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The effects of an evolutionary trade-off on scorpion diversity and feeding ecology

Yuri Simone

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Yuri Simone

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PhD

# The effects of an evolutionary trade-off on scorpion diversity and feeding ecology

Yuri Simone

Doutoramento em Biodiversidade, Genética e Evolução

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**Orientador**

Arie van der Meijden (Ph.D.), Researcher, CIBIO

**Coorientador**

Anthony Herrel (Ph.D.), Researcher, CNRS (Paris)

# Nota Prévia

Foram publicados dois artigos científicos em revistas internacionais indexadas e com arbitragem científica, como resultado dos trabalhos desenvolvidos no âmbito desta tese. Segue-se a listagem dos mesmos, nos quais o candidato consta como primeir autor em colaboração com outros autores, esclarecendo ter participado ativamente na sua conceção, obtenção e análise de dados, discussão de resultados, e elaboração da sua forma publicada.

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2. Simone, Y., Chaves, C., van der Meijden, A., & Egeter, B. (2022). Metabarcoding analysis of different portions of the digestive tract of scorpions (Scorpiones, Arachnida) following a controlled diet regime shows long prey DNA half-life. Environmental DNA, 4(5), 1176–1186. https://doi.org/10.1002/edn3.311

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# Resumo

Os padrões de evolução de características estão intimamente ligados ao contexto ecológico. As características corporais dos organismos são frequentemente complexas e formadas por vários elementos que evoluem de forma quase independente. O número destas características descreve o espaço morfológico, enquanto o grau de covariação entre as características (frequentemente referido como, integração morfológica) define o número de óptimos adaptativos que podem ser ocupados. As estruturas corporais dos animais são frequentemente utilizadas em mais de que um propósito ecológico. Quando as pressões selectivas para diferentes funções atuam simultaneamente para moldar tais estruturas, podem surgir compensações biomecânicas. Embora as compensações evolutivas podem desacelerar a taxa evolutiva de estruturas morfológicas corporais, isso não implica necessariamente que as características compensadas sejam reduzidas na diversidade morfológica complexiva. A compensação entre força e velocidade é uma compensação biomecânica ubíqua. A impossibilidade de optimizar simultaneamente força e velocidade no desempenho animal pode criar desafios evolutivos, especialmente nos sistemas musculoesqueléticos. A força e a velocidade são compensadas tanto na mecânica de alavancas como na fisiologia muscular. Como resultado, é esperada uma elevada integração entre todos os elementos dos sistemas musculoesqueléticos na optimização da força ou da velocidade. Esta compensação também tem importantes consequências na diversidade morfológica dos animais e também na dieta. Devido à compensação entre força e velocidade, as estruturas de mordedura não podem ser optimizadas para todos os tipos de alimentos, segregando as espécies nos diferentes nichos alimentares. Esta tese investiga a ligação entre compensações biomecânicas e ecologia alimentar, utilizando os escorpiões como organismo modelo, e mais especificamente as suas pinças (quelas) como características funcionais modelo. Identificar em quais contextos ecológicos o traço é utilizado e se existem diferenças no seu uso ao longo de todo o gradiente morfológico é crucial para compreender as pressões seletivas que atuam sobre os traços funcionais. A este respeito, após dois capítulos introdutórios, o no terceiro capítulo é feita uma revisão das múltiplas pressões selectivas que moldam as quelas dos escorpiões, enfatizando os papéis ecológicos na predação, defesa e disputas sexuais. É dada particular ênfase às diferenças extremas na morfologia ou uso entre espécies ou grupos taxonómicos mais elevados, ou entre os sexos, pois tais casos são mais esclarecedores para compreender os papéis dos dois sistemas distintos de armas dos escorpiões e as suas interações evolutivas. Nesta tese, focarei principalmente na predação como motor da diversidade morfológica das quelas.

Portanto, compreender a base mecanicista do equilíbrio entre velocidade e força no desempenho do fechamento do chela é importante para possivelmente inferir o seguimento de diferentes caminhos evolutivos devido ou levando a diferentes estratégias predatórias e possivelmente à dieta. Portanto, No quarto capítulo, estudam-se espécies de escorpiões representativas de dois extremos morfológicos na arquitectura das quelas. Entre os escorpiões, as pinças apresentam uma significativa diversidade morfológica associada à ecologia. Um modelo biomecânico integrando microtomografias de *sincrotron*, dados de performance e arquitetura muscular foi construído. Os resultados obtidos revelam uma forte integração da arquitectura muscular e elementos estruturais em direção a duas estratégias funcionais de desempenho: a força no fechar das pinças é optimizada em espécies de dedos curtos através da vantagem mecânica de alavancas e músculos, bem como do comprimento do sarcómero. Em espécies de dedos longos, a velocidade no fechar das pinças é optimizada, por exemplo, por meio de aceleração precoce. Embora outras pressões funcionais possam também estar em jogo, uma estratégia parece optimizada para agarrar presas enquanto a outra para esmagar presas. Estas duas estratégias divergentes impulsionadas por compensações podem ter tido impactos profundos na amplitude trófica dos escorpiões e na evolução do veneno. Para confirmar que diferentes morfologias de quelas com diferentes desempenhos de fechamento podem levar a diferentes dietas, é necessário obter informações sobre quais presas os escorpiões consomem. No quinto capítulo, desenvolveu-se uma técnica de *metabarcoding* molecular para recuperar o DNA das presas do sistema digestivo dos escorpiões, uma tarefa complicada pela baixa frequência de alimentação nos escorpiões e pela digestão parcialmente externa. Utilizando os escorpiões florestais do Vietname (*Heterometrus laoticus*), foram analisados regimes alimentares controlados e analisadas diferentes partes do tracto digestivo. Através deste método, detectaram-se todos os diferentes tipos de presas oferecidos, o que proporcionou perspectivas sobre o *timing* dos eventos de digestão dos escorpiões. Além disso o hepatopâncreas foi identificado neste estudo como a secção óptima do tracto digestivo para a detecção das presas, oferecendo uma notável meia-vida de detectabilidade de DNA até 53 dias. Estos resultados permitiram refinar a actual compreensão do comportamento alimentar dos escorpiões e ofereceram recomendações metodológicas para futuras análises moleculares da dieta. Finalmente, para obter informações sobre a dieta de escorpiões com regime de forrageamento desconhecido, a metodologia desenvolvida foi empregada em uma população natural de escorpiões apresentando dimorfismo sexual em seus quelos para testar se diferentes formatos de quelos permitiam a segregação de nicho de forrageamento.. Esta tese reúne aspectos díspares da biologia dos

escorpiões, elucidando as compensações evolutivas que estão na base das armas, biomecânica e ecologia alimentar. Ao integrar abordagens morfológicas, biomecânicas e moleculares, esta investigação fornece um quadro abrangente para compreender a intrincada interação entre forma e função na diversidade das pinças dos escorpiões.

## Palavras-chave

Modelo biomecânico - Quelas - Dieta - Conteúdo intestinal - Metabarcoding - Integração morfológica - Arquitetura muscular - Escorpiões - Armas de escorpiões - Sistema de entrega de veneno - Trade-offs

# Abstract

Patterns of trait evolution are tightly linked to their ecological context. Organismal traits are often complex and formed by several different quasi-independently evolving traits. The number and diversity of the latter describe the morphospace while the degree of covariation among traits (also referred as morphological integration) may constrain the number of adaptative optima that can be occupied.

Animal body structures are often used for more than one ecological purpose. Biomechanical trade-offs can arise when selective pressures for different functions act simultaneously to shape such structures. Although evolutionary trade-offs may slow down the rate at which a morphological body structures evolve, they do not necessarily imply that traded-off traits are reduced in their overall diversity.

The force-speed trade-off is a ubiquitous biomechanical trade-off. The impossibility of simultaneously optimizing force and speed in animal performance can pose evolutionary challenges, specifically in musculoskeletal systems. Force and speed are traded-off in both lever mechanics and muscle physiology. Consequently, a high integration among all the elements of musculoskeletal systems toward the optimization of either force or speed is expected. This trade-off has also important consequences not only for animal morphological diversity but also in their diet. Due to the force-speed trade-off, biting structures cannot be optimized for all types of food items, segregating species into different foraging niches.

This thesis investigates the link between biomechanical trade-off and feeding ecology using scorpions as model organism, and more specifically their pincers (chelae), as model functional traits.

Identifying in which ecological contexts the trait is used and whether there are differences in its usage across the whole morphological gradient is crucial to understand the selective pressures acting on functional traits. In this regard, following the two introductory chapters, the third chapter reviews the multiple selective pressures shaping scorpion chelae, emphasizing their ecological roles in predation, defence, and sexual contests. Particular emphasis is given to the extremes in the differences in morphology or usage between species or higher taxonomic groups, or between sexes, as such cases are most insightful to understand the roles of each of the two distinct weapon systems of scorpions and their evolutionary interactions.

In this thesis, I will mainly focus on the predation as driver of chela morphological diversity. Therefore, understanding the mechanistic basis of the speed-force trade off in chela closing performance is important to possibly infer the following of different evolutionary pathways due or leading to different predatory strategies and possibly diet.

Therefore, in the fourth chapter, scorpion species representing the two morphological extremes in the chela architecture were studied. Across scorpions, the chelae showed significant morphological diversity associated with ecology. A biomechanical model of chela closing integrating synchrotron microtomographic data, performance- and muscle architecture data was developed. The main findings reveal a strong integration of muscle architecture and structural elements towards two functional optima of performance: closing force is optimized in short-fingered species through mechanical advantage of levers and muscles as well as sarcomere length. In long-fingered species closing speed is optimized, e.g., by means of early acceleration. Although other functional demands may be at play, one system seems optimized for prey grasping, and the other for prey crushing. These divergent optima, driven by trade-offs, may have had profound impacts on the trophic range of scorpions, and the evolution of their venom.

To confirm that different chela morphologies with different closing performances might lead to different diet, is necessary to obtain information about which prey do scorpions consume. To this date traditional methods provided very little information about trophic habits of scorpions. Therefore, in the fifth chapter, a metabarcoding-based technique was developed to retrieve prey DNA from the scorpion digestive system, a task complicated by the low feeding frequency and external digestion in scorpions. Focusing on Vietnamese forest scorpions (*Heterometrus laoticus*), controlled dietary regimes were employed and different portions of the digestive tract were analysed. All the different prey species that were offered were also detected, providing insights into the timing of scorpion digestion. Furthermore, the hepatopancreas was identified as the optimal digestive tract section for prey detection, offering a remarkable 53-day DNA detectability half-life. These findings refined our understanding of scorpion feeding behaviour and offered methodological recommendations for future molecular diet analyses.

