrence time for toxicity signs were as follows (in min, females/males): tremors and muscle fasciculations (21/18), hypotonic rear limbs (22.5/20), abundant sialorrhea (22/26), dyspnea with alternate episodes of bradypnea and apnea (27.5/36); animals presenting bilateral exophthalmus (21/28) and spastic paralysis (39/44) always died (40/45), whereas those not presenting these signs survived. Although there is tendency in female-envenomed mice to manifest earlier signs of toxicity compared to male-envenomed animals, no statistically significant difference was found between their times of occurrence. In general, sialorrhea and dyspnea (bradypnea/apnea) occurred significantly (Kruskal-Wallis = 6.22; \( p = 0.04 \)) earlier in those mice which died after injection of either female or male venoms, compared with surviving mice.

Figure 4 presents photographs recording toxicity signs in envenomed mice such as toxic facies, sialorrhea, ocular secretion, dorsal skin contracture, Straub sign, convulsions, and ataxic movements. Between 10 and 20min, ocular secretion, dorsal skin contracture, Straub sign, convulsions, and ataxic movements were observed. Beyond the 20min time point, which corresponds to the late phase of toxicity, differences were noted in the chronology of signs in mice injected with female and male scorpion venoms. Occurrence time for toxicity signs were as follows (in min, females/males): tremors and muscle fasciculations (21/18), hypotonic rear limbs (22.5/20), abundant sialorrhea (22/26), dyspnea with alternate episodes of bradypnea and apnea (27.5/36); animals presenting bilateral exophthalmus (21/28) and spastic paralysis (39/44) always died (40/45), whereas those not presenting these signs survived. Although there is tendency in female-envenomed mice to manifest earlier signs of toxicity compared to male-envenomed animals, no statistically significant difference was found between their times of occurrence. In general, sialorrhea and dyspnea (bradypnea/apnea) occurred significantly (Kruskal-Wallis = 6.22; \( p = 0.04 \)) earlier in those mice which died after injection of either female or male venoms, compared with surviving mice.

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Table 2. Frequency of clinical manifestations induced by *Tityus nororientalis* female and male venom in C57BL/6 mice.
secretion, pasty defecation, Straub sign, convulsions, and hypotonic rear limbs.

**Mass spectrometry and electrophoretic analyses of scorpion venoms**

Figure 5 shows the results of compositional analysis of female and male *T. nororientalis* venoms using mass spectrometry via MALDI-TOF in the 5500-8000 Da range, which corresponds to the molecular mass range of long-chain neurotoxins responsible for the lethality to vertebrates and insects of buthid scorpion venoms (Rodríguez de la Vega and Possani, 2005). Overall, mass spectra are similar in females and males although differences are apparent in the lower mass range. Particularly, components (in Da) 1 (6100.00), 2 (6362.403), 3 (6502.00), and 4 (6710.00) are present almost exclusively in female venom. Component corresponding to peak 5 (6863.74) is present in both sexes but it is approximately 2-fold more abundant in the female venom. Inset of Figure 5 presents a representative polyacrylamide gel electrophoresis performed in the presence of acetic acid and urea (Wang et al, 1997) which has been shown to improve resolution of cationic components in scorpion venoms which bear similar masses (Borges et al, 2006a). Two components out of 14 bands...
stained with Coomassie Blue are shown to be female-specific components.

**Venom immunological reactivity**

A comparison of the ability of female and male *T. nororientalis* venoms to inhibit binding of anti-*T. discrepans* antibodies to immobilized *T. discrepans* venom as a measure of their cross-reactivity is presented in Figure 6. Horse anti-*T. discrepans* antivenom is the only antiscorpion antivenom commercially available in Venezuela to treat accidents in the country (Borges et al., 2008). By increasing protein concentration from 1 to 10 000 ng/ml both female and male venoms were able to reduce the amount of free antibodies available for binding to *T. discrepans* venom immobilized.

