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Venom metering in scorpions under controlled threat levels

Dissertação de Mestrado em Ecologia,
orientada pelo Professor Doutor José Paulo Sousa e coorientada pelo Doutor Arie van der Meijden
e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de mestre em Ecologia, realizada sob a orientação científica do Professor Doutor José Paulo Sousa e coorientação do Doutor Arie van der Meijden.

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Abstract

Scorpion venom is a complex mixture of toxins. A lot of work is being done on the biochemical properties of the scorpion venom and not that much of work has yet been performed on the biomechanical aspects of scorpion venom delivery.

The amount of venom released during different stings is known to be different. Scorpions, just like snakes and spiders, have the ability to meter the amount of venom they want to expel. The main reason why venom is metered is that its production is an expensive metabolic investment. In snakes, metabolic rate can be increased up to 11% during venom regeneration, and in scorpions it gets even higher, up to 39%, for milked specimens compared to the unmilked ones.

Many factors can influence the amount of venom scorpions release. Factors like the amount of total venom present in the scorpion's venom glands at that moment and the perceived level of threat to the scorpion. The level of threat is the factor that was manipulated in this study in order to test its influence on the volume of released venom. *Hadrurus arizonensis*, a species of big desert scorpion, was used in this study.

Our results show that during ten consecutive attacks to which scorpions were exposed in rapid succession, venom volume released on each attack is being controlled by the scorpion, changing the amount of released venom between the attacks. Overall, the amount of venom released on consecutive attacks decreases, but there is high inter-individual variability.

The number of dry stings, where no venom is released whatsoever, increases with the number of consecutive threats, and also varies by individual.

In conclusion, results from the study do support the first hypothesis which defends that scorpions are able to meter the amount of venom that they expel during a sting. However the second hypothesis was rejected, since it was expected that larger quantities of venom would be released as the level of threat increased and precisely the opposite was observed,

Keywords: scorpions; venom; metabolic rate; *Hadrurus arizonensis;* threat level; venom volume

Resumo

O veneno de escorpião é uma complexa mistura de toxinas. Grande parte do trabalho que está a ser desenvolvido com este animal foca-se nas propriedades bioquímicas do veneno deste organismo, fugindo dos aspectos biomecânicos de injeção desse mesmo veneno.

A quantidade de veneno que é libertada em diferentes picadas não se mantém constante e, tal como cobras e aranhas, os escorpiões possuem a capacidade de alterar e medir a quantidade de veneno que expulsam. A principal razão deste controlo, prende-se com a carga metabólica associada á produção desta mistura de toxinas. No caso das cobras, durante a regeneração do veneno, a taxa metabólica pode aumentar até 11%. Nos escorpiões o aumento pode ser ainda maior, atingindo os 39%, quando comparado com indivíduos não sujeitos a extração de veneno.

São vários os fatores que influenciam a quantidade de veneno expelida pelos escorpiões, desde a quantidade total de veneno disponível nas glândulas de veneno ao nível de perigo a que o escorpião está sujeito. Neste estudo, o nível de perigo foi o fator manipulado, de modo a testar sua influência na quantidade de veneno expelido pelos escorpiões *Hadrurus arizonensis*, espécie utilizada neste projeto.

Os resultados deste estudo demonstram que, ao longo de dez ataques consecutivos a que os escorpiões foram submetidos, a quantidade de veneno libertado foi controlada pelo organismo, havendo variação do volume expelido entre os diferentes ataques. De um modo geral, a quantidade de veneno

libertado foi reduzindo ao longo de ataques consecutivos, observando-se, no entanto, uma grande variabilidade a nível individual.

O número de picadas, durante as quais não foi observada libertação do veneno, aumentou com número dos ataques consecutivos a que os individuos foram submetidos.

Em conclusão, os resultados deste estudo confirmam a primeira hipótese de os escorpiões possuírem a capacidade de controlar a quantidade de veneno injetado durante uma picada. No entanto, a segunda hípotese foi rejeitada, pois era esperado que maiores quantidades de veneno fossem libertadas à medida que o nível de ameaça aumentasse, no entanto, foi observado precisamente o oposto.

Palavras-chave: escorpiões; veneno; taxa metabólica; *Hadrurus arizonensis;* nível de ameaça; volume de veneno

I. Introduction

1. Venom

1.1 What is venom?

Venoms are complex mixtures of neurotoxins, proteins, salts, water and some small molecules [1], [2], that are used by a large variety of organisms. They can be used for prey capture, in defensive behavior or for intraspecific competition [3]–[5].

This definition of venom can also be applied to toxungens and poisons, so what is the difference between these three mixtures? The difference resides on the methodology used to deliver it. Toxungens for instance are applied to the outside of the body of the victim without provoking any physical injury to it. Poisons are delivered by a passive strategy in which the target organism will enter in contact with the poison by itself. Poisonous organisms still might have some basic adaptations that facilitate them in poison delivery. One of the ways to do that, is to secrete a toxic mixture through the skin when being threatened [6]–[8].

The delivery of the venom resides in a specialized system or device that will inject the mixture inside the victims' body via fangs, stinger or other specialized mechanisms like harpoons, beaks, etc. [9]. This avoids any unnecessary extended physical contact with the target organism [10], [11]. This method of venom delivery predicates the creation of some kind of wound/mechanical injury to the victims' body [6], [7].

1.2 Venomous organisms

Worldwide, people encounter various organisms capable of producing venom [1], [2]. The most commonly known venomous organisms are snakes, spiders, scorpions, bees and ants. Venom is used by many species of snakes, scorpions, spiders, centipedes, jellyfish, lizards, cephalopods, fish and even some mammals like the short-tailed shrew [2], [4], [9]. But it is not only used by animals, some bacteria, fungi, protists and even some plants managed to evolve venom as a survival tool [6], [7]. Venom is a very useful tool that some organisms have at their disposal, helping them to subdue a more difficult/agitated prey [12].

Snakes are one of the most studied venomous organisms and over the years, a great attention has been given to the amount of venom that snakes release during a strike, and how it can be controlled by the animal itself. Snakes inject their venom into the target's body thought a pair of hollow fangs [13], [14]. The snake's venom-delivery system is composed of venom glands that are linked by two venom ducts to two fangs. The fangs position on the jaws is not always the same, depending of the species lineage, fangs can be located on the front of the upper jaw, like it is observed in vipers or cobras, or they can be rear fangs like in grass snakes. There is also a considerable difference in shape between anterior and posterior fangs. Anterior fangs are tubular shaped, while rear fangs never have a tubular shape, and can be slightly or deeply grooved [15], [16]. There are some species that have a posterior tooth that is not differentiated, so they are named 'non-fanged' [16].

This venom-delivery system has evolved to inject large amounts of venom in extremely short periods. The amount that is expelled is dependent on the species, on the individual and external stimuli [17]–[19].

Two groups of arachnids also known for their potent venoms are spiders and scorpions. Despite most of the spiders being predators, not all of them have venom at their disposal. Hackled orbweavers (Uloboridae) and some species of primitive mesothelids are the exceptions that have no venom glands [20].

Spider venom is used mostly for predation, with the goal of killing or paralyzing the prey. To deliver the venom, spiders have a pair of fangs that, just like in snakes, inject venom into the target's body. Fangs are used as a mechanical device to provoke a physical injury to the victim. Inside each fang is located one venom duct thought which the venom is expelled. Venom is produced in two venom glands, which can be located in two different places. In case of mygalomorphs, venom glands can be found in the fang base, while for araneomorphs, it is located in the anterior part of the cephalothorax. The use of venom is carefully regulated, and only the amount that is needed to subdue the prey is used [20].

1.3 Venom optimization hypothesis

Despite being an extraordinary tool, venom is used carefully, in order to save it as much as possible. This happens because venom production is an expensive metabolic investment. For predation purposes, venomous organisms should meter the amount the venom they want to expel, so they do not inject more venom than is necessary and thus avoid investing energy in venom regeneration. However, if they inject less venom into the prey's body, they might

be risking the prey fleeing. For defensive usage, the amount of venom used is as important as for predation. During defensive maneuvers, scorpions should be able to inject enough venom to escape the predator and not be killed. But it remains important not to inject less nor much more venom than is needed.

Despite representing only about 0,5% of body weight in snakes [3], [5], [21], [22], scorpions [3], [23] and spiders [24], the metabolic cost of venom regeneration can be significant. For instance, in snakes, metabolic rate had an increase of 11% for the 3 days after milking compared to the control group, where no venom was extracted [25]. Similar studies show the increase in metabolic rate in scorpions to be even higher (see below).

Some studies involving *Cupiennius salei,* a neotropical wandering spider, show that venom is being managed and being used only in more difficult to handle preys [3]–[5].

Pandinus imperator, the emperor scorpion, is known to change stinger usage as they grow older [3], [26]. Casper (1985) observed that young scorpions did use their stinger two or three times when subduing a prey cricket. This stinger usage was getting rarer with time, and disappeared when they got to 6-8cm long (*P. imperator* can grow up to 17cm long). As they get bigger, they get powerful enough to use only pedipalps for predation, almost never using stinger while adults [26].

A study done in 2007 by Nisani et al. used the desert grassland scorpion, *Parabuthus transvaalicus* to find out how metabolically expensive venom regeneration is. Three days after milking scorpions, metabolic rate and thus far regenerated venom were analyzed. Metabolic rate in milked scorpions was in about 39% higher than in unmilked scorpions, and it did not return to baseline

after 72 h. Three days after first milking, scorpions were milked again, in order to compare venom compositions. Venom from the first milking had approximately four-fold higher protein content then from the second milking. Therefore, after a period of 72 h the venom regeneration was not still complete. Authors of the study do caution that cost of venom regeneration during “normal” conditions, when scorpions release venom by itself, might be smaller since scorpions do expel much smaller amounts of venom during natural behavior [27].

2. Scorpions

Scorpions, (Arthropoda; Chelicerata; Arachnida; Scorpiones) are small to medium sized chelicerate arthropods, that can be found in most different terrestrial habitats, occurring from temperate forest to deserts and tropical forests [28]–[30].

There are currently known about 2000 scorpion species [31], all of the species are known to be venomous. However, only about 50 species have venom potent enough to be considered dangerous to humans. Young and elderly people are more susceptible to die from a scorpion sting. In general, most scorpions are not able to kill a healthy human adult, either for not delivering enough venom, or venom not being strong enough. Medically significant scorpion species are almost all from the family Buthidae [29], [32].