Finally, to obtain diet information in scorpions with unknown foraging regime, the developed methodology was employed in a natural population of scorpions showing sexual dimorphism in their chelae to test whether different chela shapes allowed for foraging niche segregation.

This thesis bridges disparate aspects of scorpion biology, elucidating the evolutionary trade-offs that underlie their weaponry, biomechanics, and feeding ecology. By integrating morphological, biomechanical, and molecular approaches, this research provides a comprehensive framework for comprehending the intricate interplay between form and function in scorpion chela diversity.

## Keywords

Biomechanical model, Chelae, Diet, Gut content, Metabarcoding, Morphological integration, Muscle architecture, Scorpions, Scorpion weapons, Venom delivery system, Trade-offs.

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# List of Abbreviations

| | |
|---|---|
| α-NaScTx | α Sodium channel-acting toxins |
| β-NaScTx | β Sodium channel-acting toxins |
| AICc | Corrected Akaike Criterion Index |
| ClTx | Chlorotoxins |
| COI | Cytochrome oxidase subunit 1 |
| $D_{50}$ | DNA half-life |
| ECRB | Extensor carpi radialis brevis |
| ECRL | Extensor carpi radialis longus |
| GLMM | Generalized Linear Mixed Models |
| HDP | Host defence peptides |
| $HNO_3$ | Nitric acid |
| KScTx | Potassium channel-acting toxins |
| $LD_{50}$ | Median lethal dose |
| LRT | Likelihood ratio tests |
| MA | Mechanical advantage |
| $Na^+$ channels (NaScTx) | Sodium channel-acting toxins |
| NCBI | National Center for Biotechnology Information |
| PCSA | Physiological cross-sectional area |
| PGLS | Phylogenetic partial least squares |
| RMA | Reduced major axis regression |
| Sr-µCT | Synchrotron microtomography |

# Chapter 1
# General Introduction

## 1.1 What is morphological diversity?

A crucial goal of evolutionary biology is understanding the processes underlying species and morphological diversity at micro- and macroevolutionary scale (Arnold et al., 2001; Rolland et al., 2023). Although both species diversity and morphological diversity are extensively used as indexes to quantify biodiversity, they are not synonyms (Roy & Foote, 1997). One focal evidence is that morphological diversity is not equally distributed across extant taxonomic groups. For instance, cichlids or anoles are particularly rich in both species and phenotypic diversity (Kocher, 2004; Losos, 2009), some groups of rodents are species-rich but poor in morphological diversity (Alhajeri & Steppan, 2018), marsupials are a small group but with high morphological heterogeneity (Sánchez-Villagra, 2013), and finally groups like lungfish are both species poor and morphologically homogeneous (Guillerme et al., 2020). Although many advances have been made in this field, the patterns underlying variation in both species richness and morphological diversity across major organismal groups remain poorly understood.

Morphological diversity is a quantitative description of the degree of distinctness of a multivariate set of morphological traits within a group of taxa at and above the species level (Hopkins & Gerber, 2017; Roy & Foote, 1997). To assess such diversity, it is necessary to identify morphological descriptors to quantify dissimilarities between morphologies. One widely used metric of dissimilarity is the Euclidean distance. Consequently, the set of morphological descriptors generates a spatial configuration in which each taxon is located. This multidimensional space, having morphological measures as reference axes, is defined as the morphospace (Budd, 2021). The dimensionality of a morphospace is determined by the number and the range of independently varying parameters defining the morphological trait (Vermeij, 1973). The greater the number of parameters, the larger the morphospace, meaning more available adaptative zones can be occupied (Wainwright, 2007). Nevertheless, trait morphospace is often not uniformly occupied in space and time. Many theoretical configurations are subject to physical, developmental, or phylogenetic constraints making these areas of the adaptative landscape impossible to be occupied (Alexander, 1985; Higham et al., 2021; Raup, 1966). Alternatively, highly invariant environments, like for instance the subterranean one, “trap” species in a narrow area of the morphospace around a local adaptative optimum (Sansalone et al., 2022).

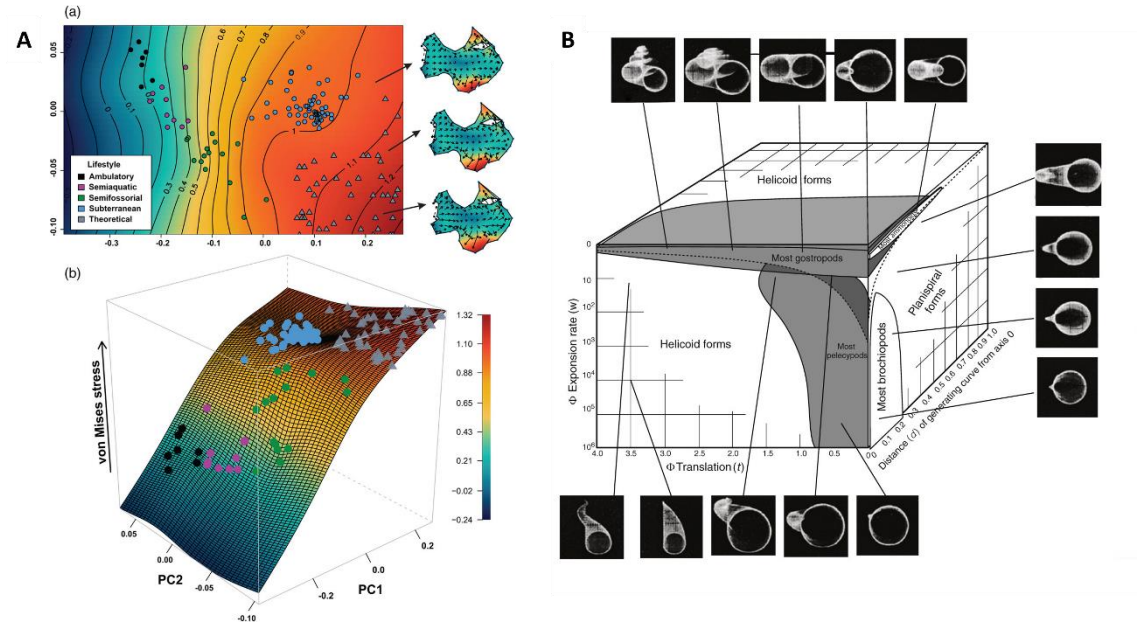


**Figure 1.1.1** Occupancy of the available morphospace in two different groups of extant taxa. In panel A the configuration of mole humeri is showed along the functional morphospace. The sampled taxa are represented with circles while triangles are theoretical configurations. The isocline from 1.0 to 1.3 are areas of high locomotion performance values while the isoclines ranging from 0.1 to 0.4 are the areas of low-performance values. Interestingly, the areas of high-performance values are occupied only by theoretical configurations of mole humeri. In B the Raup's representation of the morphospace of coiled shells is presented. In this model, shell geometry is defined by three parameters, the grey areas are the ones occupied by extant species while the white areas of the theoretical morphospace are not occupied by any species. (Image A is modified from Sansalone at al. 2022 while image B is modified from Raup et al., 1966).

In other cases, morphospace occupancy varies along the taxon's evolutionary history. In many metazoan clades, the highest level of morphological variability appears in the initial phases of their evolutionary history (Deline et al., 2018; Oyston et al., 2015). This is often due to the developing of new morphological traits (also called key innovations) allowing the exploitation of a new ecological niche and consequently expanding the currently available morphospace (Miller et al., 2023; Wainwright, 2007) and enhancing species richness. Among many, the evolution of wings is coupled to the explosive increase in insect diversity (Nicholson et al., 2014); the evolution of the lower jaw from an ancestral rostral gill arch catalysed early vertebrate evolution (Johanson et al., 2019), or in more recent times, high duty-cycle echolocation promoted the diversification of two bat families, Rhinolophidae and Hipposideridae, allowing them to detect prey in closed environments, such as rainforests (Peixoto et al., 2018). However, the magnitude of morphological diversity is not constant across time, and different ecological processes might fix or sweep out a given initial level of richness of both species and forms. The main driving forces for the origin of key innovations are both extrinsic and intrinsic factors. Extrinsic abiotic and biotic factors like climate change and

species interactions modulate the tempo and pace of the morphological diversification, while the intrinsic factors like developmental, physiological, genetic or architectural constraints determine the volume of the morphospace and consequently, the potential trait evolvability (Arbour et al., 2021; Voje et al., 2015).

### 1.1.1 Morphological diversity and functionality

In order to understand the causes and the consequences of any evolutionary pattern shaping the morphological diversity across taxa it is important to clearly define the function of the trait analysed. Function in biology might have multiple definitions (for a review about the topic see Keeling et al., 2019), nevertheless in this thesis only the mechanical and the ecological functions will be considered. Mechanical function represents *how* intrinsic factors coordinate to perform a specific task independently from any extrinsic factor. However, the mechanical output of a trait also affects the whole organism performance and the derived ecological function (i.e., its role within the ecosystem) by influencing its fitness and how environmental biotic and abiotic resources are exploited (Jax, 2005). For instance, the architecture of jaw bones and muscles determines bite force. Bite force is relevant in many ecological contexts like feeding ecology or self-defence and territoriality. The latter are crucial tasks that directly influence organismal survival and reproductive chances (i.e., fitness). Nevertheless, gathering direct data about individual fitness might be difficult and extremely time consuming. To overcome this, Arnold (1983) introduced a conceptual and statistical framework linking morphology and fitness through the means of performance.

The first step of Arnold's ecomorphological paradigm establishes that trait morphological variation must be associated with a variation in performance. This can be quantified experimentally in the laboratory or through the development of biomechanical models. In these models the complexity of biological traits is often reduced to simpler shapes to which geometrical and physical laws can be applied. For many models, morphological traits of the musculoskeletal system are approximated to lever systems converting the input force generated by muscle contraction into motion. Consequently, prediction of mechanical performance can be obtained by combining the mechanical advantage (the ratio between the in-lever and the out-lever) of the structural elements with the physiological properties of muscles. The laws of mechanics are universal and muscle physiology is rather conserved across taxa (Hooper et al., 2008; Paniagua et al., 1996). Therefore, biomechanical models are useful tools to predict trait performance in extinct taxa or in those taxa for which direct measurement of performance might be

technically challenging due to size or harmful behaviour (Heethoff & Koerner, 2007; Wroe et al., 2005).