**Figure 5.** Compositional analyses of female and male *Tityus nororientalis* venoms. Top panel, Mass spectral (MALDI-TOF) analysis of female venom; numbers indicate female-specific venom components or components present at higher abundance in females. Y-axis corresponds to Arbitrary Intensity Units (x10⁻³); X axis corresponds to molecular mass (m/z, in Da). Bottom panel, Mass spectral analysis of male venom. Inset, Urea-acetic acid gel electrophoresis (12.5%, w/v, gel) of 15 µg venom protein from female and male *T. nororientalis* specimens. Arrows indicate female-specific venom components.

**Figure 6.** Differences in immunological reactivity between *Tityus nororientalis* female and male venoms. Microplates were coated with *T. discrepans* venom (100 ng/well) and binding inhibition of anti-*Tityus discrepans* antibodies was measured after incubation with *T. nororientalis* venoms as described in Materials and Methods.
The results obtained in this work demonstrate intersexual differences in composition, toxicity and antigenicity in the venom produced by *T. nororientalis*, a scorpion species endemic to the Venezuelan northeast and responsible annually for severe envenoming cases in the area (Borges and De Sousa, 2006; De Sousa and Borges, 2009; Borges et al., 2010b). This species exhibits a marked sexual dimorphism, particularly in the length and shape of metasomal segments I to V (González-Sponga, 1996). These morphological differences are not sufficient to explain the toxicological variations reported in this work, with females producing significantly less venom than males. Such variation in *T. nororientalis* is opposite to that found in the South American spider *Phoneutria nigriventer* where the greater venom production by females is attributed to their larger body size (Herzig et al., 2002). Venom toxicity is, however, significantly higher in *T. nororientalis* females compared to males, similarly to what has been observed in *P. nigriventer* by Herzig et al. (2002).

Inspection of female and male venom composition using MALDI-TOF also indicated differences in abundance as well as the presence of female-specific venom components. Such differences may account, in part at least, for the higher toxicity of female *T. nororientalis* venom in C57BL/6 mice, although this requires further investigation. Similar results have been obtained with other arachnids, showing intersexual differences in the abundance and specificity of venom components (Binford, 2001; Herzig and Hodgson, 2009).

In the case of scorpions, Abdel-Rahman et al. (2009) have already shown that female specimens of the Egyptian *Scorpio maurus palmatus*, a species of poor medical importance, produce a significantly more complex venom than males. Such variation in *T. nororientalis* is ascribable to the sexual dimorphism, particularly for severe envenoming cases in the area (Borges and De Sousa, 2006; De Sousa and Borges, 2009; Borges et al., 2010b). This species exhibits a marked sexual dimorphism, particularly in the length and shape of metasomal segments I to V (González-Sponga, 1996). These morphological differences are not sufficient to explain the toxicological variations reported in this work, with females producing significantly less venom than males. Such variation in *T. nororientalis* is opposite to that found in the South American spider *Phoneutria nigriventer* where the greater venom production by females is attributed to their larger body size (Herzig et al., 2002). Venom toxicity is, however, significantly higher in *T. nororientalis* females compared to males, similarly to what has been observed in *P. nigriventer* by Herzig et al. (2002).

Interestingly, female *T. nororientalis* venom was significantly less reactive towards anti-*T. discrepans* antibodies compared to male venom. In females, 7-fold more venom protein (compared to *T. discrepans*) was needed to produce 50% inhibition of antibody binding to immobilized *T. discrepans* compared to 2-fold in the case of males. This can be taken to indicate differences in the bioactive surface of female *T. nororientalis* toxins involved in antibody binding and/or target receptors. It would be of interest to determine whether these differences in toxicity and antigenicity can also be found in other *Tityus* venoms, particularly *T. discrepans*. Antivenom preparation could benefit from these findings as to compensate for such intersexual variations when milking scorpion venom for immunization.

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CONCLUSIONS

Intersexual variations were found in venom production, toxicity and antigenicity in the Venezuelan scorpion species, *T. norientalis*, suggesting the existence of sex-related differences in venom composition and activity.

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STATEMENT OF COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

♀; female
♂; male
Da; Daltons
ELISA; Enzyme-linked immunosorbent assay
MALDI-TOF; Matrix-Assisted laser desorption time-of-flight mass spectrometry
LD₅₀; Medium lethal dose

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