Scorpions are the arachnids that are responsible for more cases of the envenomation in the world. In parts of South America, the Middle East, Asia,

Northern and Southern Africa scorpions cause significant morbidity and pediatric death [32]–[34].

Scorpions have inhabited the Earth since the Silurian period, and it is thought that at that time the venom glands were already present. Although already 420 million years have passed, scorpions have not changed very much in appearance [2], [31].

All scorpions fluoresce in the visible spectrum (400-700 nm) when irradiated by UV light [35]–[38]. It was first reported by Pavan and Vachon in 1954 and by Lawrence in the same year [36]. This fluorescence is developed with hardening of the scorpions cuticle; newly molted scorpions does not have this ability [35], [36].

Scorpions have an exoskeleton, which protects their internal physiology from the environment [36]. The scorpion body can be divided in two different parts, the cephalothorax (head and thorax) also named as prosoma, and the opisthosoma. On the prosoma two remarkable structures can be noted, the pedipalps, that carry the chelae or ‘pincers’. Depending on the species, they come with different shapes and sizes. The use of pedipalps can be quite different, ranging from defensive/predatory use, to digging, mating and even climbing [39].

The opisthosoma can be divided into twelve segments. The first seven form the mesosoma and the last five segments form the metasoma. At the end of the metasoma there is a structure named the telson that contains two venom

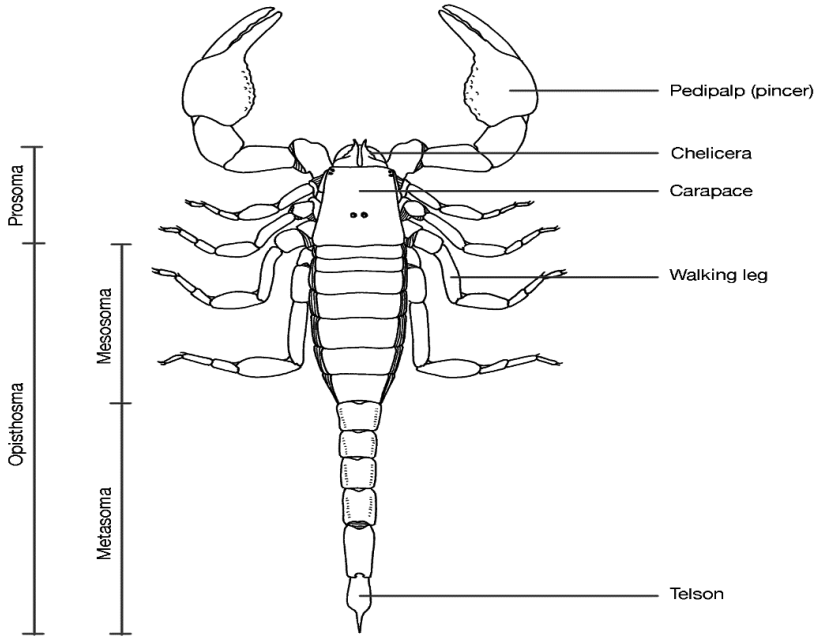

Figure 1. Scorpions morphology. From: https://www.vapaguide.info/page/24

glands and a sharp, needle-like stinger, also known as the aculeus (Figure 1). The metasoma can be curved over the scorpion's body, which happens for instance in defensive behavior or in need to sting a hard to handle prey [32].

2.1 A close look on telson

The telson is the terminal segment of the body, and it is where the venom is produced and stored. Venom production takes place in two venom glands [40], and during the expulsion, venom passes thought two separate venom ducts, through the aculeus, Figure 2 (venom ducts go further through the

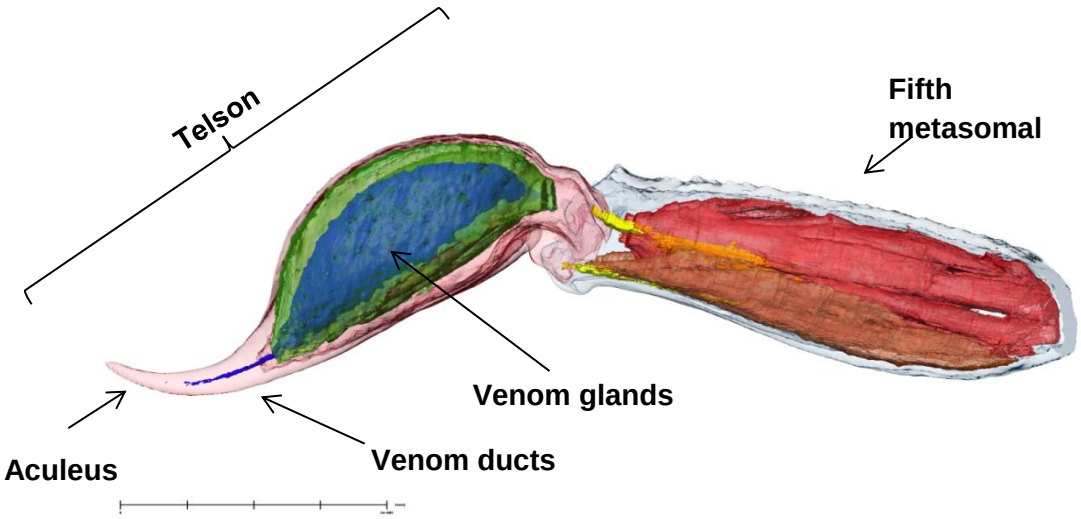

Figure 2. A rendering of µCT scan data of a scorpion's telson and fifth metasomal segment; Colored manually using Amira software. Venom gland lumen shown in blue, venom gland in green. The telson is rendered in transparent pink. The fifth metasomal segment is rendered in transparent blue, with the apodemes that attach the telsons to the abductor (red) and adductor (dark red) muscles in yellow. Scale bar is 5mm.

aculeus). Venom glands are surrounded by a layer of smooth muscle, that is responsible for the expulsion of the venom during the sting [41].

The size of the telson differs from species to species, and so does its use in defensive/offensive situations.

2.2 Prevenom, opalescent venom and viscous venom

Protein rich venom is an expensive metabolic investment. To not use this protein rich venom for all situations, scorpions are able to produce different types of venom: transparent venom, known as prevenom, opalescent venom and viscous venom, also known as milky venom. There are some differences in physical properties in these venom types. The dry mass of prevenom can have up to 80% of K^+ salt concentration and only about 10% of proteins, while milky venom can have up to 60% of protein content, and only 5% of K^+ [1].

Prevenom is a pain inducing venom due to the high K^+ concentration, so it is very useful during a defensive maneuver and it does work fine against insects, which are the main prey of scorpions. If use of the prevenom turns out to be not enough, more toxic venom will be injected afterwards. Opalescent and milky venoms are considerably more metabolically expensive, so they are used after the exhaustion of the prevenom [1], [42].

2.3 Hadrurus arizonensis

Hadrurus arizonensis is the largest scorpion found in North American deserts, and it can get up to 127mm in length [43]. But despise its size, its venom is not very potent, so this species is not considered to be dangerous to humans, with exceptions of hypersensitive persons [44]. These two factors, the size and not lethal venom, were the main reasons to choose *Hadrurus arizonensis* to be used in this master's project.

3. Previously used methods of venom quantification

In 1999, Malli et al. performed an experiment with the Tiger wandering spider, *Cupiennius salei.* It consisted of changing the intensity of the struggle of the crickets, which were used as preys. The purpose of this experiment was to see how much venom spiders would expel in response to the “fight” that crickets would give. To achieve that, one of the most common methods of venom quantification was used, Enzyme-Linked Immunosorbent Assay (ELISA). This method consists of linking monoclonal antibodies to certain venom compounds. With this method they were able to find that spiders actually injected more venom into crickets that were fighting in a more intense way [27], [45].

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MST) does allow the identification of individual venom constituents, and makes it possible to see how far certain venom compounds have penetrated into the target’s body [13].

Venom expenditure has also been measured by weighing. With this method it is possible to measure the amount of venom expelled by scorpion for predatory and defensive stings. It simply consists in comparing the weight of the scorpion or the prey, before and after venom expulsion [27].

Labeling the venom radioactively allows it to be quantified inside the prey, however it is not used nowadays due to ethical regulations and concerns [46].

An ultrasound perivascular flow probe can give a precise values for venom flow, speed and volume of the expelled venom, but it requires invasive surgery on the animal [47].

4. Project

4.1 Previous work

All of the early experiment planning was actually based on a previously

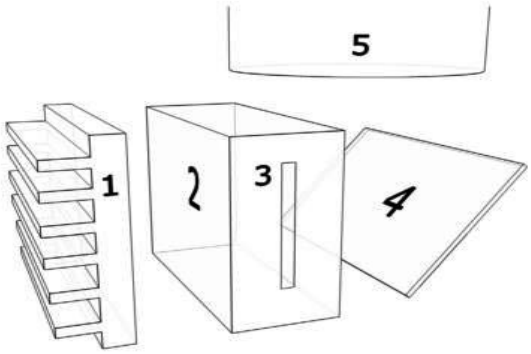

Figure 3. Schematic of venom metering setup. From: [48]; 1. LED array providing back illumination; 2. Glass chamber filled with mineral oil; 3. Metal plate with slit covered by parafilm. This is where scorpion is allowed to sting; 4. Single layer mirror; 5. Dissecting microscope with high speed video camera mounted (only lower part shown).

published paper [48]. The previous setup for venom metering allowed us to collect good data and analyze it to get some interesting results. Figure 3 shows us how it looked. Between the metal plate and the glass chamber, a parafilm membrane was stretched. This parafilm membrane had two different purposes; it held the mineral oil inside the glass chamber, and it provided a penetrable

surface for the scorpion to perforate. Before finding the slit in the metal plate, scorpions did some attempts, scraping on the plate, but no venom expulsion was observed in any of those attempts. The parafilm perforation and venom accretion into the oil were filmed with high speed video camera mounted on a dissecting microscope (5).

For this preceding work, five different scorpion species were used, and 64 events analyzed. Some interesting results came from it, providing us the confidence to proceed with this project; It was possible to see that the same specimen could actually expel different venom volumes within different stings. It did prove once more that the amount of venom that they release is not fixed and is being controlled by scorpion, which may be influenced by some external stimuli [42], [49]. For every single specimen, the procedure consisted of grabbing it with forceps by the fourth or fifth metasomal segment and placing the telson near a metal plate. Holding the telson near metal plate, would allow the scorpion to search for a spot to perforate, and after perforating the parafilm membrane, venom would start to be injected. The parafilm piercing and the venom accretion were then recorded using a high speed video camera (FasTec Imaging, San Diego, CA, USA) linked to an Olympus SZX16 microscope [48].