The second step of the ecomorphological paradigm requires the correlation of the measured or estimated performance with the ability to exploit an environmental resource. This can be obtained through field observations or ad-hoc experiments such as the success rate obtained from feeding experiments done with different sympatric prey types.

### 1.1.2 When multiple selective pressure act on the evolution of morphological diversity: Functional trade-offs

It is quite uncommon that one single selective pressure shapes the diversity of morphological traits. When multiple selective forces have contrasting directions, the optimization of a trait toward one function might be detrimental for the other. For instance, the iconic adhesive toe pads, evolved in many species of geckos, increased their clinging performance and allowed them to exploit vertical smooth habitats (Russell, 2002). However, during locomotion, toepads need to be detached prior to body displacement. This then has negative consequences on the escape capacity of these animals, as it limits sprinting performance. Toepads are thus a morphological trait that allowed the occupation of a unique and difficult niche but at the same time increased the risk of predation (Irschick et al.,2016).

Functional trade-offs occur when the benefits in fitness obtained from a functional performance come at the cost of another (Garland et al., 2022; Stearns, 1989). They are ubiquitous and might be of different nature, from allocation constraints affecting mostly life-history traits to biomechanical conflict (Garland et al., 2022). Regarding the latter, the well-known mechanical trade-off between force and speed dramatically affects musculoskeletal performance in many organisms. Due to simple lever mechanics, the increasing of the mechanical advantage leads to an increased output force and simultaneously to a decreased output speed.

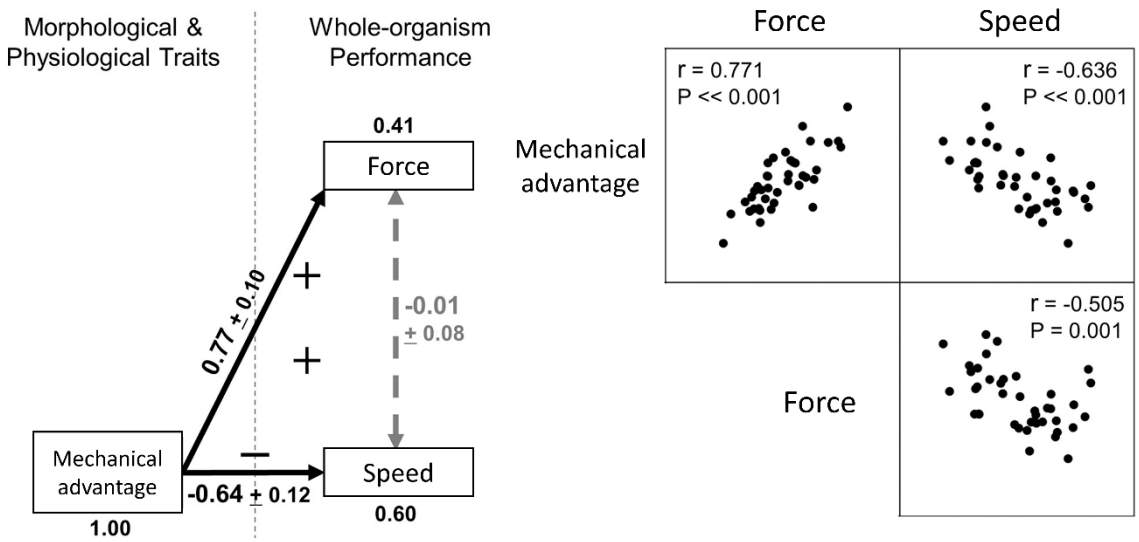


**Figure 1.1.2** Simulated data to show the effect of force-speed trade-off in closing beak performance in relation to mechanical advantage in birds. (Adapted from Garland et al., 2022).

However, also muscle contraction is similarly confronted with trade-offs. Muscles generate maximum force when the speed of contraction is zero (isometric contraction) but when muscles contract at a certain speed, the force generated is lower than that generated isometrically (Hill, 1938; Seow, 2013).

It has been argued that functional trade-offs may represent a constraint for the evolution of morphological diversity (Walker, 2007). Nevertheless, several studies found that traits that are subject to performance trade-offs tend to evolve more rapidly, showing that trade-offs can promote rather than limit morphological diversification (Corn et al., 2021; Holzman et al., 2012). Complex traits can be reduced into multiple semi-independent units defining the morphospace (Walker, 2007). For instance, the musculoskeletal elements of jaws can be reduced at the level of the structural system (the bones forming the lower jaw), and the source of power allowing jaw adduction (the muscles attached to the lower jaw). The structural elements can be additionally reduced into the length of the two lever arms and the muscles in all their hierarchical components: sarcomeres, fibres, and fascicles. When all these variables are singularly considered, evolutionary rates can be estimated for each of these variables, leading to a more informative view of the components that are diversifying the most and which ones are instead the more conserved (Holzman et al., 2012; Muñoz et al., 2017).

This reductive process not only permits investigation of the diversification rate of each component of a functional trait but also whether this diversification leads to the same performance optimum or toward multiple contrasting performance optima. In traded-off performance traits, physiological and structural elements can show a high degree of covariation (i.e. high integration) and coordination toward one single

evolutionary trajectory (Hedrick et al., 2020). As a consequence, strong integration has been generally considered as a strong limiting factor for morphological diversity. Nevertheless, even groups with highly integrated systems and traded-off performances may present high rates of diversification, especially at the extremes of the trait morphological gradient (Burress & Muñoz, 2022; Goswami et al., 2014). In some cases, this can be explained if we assume that in particular complex traits, formed by several units, the performance costs paid due the morphology of one trait can be mitigated by independent, compensatory changes in other traits (Holzman et al., 2011), maintaining in this way the overall fitness and mitigating the constraints imposed by functional trade-offs.

## 1.2. The consequences of the force-speed trade off in feeding ecology

One of the most important tasks for an animal is to get its meal. In this regard, it is not surprising that the most variable traits in animals are often associated with their feeding ecology (Corn et al., 2021; Holzman et al., 2012). Biting structures must be designed to minimize the energetic costs associated with grasping and processing food. Food items can be fast, slow, soft, or hard. Because of the force-speed trade-off in musculoskeletal systems, a biting structure cannot be optimized for all types of food items. A biting structure with high mechanical advantage would be better suitable to crush hard food items but not as suitable to grab elusive and fast prey. On the other hand, to catch elusive and soft prey, biting structures with lower mechanical advantage would be ideal. This correlation between food-type and the design of biting structure has been shown in many different taxa from crustaceans to mammals (Blanco & Patek, 2014; Herrel et al., 2002, 2005; Holzman et al., 2012; Meyers et al., 2018; Ornelas, 1994; Santana et al., 2010; Schenk & Wainwright, 2001; Yamada & Boulding, 1998).

In some cases, the relation with feeding performance and biting structure design is not as clear because diet composition is poorly studied. Only recently, molecular tools based on high-throughput sequencing of gut content (reviewed in Ando et al., 2020; Pompanon et al., 2012; Sousa et al., 2019), regurgitates (Neidel & Traugott, 2023; Symondson, 2002), and faeces (Deagle et al., 2009; Sint et al., 2015) have been developed. These new tools allowed to disentangle the diet of rare species or the trophic network of liquid feeders. The latter leave little-to-no leftovers of their food and are often characterized by very infrequent feeding events (Macías-Hernández et al., 2018; Sierra Ramírez et al., 2021; Uiterwaal & DeLong, 2020).

## 1.3. Why scorpions?

Scorpions are an ancient order of arachnids accounting for about 2700 species distributed across 22 families (https://www.ntnu.no/ub/scorpion-files/intro.php). Scorpions appear to have the most conserved, but at the same time versatile body plan; it has remained nearly unaltered since their terrestrialization approximately 320Mya ago, and yet, they are an extremely successful group that radiated across all habitats, ranging from arid and dry deserts to humid rainforests.

Despite their very conserved body plan, scorpions show a remarkable morphological variability. This is particularly evident at the level of the appendages protruding anteriorly (legs and pedipalps) and the tail-like structure (metasoma) posteriorly. Nevertheless, the reason why scorpions exhibit such phenotypic diversity remains unclear.

Only recently, the phenotypic diversity of pedipalps, metasoma and ambulatory legs has been correlated with microhabitat use, proving the existence of four distinct ecomorphs, each one characterized by a finite range of anatomical covariation among them (Coelho et al., 2022). For instance, scorpions with slenderer pedipalps and longer legs seems to be associated with sand fossorial microhabitats while scorpions with more robust pedipalps and shorter legs tends to be associated with rock-dwelling habitus.

**Figure 1.3.1** Morphological diversity of scorpions.

However, microhabitat use has a clear direct effect of phenotypic diversity only in a locomotory context (Coelho et al., 2022). Microhabitat usage is, however, likely not be the only evolutionary driver of scorpion morphological diversity. Intra- and interspecific interactions surely play an important, but overlooked, role in shaping scorpion morphological diversity.

Scorpions are exceptionally well-suited to study the interactions between functional traits, their performance, and their ecological roles because of their unique separation of functions into different body parts. This functional compartmentalization is also referred to as modularity (Melo et al., 2017; Wagner et al., 2007). It is possible to ascribe a single, unambiguous function for each morphological module in specific contexts. For instance, in a predatory context, many animals like spiders or snakes rely on a unique functional trait, the jaws, to grasp, inject venom and chew their prey. Scorpions perform each one of these three tasks by using three different body parts: they grasp the prey by using the pincers (chelae), inject venom through the stinger, and chew the prey by means of the chelicerae. Stinging performance and stinger diversity have been explored in detail in previous studies (Coelho et al., 2017; van der Meijden & Kleinteich, 2017) while chelicerae have received particular attention only for taxonomic reasons and not that much in a functional context. This might be because the external digestion alters the features of the prey body making the link between type of prey consumed and chelicera shape unclear. The external digestion, the slow metabolism and the liquid-feeding habits made it particularly difficult to understand the trophic ecology of scorpions. Scorpions are generalist predators feeding on a wide range of arthropods; however, the superficial knowledge of scorpion diet is the major obstacle for understanding how much diet contributed to scorpion morphological evolution.