Not all specimens would sting immediately, and in those cases, those specimens were held by a pedipalp with another pair of forceps. Of course doing that, the level of threat that specimens perceive would be different, and that could explain the difference in venom volumes by them released in different stings. Unfortunately, no association could be made between venom volume and handling intensity, since no notes were taken of which scorpions received the additional pedipalp stimulation, and which did not. This made it clear that an

experiment was needed in which threat level was systematically applied, and the amount of expelled venom recorded.

5. Experimental methods developed

Due to the experimental nature of the approach, we needed to develop the equipment necessary during the project. First, a mobile setup was created that would allow us to record venom expulsion as in Van der Meijden et al. [48]. However, due to difficulties in obtaining sufficient observations with this method, we finally opted for a simpler system. For completeness, I will first explain the mobile setup we developed, and the simpler method that was finally used will be explained in the materials and method (section 2).

5.1 Mobile video microscope setup

The experimental setup shown in Figure 3 was used for the venom metering, but it did have some problems. One of the major problems is that it was a fixed setup, with no possibilities to move it as freely as needed for this masters project.

To make a similar mobile setup, a relatively small metal plate, 20,5cm x 13cm, was arranged in such way, it would be possible to fix the camera, the oil chamber and a small LED to it, as shown in Figure 4.

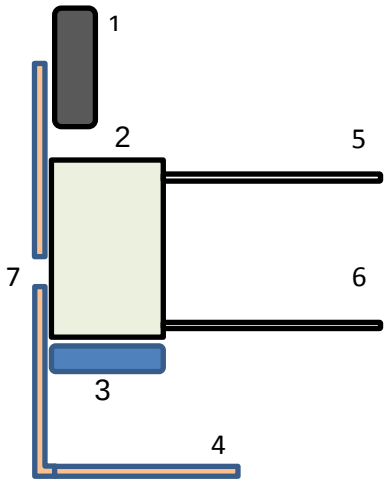

Figure 4. Shematic of new experimental setup for venom metering. 1. Microscope/Camera; 2. Oil chamber; 3. LED; 4. Metal plate; 5. Tube for air expulsion; 6. Tube for oil injection/removal; 7. Narrow slit

5.2 Camera positioning

Another big issue with the experimental setup was the positioning of the camera. As realized during the video analysis in the preliminary work, a side view is not the best view for digitizing droplet size, since scorpions would release two droplets of venom during the injection, one from each venom duct. In a large number of cases, these two droplets would merge into one, solving the problem, however if this fusion did not occur, we assumed that the second droplet (not visible on the video) would have same size as the visible one. In order to be able to see both droplets, it was decided to do the filming from the top (Figure 4) and not from the side as in the previous experiment.

5.3 Glass chamber

With the previous setup, the chamber removal could get a little bit messy, so when building another chamber, it was decided to incorporate two tubes into it as shown in Figure 4, marked with numbers 5 and 6. The upper tube, (5), used for air removal from the chamber during its filling, and tube at the bottom, (6), had the purpose of injecting and withdrawing the oil from the oil chamber. To facilitate the contact between the metal plate and oil chamber, a layer of silicone needed to be applied over the limits of the chamber that would be in contact with the plate. The use of silicone would help us to avoid the oil spilling, over the setup. A parafilm membrane placed between oil chamber and the metal plate, would have same purpose as in previous work: giving something for the scorpion to perforate.

5.4 Mobile setup

After two months designing and building the setup, the result is shown on Figure 5. When finished, this setup was used for practice with some *H. arizonensis* specimens that would not be used in my masters' project. Doing it, did allow to realize that this experimental setup could not be used for the project. Some specimens, while looking for a spot to sting, did perform various searching attempts on the metal plate, this search in most of the times did end with scorpion simply giving up from that, or getting away from the setup.

Sometimes, instead of injecting the venom thought the slit, some specimens did expel venom on the metal plate itself, as it is shown on Figure 6, behavior not observed while doing the practical work for the previous venom metering project [48].

Figure 5. Venom droplets circled on the metal plate.

Photo: Mykola Rasko

After the first behavioral trials, it was decided not to use this method for venom quantification in this masters project due to some difficulties. There were two major problems. First was the time spent on acquiring a good sting in which a scorpion would actually sting through the slit in the metal plate. The second problem was that some of the specimens would just release venom on the metal plate. This would not allow us to measure these expelled quantities. If this approach would be used, the amount of data available at the end to analyze would be quite small. So we decided to change the method in a way that we still

could get information about scorpion stings, but in a short period of time (three to four months). The methodology to be used will be explained in detail below.

6. Changes in the masters project

At the beginning, this project relied on the methodology in [48].Two months were spent on building the necessary setup, but after it was finished, when done some trials with live scorpions, we did realize that it would be difficult to collect a good amount of data. We decided that the venoms expulsion would not be filmed, and I would only focus on the volume, leaving out the velocity, acceleration of the expulsion and so on. The venom would still be collected into the oil and itsvolume measured shortly after (10-20min).

Instead of provoking the scorpions by different methods and seeing if venom by them expelled will be in different quantities, each specimen was teased for ten times consecutively. The way that scorpion threatening was done and how the rest of the project was carried out, is explained in more detail below.

6.1 Goal of the project

The goal of this project was measuring the amount of venom that scorpions would expel under different levels of perceived threat. Arie et. al., 2015, did stated that the venom released by scorpions in different stings can be expelled in different volumes, considering different stimuli that affect the scorpion at that moment. During this project, scorpions will be submitted to

different levels of threat in order to verify how does the expelled venom volumes change though different levels. Two initial hypothesis were presented.

The first one states that scorpions were expected to meter the amount of venom they expel.

The second one defends that expelled venom volumes will increase as the perceived level of threat increases as well.

II. Materials and methods

Standardized trials were performed that stimulated defensive responses from the scorpion, and the volume of the ejected venom was quantified. In order to understand which fraction of the total available venom was ejected during each trial, in a second experiment the total amount of venom available to each scorpion was also measured using electrostimulation. In a third experiment, the relative viscosity of the prevenom and opaque venom was measured.

1. Specimen selection and husbandry

Hadrurus arizonensis was the scorpion species selected. The ten specimens used were kept separately in plastic boxes. For the soil substrate granulate cork was used. Parts of paper egg boxes were provided as a shelter for the scorpions. Live specimens are kept in a climate-controlled compartment at 26ºC and with a 12 h photoperiod. All specimens were assigned identification numbers: Sc2829, Sc2830, Sc2831, Sc2832, Sc2833, Sc2834, Sc2835, Sc2935, Sc2936 and Sc2837.

Every two or three weeks, scorpions are fed with live crickets (*Acheta sp*.) but during the experiment those ten specimens selected were fed more often to avoid fatigue due to the demands of venom replenishment. A week before the experiments started, all specimens were fed equally. All of them got the same number of crickets, with relatively similar sizes, placed in their boxes.

Each week only five of those ten specimens were being used, which means these five specimens would get fatigued by end of the week due to (partial) venom depletion, Nisani et al.,(2007), have shown that venom replenishment requires a significant increase in metabolic rate. Therefore, every

week the specimens used for the experiment would receive one or two live crickets (depending of the size of the crickets available). To provide the scorpions with water, a piece of paper soaked in water was placed in a plastic tube lid, and left in a scorpion's box. Plastic lids with paper and cricket/s would be left in scorpion's box for the following weekend (for 2 days) and withdrawn right after (on Mondays).

2. Venom measuring procedures

To capture venom expelled during defensive behavior, scorpions were pressed to the substrate with a 30ml tube. This enticed the scorpion to sting into the tube. The venom was then removed from the tube and measured.

To facilitate the procedure, the venom volume measurement would not be made at the moment of its expulsion from the scorpion's telson. The venom would still be collected into the oil, and removed from it shortly after (10-20min). Immersion in oil minimizes the evaporation from these very small volumes, and facilitates their quantification (see chapter 2 from Materials and methods).

Instead of provoking the scorpions by different methods and seeing if venom expelled will be in different quantities, each specimen was challenged for ten times consecutively, hereafter called "attempts". A set of ten attempts was considered a trial. The way that this scorpion threatening was done and how the rest of the project carried out, is explained in more detail below. As mentioned before, out of ten selected specimens for the project, only five were being used each week. For that, a schedule was made, where I decided when each specimen should be used, Table I.

Table I. Scheduled showing which specimens were used in each week.

Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Sc2829	Sc2834	Sc2829	Sc2834	Sc2829	Sc2834	Sc2829	Sc2834
Sc2830	Sc2835	Sc2830	Sc2835	Sc2830	Sc2835	Sc2830	Sc2835
Sc2831	Sc2936	Sc2831	Sc2936	Sc2831	Sc2936	Sc2831	Sc2936
Sc2832	Sc2837	Sc2832	Sc2837	Sc2832	Sc2837	Sc2832	Sc2837
Sc2833	Sc2935	Sc2833	Sc2935	Sc2833	Sc2935	Sc2833	Sc2935

In the first week, specimens with Sc numbers from 2829 to 2833 were used, and in the second week these five specimens were resting, while the remaining five were being tested.

During the same week, the same five specimens were being provoked every day, and to avoid a possible habituation to the order, a simple randomization was made in Microsoft Excel.

The whole experiment lasted for eight weeks, so every specimen was used in a total of four weeks. On Mondays, no experiments were done with scorpions, but all the preparations were being made for the experiments for the rest of the week.

Ten jars were filled for about 1/3 with mineral oil. Each jar corresponds to one attempt. Lids from each jar were marked with numbers from one to ten, and were placed near the corresponding jar, so they would not get mixed during the experiments.

The scorpions were not injecting the venom directly into the jars with oil. Venom was being injected into 30ml tubes (Figure 7) which were then passed to the jars. Instead of lids, these tubes had material from white latex gloves stretched over the aperture in order to make scorpions sting thought it. The latex material was fixed to the tubes using plastic zip ties. It was important not

to stretch the latex to tight, so it would not just tear apart with the first scorpion stinging, and could allow scorpion to perform several stings per attempt.

For each attempt a jar with oil and a tube needed to be prepared, and every day, five scorpions were being used, for which ten tubes needed to be arranged (Figure 7).

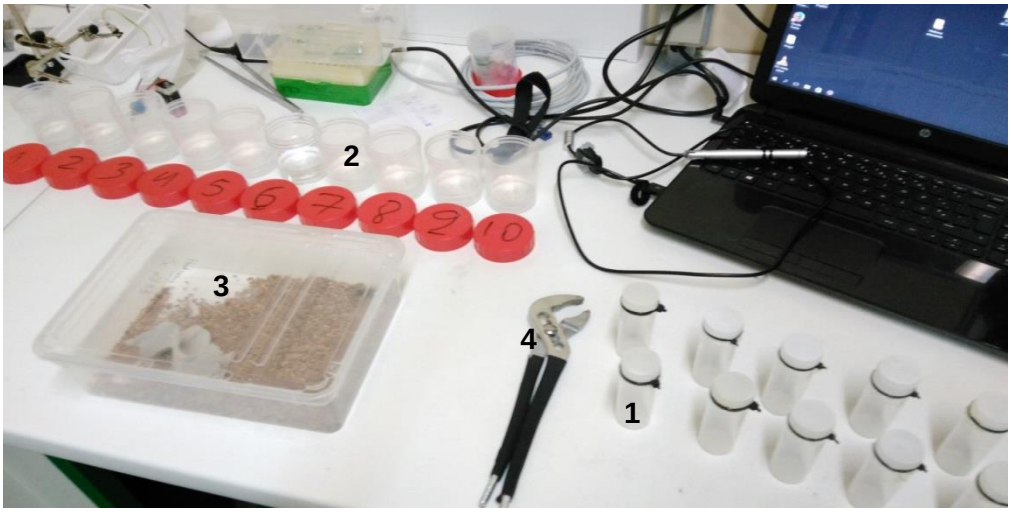

Figure 6. Desk arrangement during the experiments. **1. Tubes with latex membrane stretched over the top; 2. Oil jars; 3. Scorpion in box; 4. Forceps used to hold the tubes when presented to the scorpion. Photo: Mykola Rasko**

Each attempt consisted of placing the tube on top of the scorpion prosoma, to induce the scorpion to sting, which would not always happen. The force applied on the prosoma was not standardized, since it was being done by me and not by some electrical/mechanical device, able to apply always the same force. To monitor the time with precision, and make sure all the specimens did get an equal treatment, timed beeps were emitted by a nearby computer. These beeps were coded with in the Scratch software (MIT, Boston). Not only the time when tubes were being held on the scorpions' prosoma, but also time in between each attempt needed to be carefully controlled (**Error! Reference source not found.**8).

On Figure 8, are schematized the procedures performed on each attempt, and ten consecutive attempts needed to be performed for each scorpion, every day.

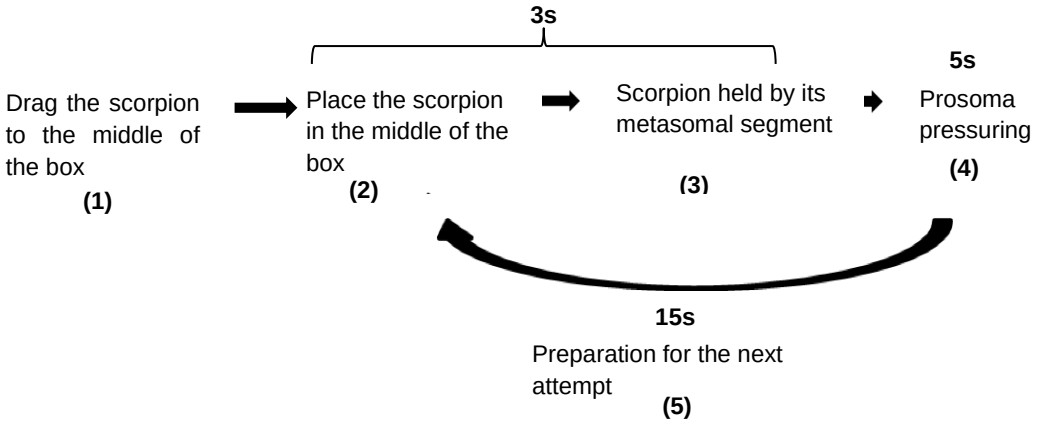

Figure 7. Schematic of procedures for one attempt (repeated ten times consecutively for each scorpion).

For each scorpion, the procedures performed were followed step by step, as they are listed below:

(1) - before performing the first attempt, scorpion needed to be grabbed by its metasomal segment (fourth or fifth), using rubber-tipped forceps, and dragged to the middle of the box and released right after. This procedure is not considered to be a part of the "attempt" procedures, it was necessary to put scorpions in a defensive posture;

(2) and (3) – first attempt begins right after step (1) is completed. Scorpion was once again grabbed by its metasomal segment and dragged to the middle of the box. This time it was not released and remained held until

three seconds had passed (Figure 8). Steps (2) and (3) were essential for the scorpion contention, and preparation for the next step;

(4) – with scorpion contained, the tube was carefully placed on the scorpion's prosoma. Its metasoma, which was still being held with forceps, is released in order to allow scorpion to sting (if willing to). To make sure that scorpion was not able to flee, the tube needed to be pressed against the prosoma, and kept like that for five seconds. The scorpion could react by performing a stinging action, could try to flee, or could not react at all.

After placing the tube on scorpions' prosoma, in most of the times, scorpions did try to sting. When the stinger was touching the plastic material of the tube, scorpions would hold on the venom release. In most cases, it was the latex perforation that induced the venom expulsion.

This venom would remain either on the latex or the tube's walls. To isolate the venom, the tube was inserted and shaken inside the oil jar. This detached the venom droplets from the tube and/or latex. The venom droplets would accumulate on the bottom of the oil jar.

(5) – the tube remained on the scorpion's prosoma for five seconds, when finished, the current attempt was considered to be over, and the preparations for the next attempt were taking place for exact 15 seconds.

Afterwards, same procedures were performed for each one of the nine remaining attempts.

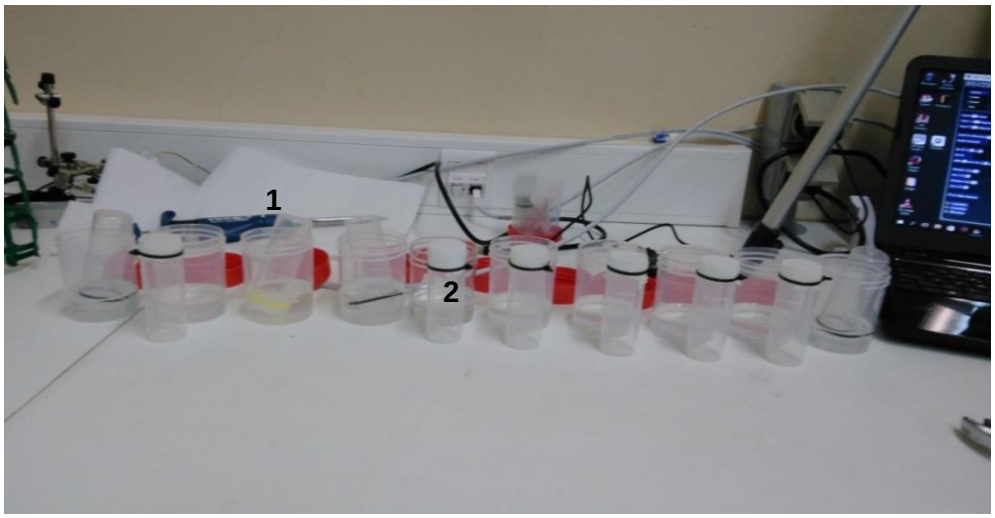

Figure 8. Oil jars and tubes after a threatening experiment. 1. Tube inside the oil jar; 2. Tube used during the experiment, but not placed inside the respective oil jar, since scorpion did not perform any sting. **Photo: Mykola Rasko**

Submerging the venom in oil ensured that the venom volume would not decrease due to evaporation, Figure 9. Being isolated in spherical droplets, venom could easily be withdrawn from the oil and then quantified. To do that, a

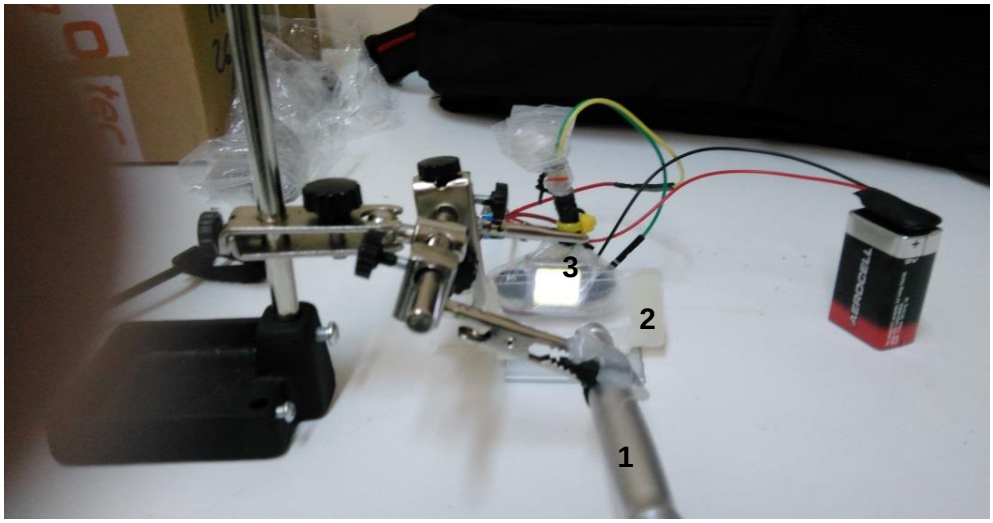

Figure 9. Venom measuring setup. 1. Microscope camera; 2. Oil chamber; 3. LED powered by 9V battery. **Photo: Mykola Rasko**

special setup was built (Figure 10).

Inside the jars with oil, venom did remain from 10 to 20 min, after which it

Figure 10. Image form a video made with microscope camera, where a droplet of venom (1) and a part of Pasteur pipette (2) are visible. Photo: Mykola Rasko

was withdrawn using a plastic Pasteur pipette. Quite often a dissecting microscope was necessary in order to spot venom droplets. Since the water-based venom is denser than the oil, the droplets would accumulate on the bottom of the jars. Inclining the jars made the droplets collect on one side of the jars, facilitating removal and ensuring that all droplets that were expelled, were also measured.

After extracting oil containing all venom droplets with a Pasteur pipette, venom then was transferred to the oil chamber. Inside of it, while sinking through the oil, the size of the droplets was recorded by a microscope camera connected to a laptop thought a USB cable. A LED was used to provide background illumination (see Figure 11).