Despite the chelae being the first body structure getting in touch with prey, little attention has been paid to the functional implications that the morphological diversity of the chelae might have on the foraging ecology in scorpions. The morphological range of chela diversity in scorpions is remarkably high. On one extreme there are species with slender, long-fingered chelae while on the opposite side there are species with very robust and short-fingered chelae (van der Meijden et al., 2010; 2012). In other pincered animals like crabs, the morphological diversity of chelae has been highly correlated to the biomechanical features of the most consumed prey (Taylor, 2001). Scorpion chelae are cantilevered systems subjected to force-speed trade-off (Simone & van der Meijden, 2018). Species with long fingered chelae are faster but weaker than short fingered ones, while species with short-fingered and more robust chelae are stronger but significantly slower than the long-fingered ones.

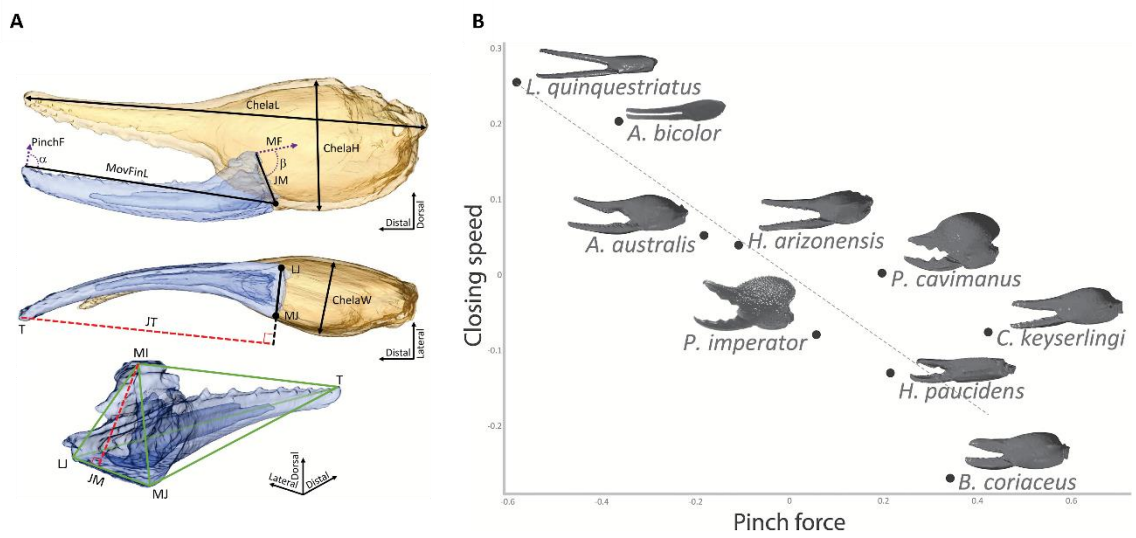


**Figure 1.3.2** In A, the main morphological measures of scorpion chelae are showed, tarsus is showed in salmon while the movable finger in blue. The graph in B shows the size-corrected maximum closing speed on the vertical axis and a negative correlation with size-corrected pinch force on the horizontal axis, indicating a trade-off between speed and force. (Images modified from Simone & van der Meijden 2018).

Irrespective of whether diet is the cause of the observed variation in chela morphology, it does impose limitations on several functions. Fast but weak chelae are not optimal for holding prey but more suitable in grasping fast prey. Likewise, strong but slow chelae are less likely to be a useful tool for grabbing fast prey but excellent tools to crush it. Since chelae are not the only modules involved in predation, these constraints might also affect the evolution of venom composition. In the species that have worse holding performance, venom should act quickly while in the species with strong crushing capacity, it may be released from this pressure and take other evolutionary trajectories.

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# Chapter 2
# Objectives and thesis outline

## 2.1 General objectives

The main goal of this dissertation is to understand the effects that the force-speed trade-off in scorpion chelae has on feeding ecology and morphological diversity. The general goals are defined as follows: 1) Review all the ecological relevant roles that scorpion chelae have with an emphasis on feeding ecology; 2) From the study of the anatomy of scorpion chelae, test whether closing muscles and structural elements of scorpion chelae show high level of functional and kinematic integration; 3) Develop a protocol to investigate diet in scorpions through high-throughput sequencing of gut content; 4) Infer possible implications that chela morphology and consequently performance might have on predatory behaviour and the evolution of the other modules implicated in prey capture.

## 2.2 Thesis outline

This thesis is divided in six chapters. Chapter 1 is an introduction to the relevant theoretical concepts to the following chapters, starting with an overview of the evolutionary processes and constraints shaping morphological diversity in space and time. Next, the conceptual framework of how morphology, performance, and fitness are linked according the ecomorphological paradigm is presented. Emphasis is given to the mechanical function of morphological traits, how it is analysed and how it is translated into a whole-organismal function within the ecosystem. A specific section is dedicated to how the effect of conflicting selective pressures generate functional trade-offs on morphological traits, particularly in a foraging context. This introductory chapter ends with the presentation of the main protagonist of this thesis: scorpions and their chela morphological diversity. Emphasis is given not only to the reasons that make these animals such good models for research in functional morphology, but also to the challenges of doing ecological studies on these common, yet extremely overlooked animals.

In Chapter 2, the dissertation outline, main objectives, and particular questions addressed in this thesis are described.

Chapter 3 provides a review of the functional performance of scorpion chelae and venom, not only in foraging context, but also in other two fundamental ecological contexts: self-defense and sexual behaviors. Emphasis is given on the differences in

morphology and performance between species highlighting the roles and the possible evolutionary trajectories of the two distinct, yet interacting, weapon systems. In this chapter, I wanted to mention other two possible drivers of scorpion morphological diversity in order to exemplify how the design of scorpion chela is an evolutionary compromise among functions. This work was published as:

- Simone, Y., & van der Meijden, A. (2021). Armed stem to stinger: a review of the ecological roles of scorpion weapons. *Journal of Venomous Animals and Toxins Including Tropical Diseases*, 27, 1–21. https://doi.org/10.1590/1678-9199-jvatitd-2021-0002

The goal of chapter 4 is to investigate the anatomical basis of the force speed trade off in two species at the extreme of the range of chela phenotypic diversity. Through the 3D volumetric reconstruction of synchrotron scans, I developed a landmark-based biomechanical model of chela abduction/adduction where muscle contraction speed and biteforce can be estimated at each degree of rotation. The musculoskeletal system was decomposed in all its components and linked to the output of the model to test the degree and the direction of kinematic integration along one single evolutionary trajectory in a system governed by force-speed traded-offs. This work is in preparation for submission in *Proceedings of the Royal Society B: Biological Sciences* with the title:

- Simone Y., Herrel A., Boistel R., & van der Meijden A. Crushers versus graspers: Biomechanical model of finger adduction reveals high integration in the musculoskeletal system of scorpion chelae.

Chapter 5 focuses on the study of scorpion diet, and it is composed of two sections. Section 5.1 is the description of the first high-throughput sequencing protocol to retrieve prey DNA from captive bred scorpions under an imposed diet. Section 5.2 includes some preliminary results of the application of the protocol described in 5.1 on a natural population of scorpions presenting strong sexual dimorphism in pedipalp shape.

The work in Section 5.1 was published as:

- Simone, Y., Chaves, C., van der Meijden, A., & Egeter, B. (2022). Metabarcoding analysis of different portions of the digestive tract of scorpions (Scorpiones, Arachnida) following a controlled diet regime shows long prey DNA half-life. *Environmental DNA*, 4(5), 1176–1186. https://doi.org/10.1002/edn3.311

Chapter 6 includes a discussion of the topics and interpretations of the results presented in this thesis, with an emphasis on the major findings and future research challenges arising from it.

# Chapter 3

## Review of the ecological roles of scorpion weapons

## 3.1 Introduction

Environments where resources are limited increase the competition between their inhabitants. Gaining an advantage in access to resources over competitors raises an individual's chances to survive and transmit its genes to the next generation. In animals, conflicts may involve a physical antagonistic struggle between individuals. The features that most define the outcome of such competitive conflicts are usually considered "weapons" (Emlen, 2014). Although many definitions of animal weapons are limited to intraspecific competitions, particularly intrasexual competitions (Andersson, 1994; Berglund, 2013; Berglund et al., 1996; Emlen, 2008; McCullough et al., 2016; Rico-Guevara & Hurme, 2019), we will here use the broader definition of a weapon as proposed by Lane (2018). In her definition, animal weapons are features that constrain the behaviour of another individual either through direct harm or other physical disruption in one or more of three fundamental contexts of usage: i) predation ii) defence iii) sexual contests. Animal weapons may thus be classified by their context of usage but could also be organized by their mode of action, form or evolutionary history (Lane, 2018). In this review, we will organize the literature on scorpion weapons by context of usage. We will first give examples of each context of usage from across the animal kingdom to provide a comparative background for the discussion of scorpion weapons.

### 3.1.1 Weapons for predation; prey capture and handling

Weapons are used in predation to seize the prey and reduce its chance to escape by restraining or incapacitating it. The grip of the restraining structures on the body of the prey may be increased by increasing friction, interlocking, penetration, or a combination thereof. Raptorial appendages are therefore often covered with spine-like structures (e.g., praying mantis Anderson, 2018; Loxton & Nicholls, 1979; Prete, 1990, mantis shrimp Anderson, 2018; Blanco & Patek, 2014; Dingle & Caldwell, 1978 or several orders of arachnids McLean et al., 2018). Birds that feed on flying insects often have bills with serrated edges (Gosner, 1993; Ornelas, 1994). Similarly, sharp teeth, powerful mandibles and claws allow a firm grip on prey by penetrating it (Anderson, 2018). Some species instead resort to chemical secretions to reduce the mobility of a prey. Secretions may be sprayed onto the body of the prey and glue it to the substrate (comprehensively reviewed in Betz & Kolsch, 2004). Other secretions, venoms, are

injected and act on the nervous system, paralyzing or killing the victim (Casewell et al., 2013).