In order to know how much volume that droplet represents, a calibration for the videos was made. To do that, a piece of millimeter paper was placed inside of the oil chamber, and a photograph of it taken. This calibration needed to be done every time oil chamber or the whole setup was moved, in order to have precise information when measuring the venom droplets.

Both the calibration and the video analysis were done in ImageJ [50]. Each video was analyzed, and a circle was drawn manually around each venom droplet. With the calibration of the video, it became possible to know the surface area of the drawn circle. This area then was used to calculate the radius and the volume of the droplet.

If more than one venom droplet was observed in the video, the volumes were summed.

3. Total venom volume quantification

The information about total amount of venom produced by each specimen is important to compare it to the amount of venom that each scorpion expels during strikes, giving the possibility to calculate the percentage of venom released during strikes. To get that total amount of venom, a few steps were taken to make the milking procedure safe for both the scorpion and the person extracting.

Scorpions needed to be completely still during this venom collection, in order to join a glass capillary tube to the animal's stinger, so the venom could directly pass from the stinger to the tube.

3.1 Capillary tube calibration

The amount of venom produced and stored by scorpions is extremely small, and capillary tubes allowed me to measure these small volumes. However, in order to be able to use the length of a column of venom inside a capillary tube as a measurement method, a calibration of those tubes needed to be performed.

This calibration was made by filling tubes with fixed water volumes, Table II. These volumes were controlled by using a micropipette. Water was pipetted onto a plasticized millimeter paper, and then absorbed by capillary tubes. Once water is inside the capillary tube, the length of the water column inside of the tube is measured to the nearest 0.01mm using digital calipers. Capillary tubes of 75mm/80µl (VITREX Medical, Herley, Denmark) or 75mm/18µl (HIRSCHMANN Laborgeräte GmbH, Eberstadt, Germany).

For each of four tubes a scatter plot was made, Figure 12. A regression line was added for each scatter plot and the slope (mean value of 1,041µl/mm) was used to convert venom column length to volume.

Table II. Volumes used for 75mm/80µl capillary tubes calibrations and the length it occupies inside the tubes, measured with calipers(mm) and millimeter paper(mm).

Tube 1		Tube 2		Tube 3		Tube 4	
volume(µl)	calipers(mm)	volume(µl)	calipers(mm)	volume(µl)	calipers(mm)	volume(µl)	calipers(mm)
1	1,07	1	1,25	1	1,11	1	1,07
5	4,6	5	5,07	5	5,12	5	5,15
10	9,47	10	10,03	10	10,07	10	10,54
20	18,22	20	19,37	20	19,01	20	19,96
30	28,87	30	29,26	30	28,49	30	29,87
40	37,21	40	38,79	40	37,95	40	39,75
50	46,61	50	48,6	50	46,86	50	49,39
60	55,97	60	58,46	60	56,32	60	59,43
70	65,19	70	68,13	70	65,86	70	69,84

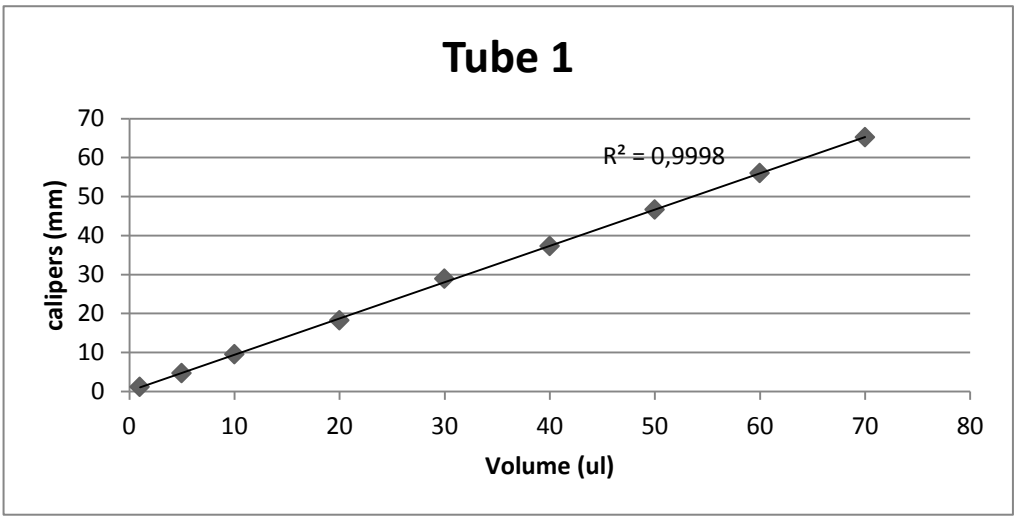

Figure 11. Scatter plot for tube 1; same procedure was made for three remaining tubes

For the second milking a different type of capillary tube (75mm/18µl) was used by mistake, so a new calibration needed to be made. The same procedures were used for the calibration of these capillary tubes, as for the previous ones. After obtaining four regression slopes, one for each tube, a mean was calculated, and obtained 0,2445µl/mm.

3.2 Anesthetizing

In order to be able to extract all of the venom contained in the scorpions' venom sacks, scorpions must be anesthetized. Despite *Hadrurus arizonensis* being adapted to survive in a very tough environment, the desert, it is still very delicate and can easily be injured if handled in a wrong way. Therefore, it must be anesthetized using isoflurane, which must be inhaled before it is restrained for venom extraction. A piece of paper was soaked in isoflurane and placed in a 50ml Falcon tube, which in it turn was placed in a transparent air-tight plastic

box together with the scorpion. The box was then tightly closed and remained that way, until scorpion stopped moving. Isoflurane does not appear to cause any direct negative or long term effect on the scorpion, however during this procedure scorpion might injure itself. Scorpion during undergoing anesthesia can perform several stinging attempts, in which it actually can injure itself (bleeding observed in some cases).

It is very important to perform electro stimulation right after anesthesia, since scorpion will start to wake up in a couple of minutes. Time spent unconscious will depend on how long they have been held in the box with isoflurane, specimen size, and possibly some other factors. Every specimen was monitored during anesthesia.

3.3 Electrostimulation

Electro stimulation was done right after the anesthesia performed on scorpions. Electro stimulation was used to extract the total amount of venom from the scorpions. Venom release was stimulated with a square wave with an amplitude of 18V, a frequency of 45Hz and a duty factor of 9% applied to the metasoma.

3.4 Total volumes calculations

Once having the scorpions' venom in the capillary tube, the length it occupies inside of the tube was measured and the volume calculated using the value obtained from the calibration curve.

Each specimen was milked three times. Once before the experiment had started, and twice after it was finished. Two specimens however were only milked twice, since it was decided to use them for the masters project right before the experimentation part has started (Sc2935 and Sc2936). The highest volumes were then selected for each specimen, and it would represent the total amount of volume that that specimen has.

Table III. Venom volume values from each specimen and specimens' prosoma lengths (mm).

	1st(µl)	2nd(µl)	3rd(µl)	Highest volume(µl)	Prosoma length(mm)
Sc 2829	22,90	15,28	18,74	22,90	9,8
Sc 2830	32,79	20,17	28,11	32,79	13,68
Sc 2831	19,78	8,31	18,22	19,78	11,93
Sc 2832	24,98	14,91	24,46	24,98	12,19
Sc 2833	30,19	26,40	24,98	30,19	13,46
Sc 2834	41,64	21,51	17,70	41,64	14,14
Sc 2835	15,62	20,90	8,33	20,90	11,8
Sc 2837	18,74	16,50	17,70	18,74	13,67
Sc2935	28,85	31,75		31,75	12,38
Sc2926	27,87	39,56		39,56	12,73

Prosoma length was measured for each specimen from digital photographs using the program ImageJ [50]. These measurements were used

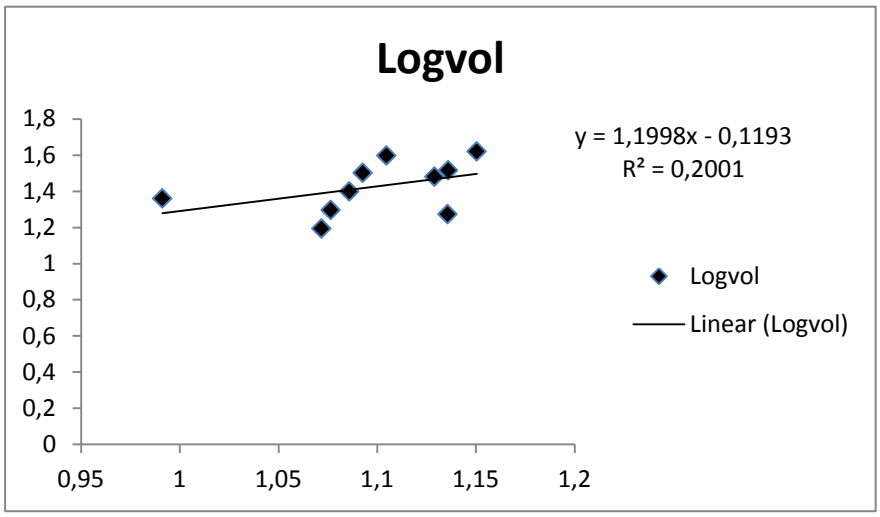

Figure 123. Linear regression of venom volume (vertical axis) against prosoma lenght (horizontal axis). Log10 transformed values were used

to verify if total venom volume is related to body size. All volumes and prosoma lengths are shown in Table III.

In order to linearly plot a length value with a volume value, the log10 transformed values were plotted. As it is possible to see in Figure 13, the R^2 is quite low, meaning that the amount of volume produced by a scorpion is not strongly related to its size.

3.5 Viscosity measurements

As mentioned in the introduction, there is a considerable difference in protein concentration between prevenom and milky venom. While extracting total venom from scorpions, was noted that sometimes, the prevenom and milky venom stayed separate inside the capillary tubes, so it was decided to perform a small side project, where we would test the viscosity of the extracted venom.

To achieve this goal some extracted, into capillary tubes, venom was selected. Only capillary tubes with venom were selected in which it was possible to distinguish between prevenom, opalescent and milky venom.

For the experiment, a FasTec Troubleshooter high speed video camera (FasTec Imaging, San Diego, CA, USA) was mounted on a Motic stereo microscope, SMZ-168 series, with a purpose of recording a Stainless Steel

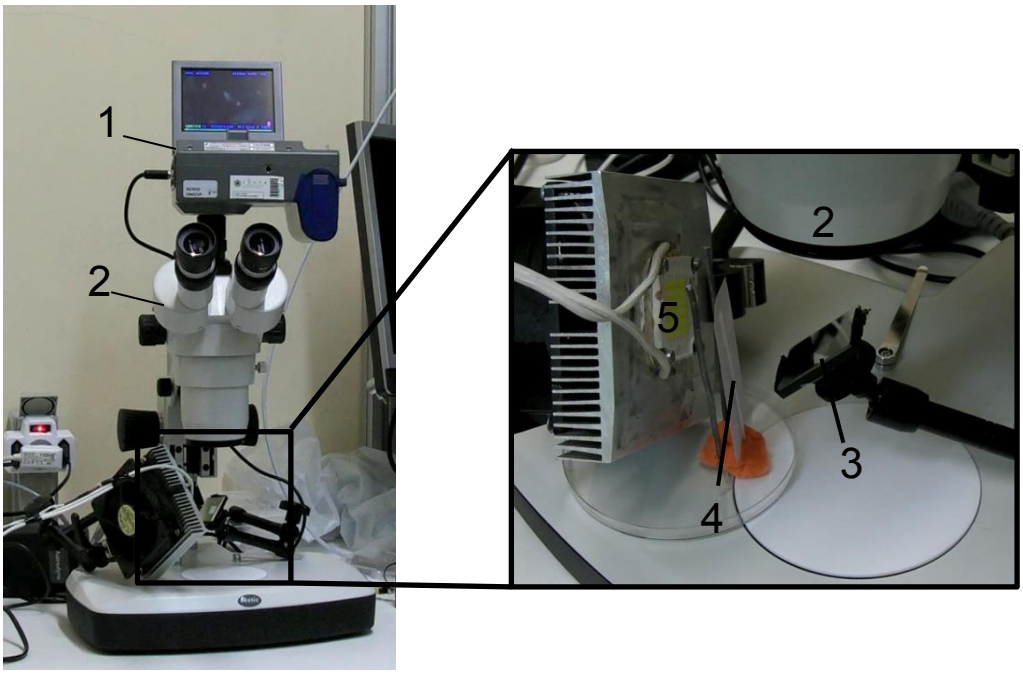

Figure 13. Experimental setup for venom's viscosity measurement. 1. High speed video camera; 2. Microscope; 3. Single layer mirror (providing side view of the capillary tubes); 4. Capillary tube containing scorpion's venom; 5. LED array providing back illumination. **Photo: Mykola Rasko**

Microsphere 92-110um (Cosperic LLC, Santa Barbara, CA USA) going down though the venom (Figure 15).

A video was being recorded, at ten frames per second, as soon as the metal bead started to go down through the venom. With a single layer mirror allowing the video recording from a side view, and a LED providing back illumination, it was possible to have a clear image of the metal sphere inside the capillary tube.

In order to have a control video, another video recording was made where the metal sphere was dropped into a capillary tube filled with water.

To calibrate the videos, behind each capillary tube, a slice of millimeter paper was placed.

Video analysis was made in ImageJ [50]. Frame by frame, a single dot was placed on the center of the metal sphere, and its “x/y” coordinates registered.

By knowing the coordinates of the metal sphere in each frame and applying the Pythagoras' theorem formula, it was possible to calculate the distance that the metal sphere “travelled” between each frame.

By knowing the total number of frames of the video, and the amount of time that took to metal sphere to travel from the top to the bottom of the capillary tube, it was possible to calculate how much time it is spent between each frame. Along with the distance information, it was possible to calculate the velocity and the acceleration of the metal sphere.

III. Results

In a period of two months, 107 trials were performed, and 1070 attempts registered. As explained before, some of the attempts needed to be considered as failed attempts, and out of 1070 attempts, 302 were considered failed. The rest of them, 768, were considered valid attempts. Included in valid attempts, there are attempts during which scorpions did sting, and the ones in which scorpions did not perform any stinging action. In about 209 attempts, scorpions did not do any stinging, which represent almost 1/3 of total valid attempts, Figure 16.

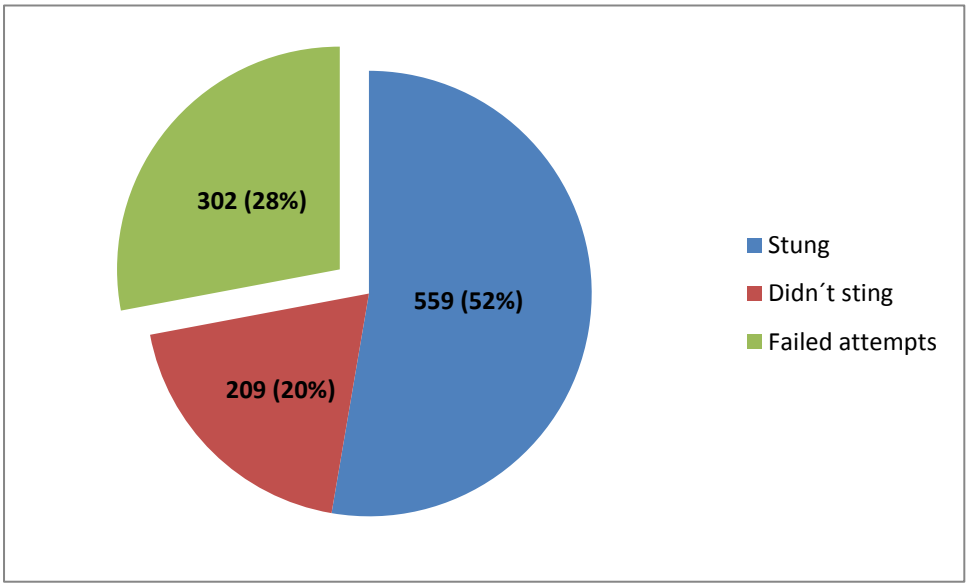

Figure 14. Diagram representing final results; showing the number of failed attempts (green) and valid attempts (blue and red), in a total of 1070 attempts performed during the experiment.

Out of 559 attempts in which scorpions did sting, 152 (28%) were considered as a dry sting, meaning that during those stings no venom was released (Table IV).

Table IV. Values on each specimen: total number of stings; trials performed; stings with venom released; dry stings and total amount of venom released during the experiment.

Individual	Stings	Trials	Sting with venom released	Dry stings	Total volume (µl)
Sc2829	60	12	39	21	12,39
Sc2830	31	10	10	21	4,51
Sc2831	66	11	55	11	9,46
Sc2832	41	10	33	8	22,16
Sc2833	39	7	24	15	3,05
Sc2834	71	12	43	28	7,76
Sc2835	61	10	48	13	18,35
Sc2936	58	12	33	25	3,59
Sc2837	74	12	69	5	40,25
Sc2935	58	11	53	5	65,07
Total	**559**	**107**	**407**	**152**	**186,59**

Table V. Values per attempt; stings; average venom volume released and standard deviation; number of dry stings and what fraction dry stings represent considering the total amount of stings

	Attempts									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sting	45	53	61	53	59	54	59	55	62	58
Average vol-attempt(µl)	0,94	0,43	0,62	0,31	0,23	0,13	0,20	0,16	0,19	0,24
Standart DV	1,73	0,66	1,81	0,56	0,6	0,22	0,34	0,38	0,52	0,80
Dry stings	7	6	14	5	18	18	17	20	19	28
Fraction dry stings	0,16	0,11	0,23	0,09	0,31	0,33	0,29	0,36	0,31	0,48

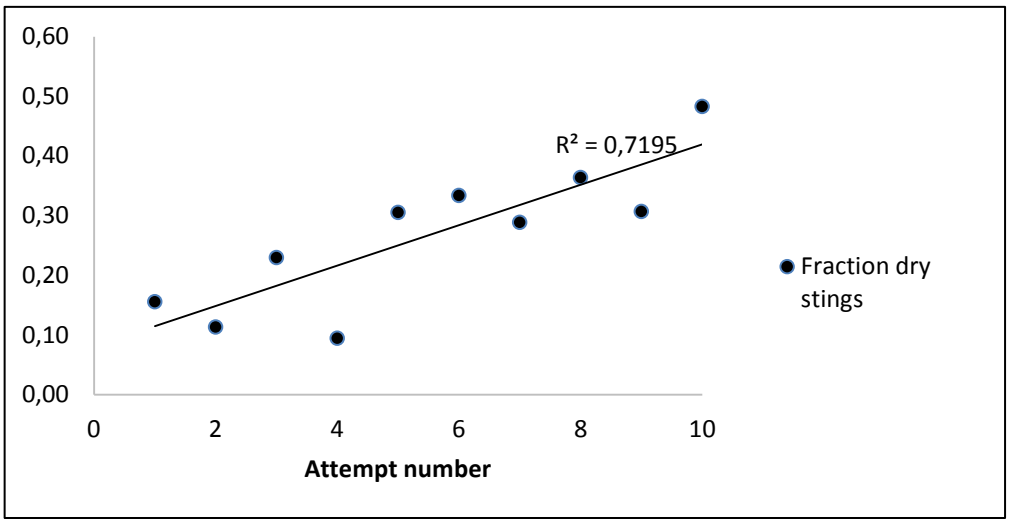

Figure 15. Scatter plot ; fraction of dry stings per attempt.

As can be seen in Figure 17, the number of dry stings increases with the attempt number.

In Table V, the values for the average venom volume per attempt do show quite a difference between the venom volumes released at first attempts and the last ones.

Figure 18 shows the venom volume released in each attempt. The average venom volume released appears to be going down, as the number of attempts goes up. The first attempt is the one with the higher average venom

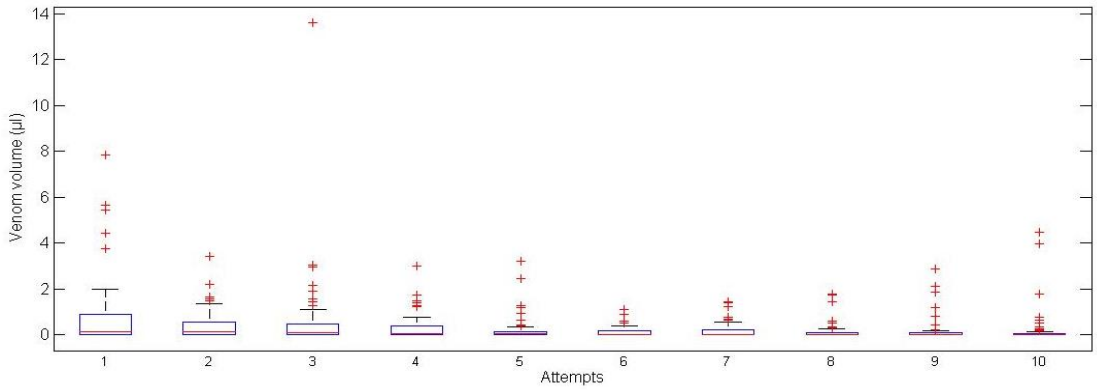

Figure 16. Box plot of venom volumes released on each attempt, pooled for ten specimens.

volume value observed, 0.94 ± 1.73µl. From that point the amount of venom released is seen to be going down, still remaining high in attempts two, 0.43 ± 0.66µl, three 0.62 ± 1.81µl and four 0.31 ± 0.56µl. However, some outliers are visible, so a boxplot was made for each specimen separately, in order to see how each specimen reacted to the attempts individually (Figure 19 and 20).