### 3.1.2 Weapons for defence

The second main context of usage for animal weapons is defence. Inducing pain or other noxious experiences is one of the most efficient strategies to deter predators from pursuing their intention to assault (Schmidt, 1986). To be effective, pain must be caused as quickly as possible, preferably before the predator has inflicted harm on the defending animal. Examples of active counterattacks through mechanical means are bites (most mammals Caro, 2005, squamates Green, 1988, arthropods Eisner et al., 2005) or pinches (e.g. crustaceans Wasson & Lyon, 2005, arachnids including scorpions van der Meijden et al., 2013, insects Eisner et al., 2005; Emlen, 2008), scratching (amphibians Blackburn et al., 2008, mammals Caro, 2005, squamates Green, 1988), stabbing (e.g. ungulates Emlen, 2008; Stankowich & Caro, 2009, swordfish Penadés-Suay et al., 2017), urticant bristles (e.g. spiders Battisti et al., 2011, millipedes Eisner et al., 1996, 2005) and flagellation (e.g. squamates Arbour & Zanno, 2018; Green, 1988). Passive mechanical defences may include urticant setae (e.g. lepidopteran larvae Battisti et al., 2011), spines (e.g. Echinoderms Anderson, 2018; Inbar & Lev-Yadun, 2005, mammals Anderson, 2018; Stankowich & Campbell, 2016) and fishes (Anderson, 2018; Reimchen, 1988) or hard and pointed scales (e.g. squamates Agosta & Dunham, 2004; Broeckhoven et al., 2015; Pianka & Pianka, 1970) that can severely harm a predator if it attempts to handle, bite or ingest it (Broeckhoven et al., 2015; Reimchen, 1988), or at least increase handling time to a point to be unprofitable for the predator (Pyke, 1984).

Beyond causing mechanical damage, a uniquely rapid and remote way to cause pain is by electric shock (e.g., eels Catania, 2019, electric rays Bennett et al., 1961). Predators can also be deterred by substances that cause pain or are otherwise noxious. Such secretions may be sprayed towards the predator/attacker, like in spitting cobras ( Westhoff et al., 2005; Guido Westhoff et al., 2010), bombardier beetles (Eisner, 1958; Eisner & Meinwald, 1966; Emlen, 2014), vinegarroons (Eisner et al., 1961; 2005), scorpions (Newlands, 1969; Nisani & Hayes, 2015) and some species of millipedes (Eisner et al., 1978) and ants (Touchard et al., 2016). Alternatively, noxious chemicals may be secreted from glands located in the skin, like in many amphibians (Daly, 1995; Nelsen et al., 2014). Finally, noxious secretions may be delivered into the predator's body through specialized structures like stingers (e.g. hymenopterans Schmidt, 2019, scorpions van der Meijden & Kleinteich, 2017; Zhao et al., 2016 or stingrays Barbaro et

al., 2007), fangs and mouthparts (e.g. centipedes Undheim et al., 2015, spiders Foelix, 2010 and snakes du Plessis et al., 2018), spines like in many fishes (Anderson, 2018; Diaz, 2015; Gwee et al., 1994) and nettle cells in cnidarians (Jouiaei et al., 2015) and bony protrusions in amphibians (Heiss et al., 2010; Jared et al., 2015). In these cases, mechanical damage is augmented with a chemical agent.

Other secretions instead have the objective of repelling or confounding rather than hurting a predator. Many insects and arachnids secrete unpalatable quinone or phenolic-based substances (Eisner & Meinwald, 1966; Eisner et al., 2005). Squamates and mustelids can release repelling secretions (Arbuckle et al., 2013; Sherbrooke & Middendorf, 2001; Wright & Weldon, 1990), while cephalopods and sea hares confound attackers by spraying ink (Derby, 2014; Kicklighter et al., 2005).

### 3.1.3 Weapons used in sexual contests

Weapons that often develop as secondary sexual characters are used to obtain or defend reproductive resources, and/or to coerce sexual partners. In dyadic fights, morphological weapons are used in stabbing (e.g., many bovids Emlen, 2014; Geist, 1966; Lundrigan, 1996; Stankowich, 2012; Stankowich & Caro, 2009, narwhals Graham et al., 2020; Silverman & Dunbar, 1980, walruses Miller, 1975, elephants Emlen, 2014, rhinoceros beetles McCullough et al., 2014) ramming or pushing (many bovids Emlen, 2014; Geist, 1966; Lundrigan, 1996; Stankowich, 2012, dung beetles Emlen, 2008, 2014), flipping the opponent over (e.g. stag beetles Emlen, 2008, 2014; Goyens et al., 2016, tortoises Mann et al., 2006) or grappling (cervids Emlen, 2014; Geist, 1966; Lundrigan, 1996; Stankowich, 2012, crabs Levinton & Allen, 2005, arachnids McLean et al., 2018; Tallarovic, 2000). The use of chemicals weapons to solve intra-sexual contests is particularly rare and only known in platypus (Wong et al., 2012), amphipods (Takeshita & Wada, 2012) and loris (Nekaris et al., 2020; Rode-Margono & Nekaris, 2015).

### 3.1.4 The weapons of scorpions

Scorpions belong to one of the eleven extant orders of arachnids and are easily recognizable from the other members of the arachnid class by their special set of weapons. Whereas many animals have a single weapon, scorpions possess two separate weapons systems. The pincers or pedipalps, which are oral appendages located at the front of the body, and the venom-carrying stinger or telson at the caudal end of the body. They each have a different mode of action: mechanical and chemical respectively. Both weapons are used in all three contexts of usage: predation, defence

and sexual contests. The approximately 2,700 species currently described (Https://Www.Ntnu.No/Ub/Scorpion-Files/) use these two weapons systems in different ways or to different degrees in each of the three contexts of usage, which is reflected in their morphological diversity.

Scorpions are probably among the most ancient arthropods who made a full transition from water to a land-living lifestyle (Dunlop et al., 2013; Howard et al., 2019; Wendruff et al., 2020). In addition, their body plan almost did not change since the Silurian (443-419 Mya) (Waddington et al., 2015). They successfully colonized all the continents except Antarctica, which illustrates their extraordinary capacity to adapt to different and sometimes hostile environments, and the versatility of their body plan.

## 3.2 Scorpion weapons

Knowing the inner structure of an anatomical feature is fundamental to understand its performance and limitations. In this section, we will first review the anatomy literature on the two weapons systems of scorpions: their pincers or pedipalps, and venom delivery system consisting of the telson at the end of the flexible metasoma. Particular focus will be given to the musculoskeletal system and how is linked with the structure's role as a weapon in different contexts. We will only mention other, non-weapon functions, such as the sensory function of both the pedipalps and metasoma, in passing. A separate paragraph will be then dedicated to the production, composition and evolution of scorpion venom.

### 3.2.1 Overall anatomy

As in all arachnids, the scorpion body can be divided in two tagmata: prosoma and opisthosoma (See Figure 3.2.1). The prosoma works functionally like a head, containing the several sensory organs and major ganglia of the nervous system. All six pairs of appendages are attached to the prosoma. The first pair are used for feeding and forms the mouth parts or chelicerae. The second pair forms the pedipalps ending in the pincers or chelae. The other four appendages are the walking legs, used for locomotion. The body beyond the prosoma, the opisthosoma, is subdivided in mesosoma and metasoma. The mesosoma is the anterior portion of the opisthosoma and contains the sexual organs, the specialized sensorial pectines, four pairs of book lungs, the cardio circulatory system, and the post-prosomal portion of the digestive tract. The metasoma is a tail-like elongation of the body containing the hindgut and carrying the venomous stinger (telson).

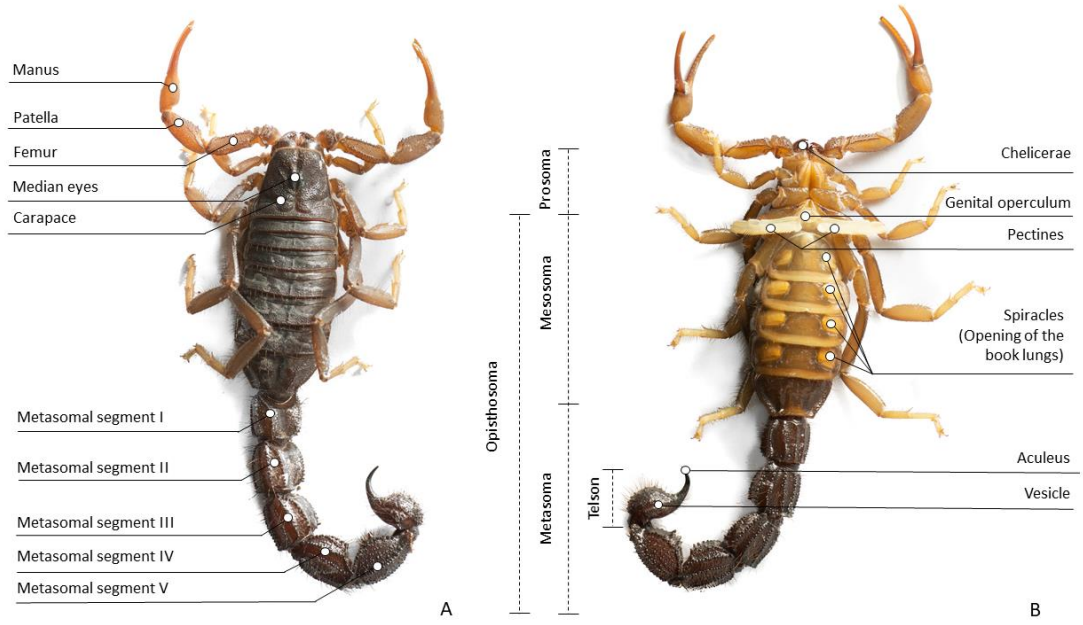


**Figure 3.2.1** Overall anatomy of a scorpion (*Parabuthus transvaalicus*, Buthidae). A) Dorsal view. B) Ventral view.

### 3.2.2 The pincers

The pedipalps are modified appendages developing very early in embryogenesis. Initially situated posterior to the cephalic lobes, pedipalp lobes gradually move anteriorly during the later stages of the embryonic development. After the posterior-anterior migration, the segments forming the pedipalps are recognizable (Farley, 1998; 2001).

The last two segments of the pedipalp, namely the manus (or tibia) and the movable finger (or tarsus) form the chela. The manus includes the fixed finger and most of the closing muscles (Dubale & Vyas, 1968; Gilai & Parnas, 1970; Mathew, 1965) (See Figure 3.2.2). The movable finger acts as a first-class lever system; it rotates on a fixed axis formed by two joints located at the antero-ventral side of the manus, determining the axis of rotation for the opening and closing of the chela. Although scorpion musculature was first described by Lankester (1885), pedipalp musculature was not included. Gilai and Parnas (1970) reported the existence of three main bundles of closing muscles in the manus of *Leiurus quinquestriatus* (Buthidae). Dubale et al (1968) recognized eight muscle bundles in the “tarsus depressor” muscle of the manus in a single specimen belonging to the genus *Heterometrus* (Scorpionidae). Another closing muscle is located in the next proximal segment, the patella (Dubale & Vyas, 1968; Gilai & Parnas, 1970; Snodgrass, 1952). This muscle is composed of long fibered bundles which are mechanically connected to the movable finger by a long ligament (Gilai & Parnas, 1970). In the patella, muscles that adduct and abduct the chela in the frontal plane are also present (Bowerman & Larimer, 1973).