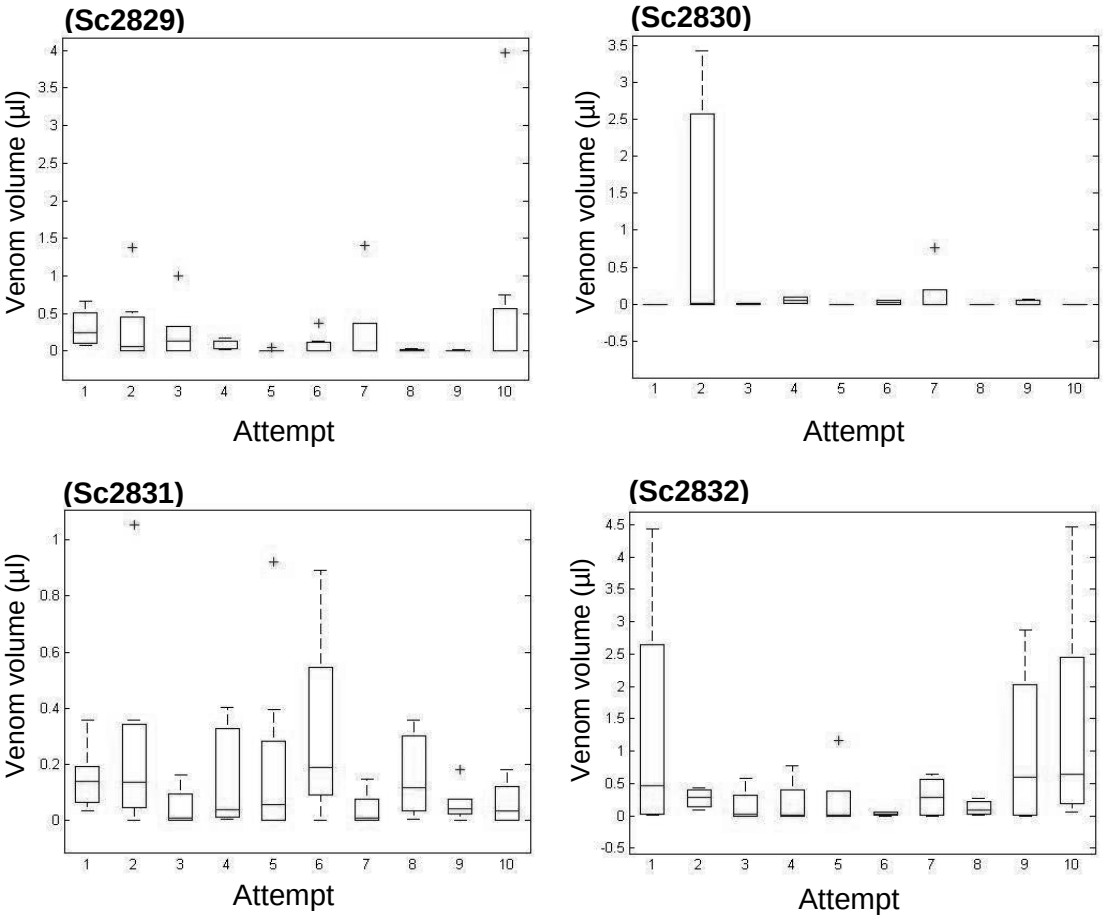

Figure 17. Box plots of venom volume – attempt, performed for specimens Sc2829, Sc2830, Sc2831, Sc2832.

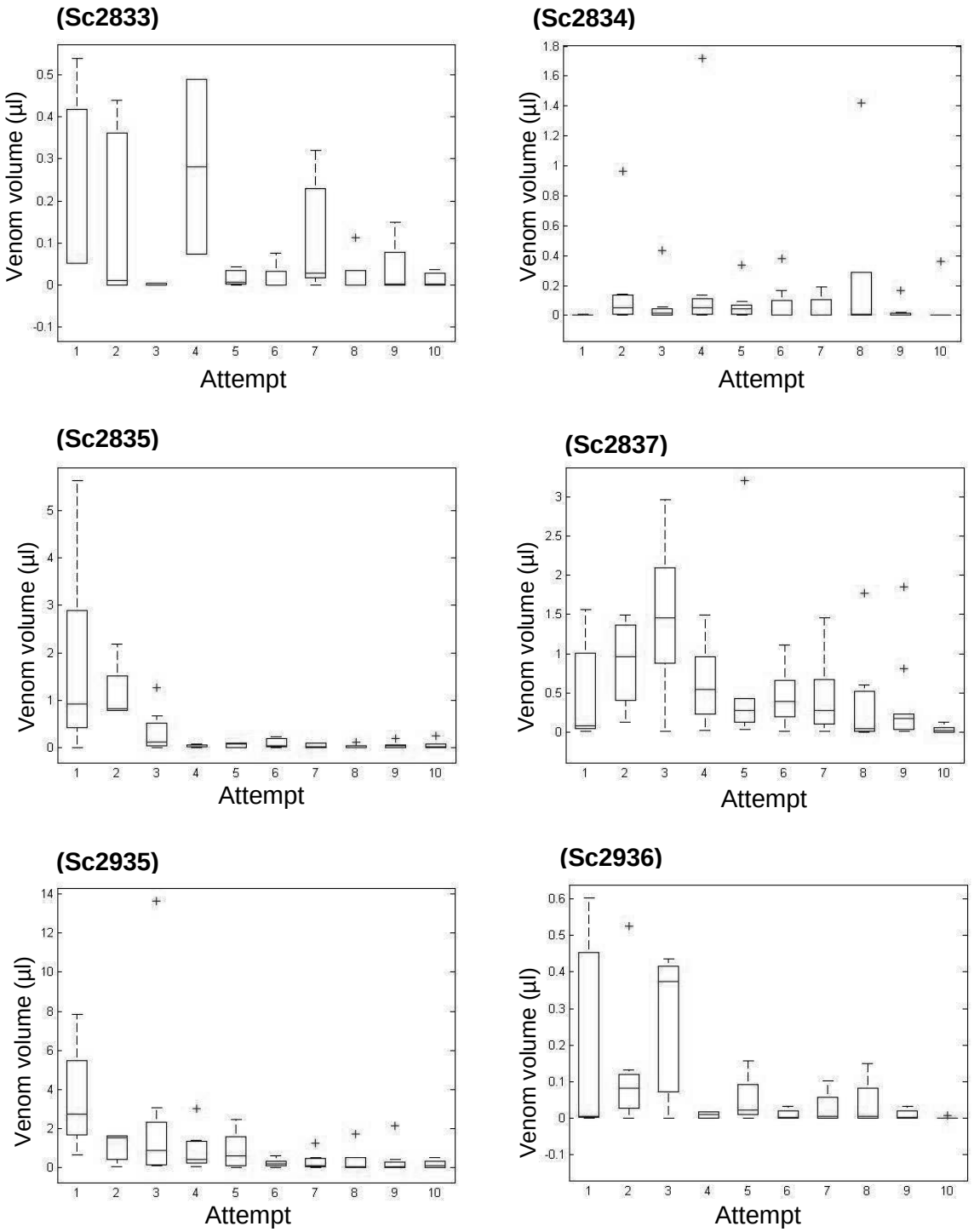

Figure 18. Box plots of venom volume – attempt, performed for specimens Sc2833, Sc2834, Sc2835, Sc2837, Sc2935 and Sc2936.

To know what contributed to different venom volumes expelled by scorpions, a Linear Model was used.

Several independent variables were analyzed against the dependent variable “expelled venom volume”. To account for possible fatigue or habituation, “Date” was included as an independent variable. A variable was added to take into account the differences existent on individual level between different specimens.

The formula was the following one: Volume ~ Attempt + Date + as.factor(Individual).

The results of the linear model along with standardized values are shown in Table VI.

Table VI. Linear model results; variables marked with “*” are the more significant ones, having low P values; (more “*” correspondsto higher significance).

Coefficients:

	Estimate	Estimate(stand.)	Std, Error	t value	Pr(>|t|)	
(Intercept)	27,6430062	-0,15365	41,7430265	0,662	0,5081	
Attempt	-0,061782	-0,18604	0,0131543	-4,697	3,35E-06	***
Date	-0,0006392	-0,02696	0,0009845	-0,649	0,5164	
[T,2830]	-0,0496553	-0,05272	0,1941614	-0,256	0,7982	
[T,2831]	-0,0850494	-0,09031	0,1589727	-0,535	0,5929	
[T,2832]	0,349481	0,37109	0,1779009	1,964	0,05	*
[T,2833]	-0,1201443	-0,12757	0,1807075	-0,665	0,5064	
[T,2834]	-0,0781728	-0,08301	0,1542237	-0,507	0,6124	
[T,2835]	0,1315699	0,1397	0,1600842	0,822	0,4115	
[T,2837]	0,3758485	0,39908	0,1529823	2,457	0,0143	*
[T,2935]	0,9295344	0,987	0,1618813	5,742	1,55E-08	***
[T,2936]	-0,1000043	-0,10619	0,1620288	-0,617	0,5374	

Same model and same independent variables were used to find how the amount of dry stings is influenced. The formula and the results are shown in Table VII.

Table VII. Linear Model; dry stings are represented as a dependent variable; Attempt and Date, represent the independent variables; the number of "*" represents the significance of the variable.

Coefficients:

	Estimate	Std, Error	t value	Pr(>|t|)	
(Intercept)	3,76E-02	1,82E-02	2,066	0,03897	*
Attempt	8,07E-03	1,59E-03	5,090	3,84E-07	***
Date	-4,78E-05	2,79E-04	-0,171	0,86388	

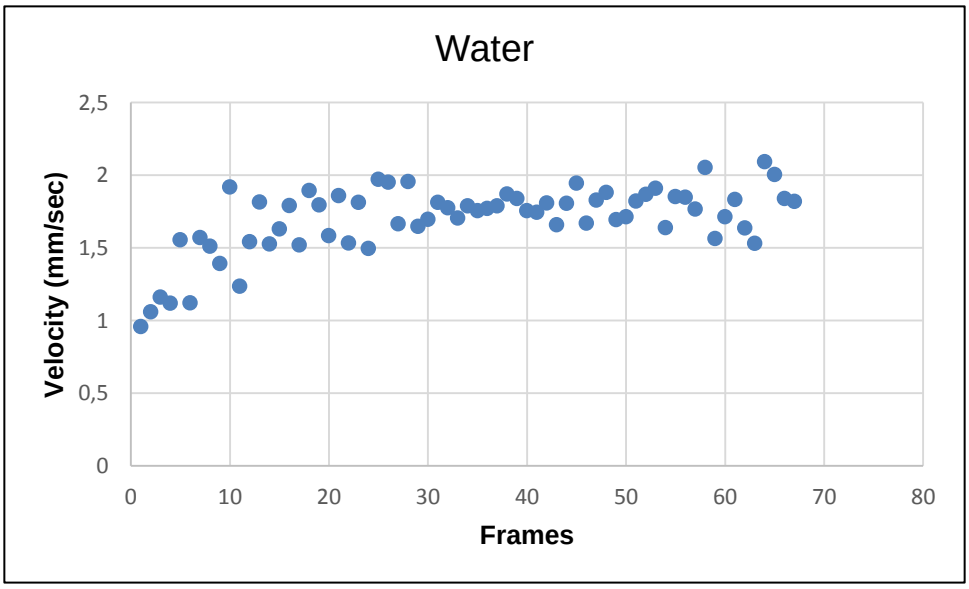

Figure 19. Velocity of the metal sphere inside a capillary tube filled with water.

For the viscosity experiment, were analyzed two different videos, one with the metal sphere inside a capillary tube filled with water (Figure 21) and in the second video was recorded the metal sphere moving inside the capillary tube containing scorpion's venom (Figure 22).

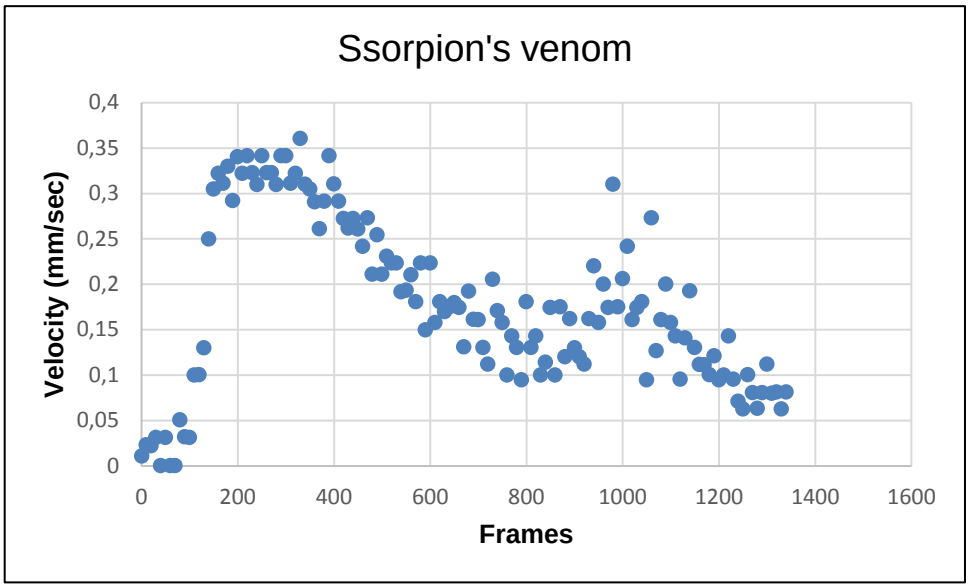

Figure 20. Velocity of the metal sphere inside a capillary tube filled with scorpion's venom.

IV. Discussion

The results of this study do support the hypothesis established at the beginning that scorpions expel different venom volumes, depending on the situation.

From the 768 valid attempts, 209 consisted of scorpions not stinging at all and 559 where scorpions were observed performing a stinging action. But, as shown in Table 5, not during all of the 559 stinging attempts there was venom released, in fact, in about 27% of them appear to be dry stings. During these attempts scorpions did performs one or several stinging actions, but when analyzing the oil jars where venom should have been accumulated, those jars did not have any venom in it. These results show that scorpions can perform stings and do not release any venom. This behavior was already observed in some studies with rattlesnakes [17], [47] and scorpions like *Parabuthus transvaalicus* [49].

Since the goal was to observe how scorpion behavior changes with the threat level the number of dry stings was observed to go up, as the number of attempts got higher. During the whole experiment, through the ten attempts scorpions did continue to sting. However, the venom expulsion did decrease considerably, when getting close to the tenth attempt. The tenth attempt does have the higher fraction of dry stings observed, of about 0.48. This is much higher than in the first attempt, where the fraction of dry stings was about 0.16 (Table V).

When observing the box plot of venom volumes per attempt of all of the specimens altogether (Figure 18) it is possible to see that the volumes are getting smaller with the attempts. However, when analyzing all ten specimens separately and making a different box plot for each one of them (Figures 19 and

20) this clear pattern of venom volume going down with the attempt number is not that clear. Not all of the specimens release more venom at the first attempt, and the slowly decrease it. In Figure 19, boxplot of specimen Sc2832, does show that this specimen did release a big venom volume at first attempt, 2.5 ± 2µl, however the ninth and tenth attempts do have as well a big amount of venom released, 2 ± 1µl and 2.5 ± 2µl, respectively. Box plot of the Sc2831, does show an increase in volume expelled for middle attempts, and then goes down again. This shows that individually, each specimen is different from each other, and when afterwards the data was analyzed, this factor was taken into account.

Some factors like the attempt number, the date when the experiment was performed and individual differences between the specimens, were considered the main variables that could have influenced the venom volumes expelled by scorpions. In Table VI, the results are shown, where the attempts appear to be significant for the quantity of venom expelled by scorpions, with a p value of 3.35e-06. This means that scorpions did vary the amount of venom expelled, considering the attempt that they were submitted to.

Since the experimental work lasted for two months, there was a possibility that scorpions eventually could get used to the procedures, and maybe changed their behavior and expelled venom volumes. The long duration of the experiment could also provoke fatigue in scorpions, making them react differently by the end of it. However, after analyzing it, no significant influence was observed by date over the expelled venom volume, with a p value of 0.52 (Table VI).

Individual differences do show a strong relationship between the amounts of venom expelled and different specimens. One specimen does appear to have a strong relationship with the overall results.

In order to investigate why does Sc2935 has such a strong signal, some basic information was gathered about it, like its gender, size and its age, but no obvious differences to the other specimens were found there.

Since all of these variables are in different scales, a standardization of them was performed, and another liner model done, but the outcome did not change, still a strong signal observed for the attempts and for the individuals, with no significant influence observed for the date.

The reason this methodology of scorpion threatening was used, was to simulate a predator attack that occurs in a natural environment. For instance, grasshopper mouse or fennec fox do prey on scorpions, and they attack it by investing on scorpions several times, grabbing it by the telson in an attempt of immobilizing it. This was what motivated me to perform several attempts on each scorpion in a short period. Each attempt was considered to elevate the threat level for the scorpion, so it was expected to observe more venom expelled at last attempts, which was not the case. By analyzing the data, it is clear that in fact scorpions did expel more venom at the first attempts, which was not expected.. So instead of injecting more venom as the threat to scorpions life persist, these arthropods might inject a large amount of venom at the beginning. Another possibility is that maybe the methodology used to increase the threat level, was not appropriate one, and some different approach needs to be done. On the other hand, the methodology could be good, and the venom volume decreasing could be related to the scorpions itself. Meaning that,

while perceiving a possible threat, scorpions would release as much venom as they could on the first opportunity, which in this case, was the first attempt. Since the effects of the venom are not be immediately perceived, and it can take some time, so it would be wise if the initial injected venom volume would be higher, so when the effects start to be noticed, they could be felt in a more intense and persistent way.

Some specimens, Sc2829, Sc2832 and Sc2834 appear to be releasing more venom not only at the first attempts, but at the last attempts as well. This means that maybe the number of attempts performed in each trial could not be sufficient, and possibly an increase in the attempts number could be seen for the future work.

The viscosity experiment did provide us with some interesting results. When observing Figure 21, it is visible that the velocity of the metal sphere inside a capillary tube filled with water is quite constant, about 1.5-2mm/sec.

When observing Figure 22, which represents the velocity of the metal sphere inside a capillary tube containing scorpion's venom, it is possible to see that the velocity does fluctuate, and does not remain constant as in the case of water.

The scorpion's venom that was collected into the capillary tube, was not homogeneous. It was possible to distinguish between the prevenom and the milky venom. With the prevenom on the top, when metal sphere dropped, it was expected that the velocity of the sphere would be higher at the beginning, while going though prevenom. After reaching the milky venom, it was expected the velocity of the sphere to decay considerably, since the milky venom is much

more viscous than the prevenom. As explained in the introduction, milky venom has higher protein content, what gives its milky color and the viscosity.

When analyzing Figure 22, it is possible to see that in the first 250-300 frames, the metal bead reaches its highest velocity, and starts to slow down afterwards. The metal sphere reached its maximum velocity while going through the prevenom, and when the protein content of the venom started to get higher, changing from prevenom to venom, the velocity of the sphere started to decay, going from 0.35mm/sec (prevenom) to almost 0.05mm/sec (milky venom).

Despite only having one video for the venom viscosity and one for the calibration with water, a strong signal can be observed in velocity changes of the metal bead inside each capillary. Analyzing more videos of the metal bead “fall” though the venom, would allow to have a more solid image of the differences between the prevenom and the milky venom.

V. Conclusion

This study allowed us to have some clearer image on how scorpions manage their venom when found in a threating situation, where the threatening is persistent through time. No similar work has been done previously, what makes it difficult to do compare results obtained in this study to something else.

Future work should be guided towards improvement of the experimental procedures and maybe some thinking about other ways to simulate a predator attack on scorpions.

Other species should definitely be analyzed, with the priority going to medically important species, in order to be able to spot differences in the behavior, if there is any.

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