Contrary to crustaceans (Govind et al., 1987; Yamada & Boulding, 1998), scorpion chelae do not have opening muscles (Dubale & Vyas, 1968; Gilai & Parnas, 1970; Mathew, 1965), and the movable finger abduction is due to the elastic recoil of resilin in the joint (Govindarajan & Rajulu, 1974; Mathew, 1965; Sensenig & Shultz, 2004; Snodgrass, 1952). The increasing of hydraulic pressure in the manus and an elastic snap-like recoil given by sclerotized plates (arthrodial sternites) located the dorso-posterior interface of the movable finger and the manus (Alexander, 1967).

On the surface of the cuticle of the chela, several hair-like structures with chemo- and mechano-sensorial functions are present: trichobothria, located on the whole surface of both chelae, and a little group of sensilla on the tip of the fixed finger, known as the constellation array. Trichobothria are important for environmental sensing and detection of air-borne stimuli (Hoffmann, 1967), while the constellation array seems to play a role in the detection of chemical cues (Fet et al., 2006; Nisani et al., 2018). Furthermore, trichobothria placement patterns are extensively used as important taxonomic traits (Soleglad & Fet, 2001; Vachon, 1972, 1974). The cuticle on the sides where the fingers come into contact have rows of metal-enriched and hardened denticles (Schofield et al., 2003), most likely friction-enhancing and grip-improving structures (Dubale & Vyas, 1968; Stockmann, 2015) which, much like trichobothria, are widely used for taxonomic identification (Stahnke, 1970).

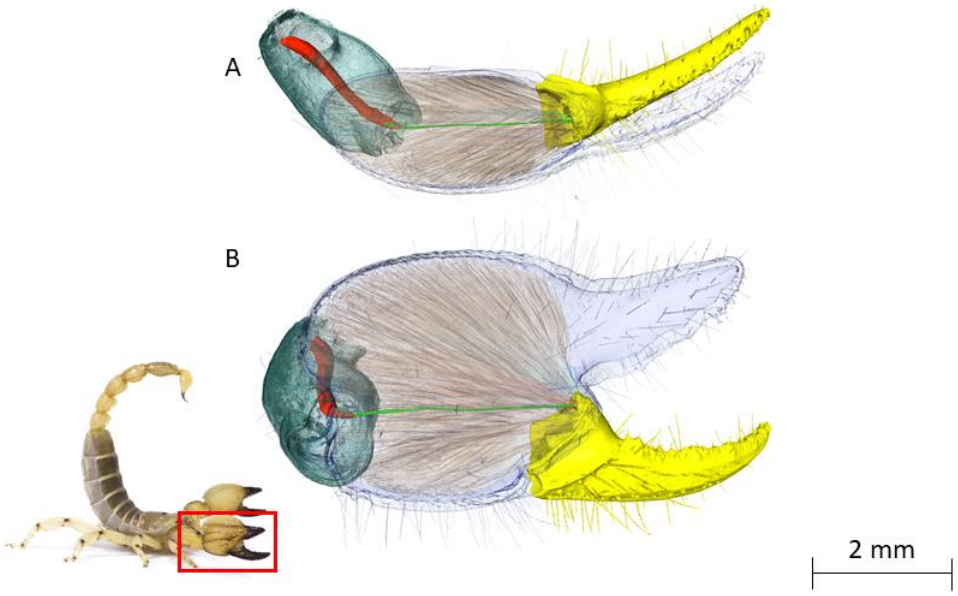


**Figure 3.2.2** Rendering of the chela closing muscles of the species *Scorpio maurus* (Scorpionidae) from synchrotron scan data. A) Dorsal view. B) Lateral view. Apodemes connecting the closing muscle in the manus to the movable finger are not shown. Only the ligament connecting the closing muscle in the patella to the movable finger is shown. Movable finger (yellow), cuticle of the manus (transparent blue), manus closing muscles (transparent orange), closing muscle in the patella (red), ligament connecting the closing muscle in the patella to the movable finger (light green), cuticle of the patella (dark green).

### 3.2.3 Venom delivering system

The unique metasoma is undoubtedly a scorpion's most distinctive feature. Evolved for both predatory and defensive purposes (Sharma et al., 2014), this five-segmented structure carries the venomous telson, which consists of the venom vesicle containing two paired venom glands, and the sharp aculeus (see Figure 3.2.3).

Metasomal segments develop later than anterior appendages due to the anterior-posterior migration of the growing region (Farley, 1998; 2001). In the early stages of development, the metasomal segments are flattened and undifferentiated and the telson is rounded and bilobed. In the later stages of the development, the metasomal segments swell and the telson tapers distally to form the aculeus (Farley, 2001).

The joint architecture and muscle organization together give the metasoma a high degree of freedom of movement (Bowerman, 1972b) which is fully achieved after the second instar (Farley, 2001).

The metasoma musculature has been described in (Lankester, 1885; Snodgrass, 1952) and partially by (Bowerman, 1972a). The last segment of the mesosoma contains antagonistic muscles causing protraction and retraction of the metasoma (respectively, the *median antero-posterior muscle* and the *arthrodio-tergal rectus muscle*), and two muscles originating dorso-laterally and inserting ventrally at the base to the first metasomal segment (the *arthrodio-tergal obliquus muscles*). These last two antagonistic muscles allow the metasoma to be moved laterally. The metasomal segments I, II and III possess the same muscle distribution: ventrally, a large medial bundle (*median antero-posterior muscle*) and two lateral muscles, (*lateral arthrodio-sternal muscles*). All these muscles originate on the anterior segment and insert on the posterior one. Dorsally, there are four muscles originating on cuticle of the anterior segment and inserting at the base of the posterior segment. Two large medial bundles (*arthrodio-tergal rectus muscles*) and two small oblique ones are located at the side of each segment (*arthrodio-tergal obliquus muscles).* Segment IV and V each have a different configuration. Metasomal segment IV presents ventrally both the *median antero-posterior muscle* and the two *lateral arthrodio-sternal muscles*. Dorsally, only the two *arthrodio-tergal rectus muscles* are present but differently from the more anterior segments. These muscles are much more elongated and narrower. These muscles have the function to flex and extend the metasoma (Bowerman, 1972a). The V segment has only a pair of dorsal flexor muscles and a pair of ventral retractor muscles responsible for the movement of the telson (respectively named *arthrodio-tergal rectus muscles lateral* and *arthrodio-sternal*

*muscles*) (van der Meijden & Kleinteich, 2017) (Figure 3.2.3). The telson consists of a bulbous structure, which contains the two venom glands, and which narrows into the aculeus, the curved and sharp stinger through which venom is injected.

The cuticle of the stinger and metasoma is thick and covered by granules and several sensory hair-like structures (Babu & Jacobdoss, 1994; Foelix et al., 2014; Vachon, 1974). In some scorpion species, many cuticular pits containing chemosensory-like setae are present on the ventral and lateral sides of each metasomal segment. This suggests a possible sensory function of the metasoma in these species, similar to the antennae of insects (Fet et al., 2003). Soleglad et al. (2006) identified minute rows of denticles located laterally at the base of the aculeus in some species, called laterobasal aculear serrations. The function of these denticles is still unknown. Some species belonging to three different families (Buthidae, Diplocentridae and Vaejovidae) can present a sub-aculear tubercule (González-Santillán & Prendini, 2015) with important taxonomic value (Stahnke, 1970) but unknown function (van der Meijden & Kleinteich, 2017). Lourenço (2020) suggested that these sub-aculear tubercles may serve as a protection against breakage for particularly long and slender aculei, despite simpler reinforcing strategies, such as aculei with a thicker base, already exist within extant scorpions (van der Meijden & Kleinteich, 2017).

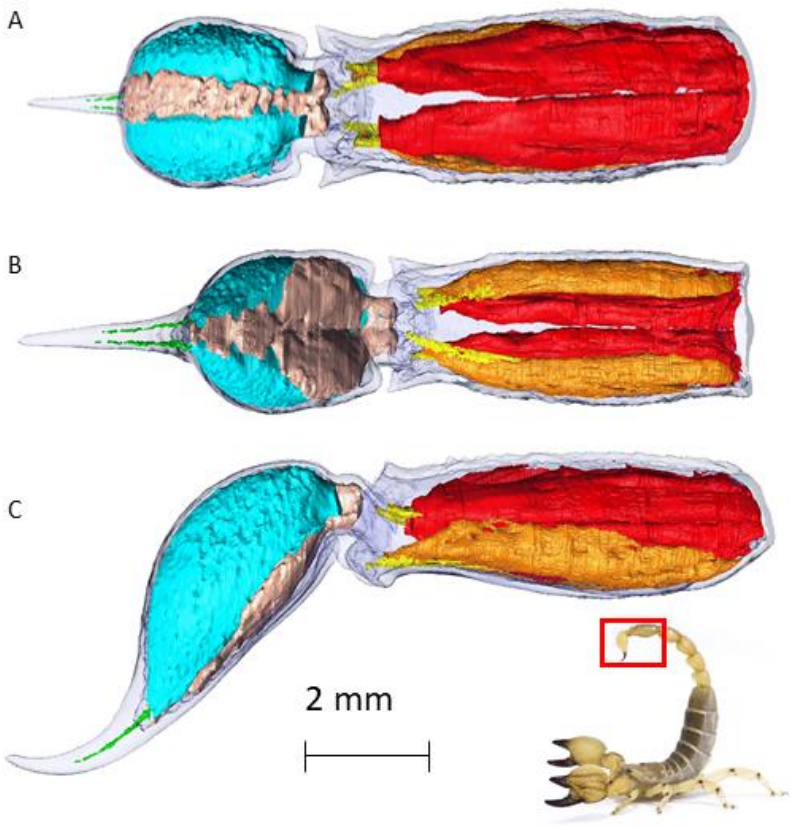


**Figure 3.2.3** Rendering of the internal anatomy of the V metasomal segment and telson from different points of view. A) Ventral view, B) dorsal view, C) lateral view. Note that the telson is dorsoventrally inverted in defensive posture, as in the

inset photograph. Cuticle of both telson and V metasomal segment is in transparent blue. Within the V metasomal segment there are a pair of dorsal flexor muscles (arthrodio-tergal rectus muscles in orange) and the pair of ventral retractor muscles (lateral arthrodio-sternal muscles in red) and the apodemes (yellow) connecting the two antagonistic muscles to the base of the telson. Within the telson are the paired venom glands (cyan) each ending with its venom duct (green) and separated by a layer of muscles (salmon) responsible of the squeezing of the venom gland against the cuticle and permitting the venom to flow inside the gland lumen. The species used for this µCT scan is *Neochactas delicatus* (Chactidae), but the species shown in the inset is *Scorpio maurus* (Scorpionidae).

### 3.2.4 Venom production and secretion

Scorpion venom is produced in the secretory epithelium of the two non-communicating venom glands located in the telson. These glands are surrounded by muscle layers radially, while their lateral portions are directly in contact with the endocuticle of the telson (see Figure 3.2.3). This arrangement is quite conserved in all scorpion families (Keegan & Lockwood, 1971; Mazurkiewicz & Bertke, 1972; Pawlosky, 1924; Sherwan, 2015; Taib & Jarrar, 1993).

Pawlosky (1924) divided the scorpion venom glands into primitive and complex, depending on the absence or presence of folds of the secretory epithelium, respectively. Between the muscles and the venom secretory system, a complex matrix of connective tissue is interposed. In the contact area between the lateral telson cuticle and the secretory epithelium, a single or multiple layers of cuboid cells are visible. Interior to the connective layer and the cuboid cells lies the basal membrane where the conically shaped secreting cells are located (Keegan & Lockwood, 1971; Mazurkiewicz & Bertke, 1972; Pawlosky, 1924; Sherwan, 2015; Taib & Jarrar, 1993). The basal part of these cells contains all the organelles, while the apical part is in touch with the lumen of the venom gland and contains several types of toxin-containing granules. In scorpions, toxin secretion is an apocrine mechanism (Jarrar & Al-Rowaily, 2008; Keegan & Lockwood, 1971; Mazurkiewicz & Bertke, 1972; Taib & Jarrar, 1993; Zlotkin et al., 1978), meaning that a portion of the cytoplasm is also secreted into the lumen of the venom gland. Secretory cells seem to be highly specialized in producing one single type of toxin (Taib & Jarrar, 1993). Each secretory cell contains granules of only one size and type, confirmed by their uniform reaction to laboratory staining techniques (Keegan & Lockwood, 1971; Yigit & Benli, 2008). Additionally, these different types of granules can be selectively stained. The high diversity of these granules in terms of their reaction to histological stains confirms that different cells produce a different product or mixture of products (Taib & Jarrar, 1993; Yigit & Benli, 2008).

The contraction of the muscles surrounding each venom gland causes the squeezing of the whole gland against the cuticle wall, and the consequent conveying of the venom produced by each gland into a cuticular duct (Snodgrass, 1952). Both ducts pass through almost the whole length of the aculeus, ending independently before the tip of the aculeus. The ovoidal openings, similar to those of hypodermic needles, are found on the dorsal side of the aculeus (Foelix et al., 2014; Halse et al., 1980; Polis, 1990; Yigit & Benli, 2008, 2010; Zhao et al., 2016). Some authors reported that in *Androctonus crassicauda* (Buthidae) (Jarrar & Al-Rowaily, 2008), *Centruroides sculpturatus* (Buthidae) (Mazurkiewicz & Bertke, 1972) and *Leiurus quinquestriatus* (Buthidae), the ducts fuse in the terminal part of the aculeus and end in a unique pore.

#### *3.2.4.1 Composition and main toxin families*

When extracting venom from scorpions, especially when electrostimulation is avoided, it is possible to observe a transparent to milky-opalescent transition in the venom coming out of the aculeus (Inceoglu et al., 2003; Nisani & Hayes, 2015; Yahel-Niv & Zlotkin, 1979; Zlotkin & Shulow, 1969). The transparent portion of the venom is generally referred to as pre-venom, while the milky, opalescent portion is considered to be the “true” venom (Inceoglu et al., 2003). The pre-venom and true venom present differences in chemical composition, with pre-venom being richer in ions than the true venom, but not in proteins (Inceoglu et al., 2003; Yahel-Niv & Zlotkin, 1979). These differences in composition between pre-venom and true venom are likely related to the differences in their appearance. The two fractions also cause different physiological effects when injected, with pre-venom apparently being less toxic than the overall more effective true venom, but still able to induce paralysis and pain (Inceoglu et al., 2003). The dichotomy between pre-venom and true venom may be an oversimplification of a continuous or semi-continuous range, with some authors identifying more than two types of venom (Yahel-Niv & Zlotkin, 1979).

The protein composition of scorpion venom is particularly complex, with more than 4,500 toxins identified so far across all studied species (Grashof et al., 2019). Nevertheless, scorpion venoms are not only rich in proteins, but also in nucleotides, amines (serotonin or histamine) and mucopolysaccharides, probably due to the apocrine secretion mechanism of the venom gland cells. However, the role these molecules play in toxicity has yet to be clarified. Previous studies have mainly focused on the peptide components of scorpion venoms. Although a complete overview of the scorpion venom literature is outside the scope of this review, in this paragraph we will highlight some of the best known and important classes of bioactive peptide compounds present in scorpion venoms. These cover about the 75% of the total transcripts obtained from

venoms of 37 species belonging to seven different families. The remaining quarter of venom compounds is composed of molecules for which either structural domains and/or function are unknown (Cid-Uribe et al., 2020). For a more comprehensive review of scorpion toxins refers to (Cid-Uribe et al., 2020; Possani et al., 1999; Quintero-Hernández et al., 2013; Rodríguez de la Vega et al., 2010; Rodríguez De La Vega & Possani, 2005).

Four main groups of bioactive compounds are currently known to be present in scorpion venoms: ion-channel binding peptides, enzymes, protease inhibitors and host defence peptides (HDP). Proteomic and transcriptomic analyses performed on venoms and venom glands of scorpions belonging to different families have shown that the most abundant fraction of peptides in scorpion venoms is represented by the superfamily of ion-channel binding toxins. Short-chain ion-channel binding toxins (from 20 to 50 amino acid residues) are specifically active on voltage-gated $K^+$ channels. Depending on their length and their folding differences, this class of peptides is further subdivided into several subfamilies. A dedicated database of scorpion toxins active on $K^+$ channels (KScTx) is available at Kuzmenkov et al. (2016). The peptides that bind to voltage-gated $Na^+$ channels (NaScTx) have longer chains (50 to 80 amino acid residues). These are classified into α-NaScTx and β-NaScTx, depending on the receptor site they bind to. Calcium channels are targeted by specific scorpion venom peptides known as calcins. These peptides generally compete with the natural ligands of the $Ca^{2+}$ channels affecting muscular contraction (Quintero-Hernández et al., 2013). The last members of the family of the ion-channel binding peptides are chlorotoxins (ClTx), altering the conductance of $Cl^-$ channels. The toxicological effects of chlorotoxins have been poorly studied so far, but many studies have focused on the potential medical applications of this class of small peptides, especially for the imaging and treatment of aggressive forms of brain cancer generally known as glioma (Cohen et al., 2018; S. Lyons et al., 2002; Zhao et al., 2019).

Another abundant class of active components of scorpion venoms are the enzymatic toxins. This class of molecules is not as abundant as in other venoms (e.g., snake venom), but its contribution to scorpion venom toxicity is not to be neglected. Phospholipases disrupt the phospholipids in the membranes of cells, causing the lysis of haemocytes and the development of oedemas and release of pro-inflammatory compounds (Krayem & Gargouri, 2020). Another class of enzymes contained in the venom of several scorpion families are the metalloproteases. These proteases are characterized by a bivalent metallic ion (usually the zinc cation) and promote the hydrolysis of proteins present on the cellular membrane. One of the most extensively studied scorpion venom metalloproteases is Antarease. Isolated for the first time from the venom of the Brazilian scorpion *Tityus serrulatus* (Buthidae), this enzyme causes the

hydrolysis of the protein regulating the cleavage of the transport vesicles, thus affecting extra-cellular transports, especially in secretory organs like the pancreas (Ortiz et al., 2014). Hyaluronidases represent another class of enzymes widely present in scorpion venom. These enzymes allow a rapid diffusion of the other bioactive compounds through tissues by degrading the extracellular matrix (Bordon et al., 2015). Even if many of the symptoms generated by scorpion envenomation have a neurotoxic nature, envenomation by species belonging to the medically important genus *Hemiscorpius* (Hemiscorpiidae) represent an important exception. The venom obtained from species belonging to this genus is more similar to snake venom because of the large fraction of enzymes and proteins, and the relatively small fraction of small peptides with affinity for voltage-gated ion channels (Dehghani et al., 2018; Kazemi-Lomedasht et al., 2017). Venoms produced by *Hemiscorpius* species mainly cause cytotoxic effects and can cause fatal envenomation while causing little to no pain (Mirakabadi, 2013).

Protease inhibitors are important components of scorpion venoms that selectively degrade the envenomated organism's proteases, thus preventing the degradation of the venom peptides injected into the organism's body, and thereby increasing venom efficacy and efficiency (Ma et al., 2016).

Host Defence Peptides (HDPs) are a large family of small peptides that has been found in all arthropods (Sabiá et al., 2019). They are related to the innate immune defences of these animals, as most of these peptides have antimicrobial action (Harrison et al., 2014). This class of proteins is generally divided into two main groups, according to the presence or absence of cysteines in the aminoacidic residual chain (Cid-Uribe et al., 2019). The effect of this relatively abundant class of peptides on scorpion venom toxicity is still not clear, but cases of haemolysis due to HDPs found in the venom of *Pandinus imperator* (Scorpionidae) have been reported (Zeng et al., 2013), possibly suggesting a disruptive effect on blood-clotting mechanisms.

### 3.2.5 Scorpion venom evolution

Venoms evolved independently several times in only a small number of animal taxa (Casewell et al., 2013) where it may be used differently. Venom may have different ecological roles, and the main drivers of its evolution can be different among taxa. In snakes, for instance, it is widely accepted that venom evolved mainly for predatory purposes (Daltry et al., 1996; Lyons et al., 2020; Ward-Smith et al., 2020). Venom may have evolved due to other demands in different groups. Self-defence has been proposed as driver of venom evolution in fishes and wasps (Harris & Jenner, 2019; Walker et al., 2019), intra-specific competition in venomous mammals (Rode-Margono & Nekaris,

2015), possibly mating behaviour in scorpions (Francke, 1979; Polis, 1990; Tallarovic et al., 2000) and even for an antimicrobial function in bees (Baracchi et al., 2011).

The origins of scorpion venom represent an ongoing debate. One of the most widely accepted hypotheses is that toxins may have originated from innate, non-toxic peptides, after a process of gene duplication and neofunctionalization and/or exon shuffling (Brust et al., 2013; Hargreaves et al., 2014; Lynch, 2007; Pineda et al., 2020; Vonk et al., 2013; Wang et al., 2017; Whittington et al., 2008; Wong & Belov, 2012; Zhang et al., 2015). For example, some toxins belonging to the KScTx group present a very high level of structural similarity with defensins and HDPs are associated with the innate immune system of arthropods (Bontems et al., 1991; Froy & Gurevitz, 2004; Meng et al., 2016; Zhu et al., 2014). The high similarities between these KScTx toxins and defensins has been used to generate toxigenic compounds able to bind $K^+$ channels by modifying a key sequence in defensins (Meng et al., 2016; Zhu et al., 2014). The opposite transition, from KScTx to defensin has been observed through experiments of mutagenesis of the genes coding for potassium voltage-gated channel-binding peptides (Zhu et al., 2010). Interestingly, many toxins with high structural similarity with defensins have been found in other venomous taxa as well (reptiles and mammals), increasing the interest in this class of peptides as an evolutionary ancestor of many different toxins (Fry et al., 2009; Whittington et al., 2008).

### 3.2.6 Scorpionism

"Scorpionism" is the word that is commonly used to refer to fatal envenomation caused by scorpion stings (Lourenço & Cuellar, 1995). Annually, around 1.2 million people are stung by scorpions worldwide, and around 3,250 incidents result in fatal envenomation (Chippaux & Goyffon, 2008). Incidents are mainly concentrated in tropical countries, where scorpionism is an important but still neglected health issue. The hotspots of scorpionism are in Saharan Africa, the southern and eastern regions of Africa, the Middle East (mainly Iran and Turkey), south India, Mexico, Brazil, and the Amazonian basin area (including the Guianas, Venezuela, and northern Brazil) (Chippaux & Goyffon, 2008; Santos et al., 2016). Ward (2018) classified 104 species as potentially harmful (101 Buthidae, 2 Hemiscorpiidae and 1 Scorpionidae), but for only 32 of these fatalities were reported.

## 3.3 Ecological role of scorpion weapons in feeding, defense and intraspecific agonism

In this section we will review how both scorpion weapon systems are used in three main contexts of usage: feeding, defence and reproduction.

### 3.3.1 Scorpion weapons in feeding

Scorpions are nocturnal generalist predators feeding on a wide spectrum of different prey, consuming mostly arthropods, but also including small mammals and reptiles (Castilla, 1995; McCormick & Polis, 1982; Polis, 1990; Polis & McCormick, 1986). To our knowledge, only two scorpion species are known to have a somewhat specialised diet, apparently preferring spiders as prey items (Main, 1956; Toscano-Gadea et al., 2006). Data about scorpion diet and feeding ecology in the wild is generally sparse (Davison et al., 2020; Kaltsas et al., 2008; Mcreynolds, 2020; Miranda et al., 2015; Polis, 1979; Rodríguez-Cabrera et al., 2020). Therefore, most of the diet data is based on observations of wild or captive scorpions. Despite this lack of data about diet and feeding ecology, feeding behaviour has been studied in almost all scorpion families. Scorpions have very limited vision (Polis, 1990) and prey localisation mainly relies on the detection of vibrations and chemical cues. To detect soil-borne vibrations, scorpions rely on slit sensilla, which are mechanoreceptors present on the tarsi of their walking legs (Barth & Wadepuhl, 1975; Brownell & Van Leo Hemmen, 2001; Brownell & Farley, 1979a, 1979b; Couzjin, 1975), and the chemo-mechanic receptors on the pectines, which are also used to detect chemical cues (Abushama, 1964; Brownell & Farley, 1979a; Carthy, 1966; Cloudsley-Thompson, 1955; Edmunds & Sibly, 2010; Foelix & Müller-Vorholt, 1983; Han et al., 2017; Mineo & Del Claro, 2006; Warburg, 1998; Wolf, 2017). Scorpions seem not to use trichobothria to locate their prey by vibration (e.g., a walking prey) (Murayama & Willemart, 2019).

Once the prey has been detected, scorpions always use their chelae to grab it. In experiments where both chelae were blocked with wax, scorpions managed to grasp the prey using only the chelicerae (Colombo, 2015), showing remarkable plasticity of their predatory behaviour repertoire (Simone et al., 2018). Once the prey has been grasped, scorpions may or may not use the stinger to inject venom in their prey to subdue it (Bub et al., 1979; Casper, 1985; Polis, 1979; Rein, 1993, 2003; Stahnke, 1966). Stinger use in scorpion feeding behaviour is highly correlated with prey size (Edmunds & Sibly, 2010; Pocock, 1893; Rein, 1993; Rosin & Shulow, 1963), resistance (Albín & Toscano-

Gadea, 2015; Edmunds & Sibly, 2010; Rein, 2003; Stahnke, 1966), the ontogenetic state of the scorpion (Casper, 1985; Cushing & Matherne, 1980) and chela morphology, with species with robust chelae seldom using the stinger to subdue their prey, using only crushing force to incapacitate the prey (Casper, 1985; Jiao & Zhu, 2009; Mcdaniel, 1968; Schultze, 1927; Stahnke, 1966; Williams, 1987).

When a scorpion stings the prey, the telson is projected anteriorly with the metasoma, and the aculeus repeatedly touched to the body of the prey until a soft spot suitable for piercing is found (Albín & Toscano-Gadea, 2015; Bub et al., 1979; Fabre, 1923; Polis, 1979; Sarhan et al., 2013; van der Meijden & Kleinteich, 2017). Several authors described that, after the first sting, scorpions remain motionless for several minutes, most likely waiting for the neurotoxic effects of the injected venom (Bub et al., 1979; Edmunds & Sibly, 2010; Sarhan et al., 2013; Stewart, 2006). If the prey keeps struggling, further stinging events can be observed (Sarhan et al., 2013; Simone et al., 2018; Southcott, 1955). Once the prey is successfully incapacitated, scorpions use their chelae to further manipulate it, with several studies showing that scorpions prefer to orient the prey with the head towards their chelicerae before starting to consume it (Alexander, 1972; Bub et al., 1979; Polis, 1979; Sarhan et al., 2013; Schultze, 1927).

The venom is mainly used for prey incapacitation rather than killing the prey. In many insect prey, the loss of muscle control subsequent to scorpion venom injection is evident (Eitan et al., 1990; Zlotkin et al., 1985; 1987). Two different types of paralysis induced by the injection of scorpion venom have been described: one is characterized by involuntary contractions of the muscles, while the other is a flaccid paralysis through inhibition of muscle contractions (Zlotkin et al., 1971). These neurotoxic effects are mainly provoked by toxins with high affinity to ion-binding voltage-gated channels (Arnon et al., 2005; Cohen et al., 2007; Gordon et al., 1996; Gurevitz et al., 2007; Liu et al., 2005; Possani et al., 1999; Quintero-Hernández et al., 2013; Strugatsky et al., 2005; Zhang et al., 2015). Within the members of the two main families of NaScTx, for example, we can find specific toxins that are highly toxic only to insects (Borchani et al., 1997; Eitan et al., 1990; Froy et al., 1999), toxins that have a high affinity to murine sodium voltage-gated channels (Gordon et al., 1996; Jover et al., 1978; Legros et al., 2005), and toxins that show similar affinity for both insect and murine ion channels (Gordon et al., 2003; Kopeyan et al., 1993). This differential affinity of venom compounds, and the fact that scorpions are both prey and predators, can potentially explain the differences in toxicity that scorpion venom have on different target organisms (van der Meijden et al., 2017). The calculation of the median lethal dose ($LD_{50}$), the dose of a venom needed to kill 50% of the test population, is a technique widely used to quantify venom potency (Trevan, 1927). As scorpions are both prey and predators, measuring the $LD_{50}$ on

different target organisms is needed to investigate the toxicity of scorpion venom for both defensive and offensive purposes. Zlotkin and colleagues (1971) calculated the $LD_{50}$ of the venom of several species of buthids on two different target organisms and found that when venom from the species *Buthus occitanus paris* (Buthidae) was injected into fly larvae, the $LD_{50}$ calculated was the lowest (i.e., highest toxicity), while the same species had the highest $LD_{50}$ in mice. Similar results have been provided by numerous other studies (Kopeyan et al., 1993; van der Meijden et al., 2017; van der Valk & van der Meijden, 2014; Zhu et al., 2016; Zlotkin et al., 1971), showing that $LD_{50}$ results are only indicative of relative toxicity in the species that was tested, and provide little indication of toxicity in other, even relatively closely related species. Studies on the ecological relevance of scorpion venom should therefore be carried out on the presumed natural target species.

Venom is considered a fast-changing phenotype (Amazonas et al., 2019). Snake venom, for example, has been seen to change in composition depending on factors like alterations in the animal's physiological state and diet (Casewell et al., 2020; Mackessy, 2010). In recent years, changes in scorpion venom composition and production following diet alterations have been recorded (Berto Pucca et al., 2014; Tobassum et al., 2018). Pucca (2014) observed different peaks in venom profiles obtained from scorpions belonging to the same species fed with different types of prey, suggesting rapid adaptation of venom composition to different prey types. Similarly, Tobassum (2018) divided scorpions belonging to the same species into groups and fed each group with a different type of prey, observing significant differences in the volume of venom extracted from each group after the same starvation period, suggesting that some prey items are preferable when higher volume of milked venom is required.

In the species *Centruroides vittatus* (Buthidae), venom toxicity and composition change depending on the ontogenetic state of the animals. Juveniles appear to have less deadly (higher $LD_{50}$) venom than the adults, at least when using crickets as a target species. This may be mediated by a quantitative rather than qualitative change in expression of the different toxins with ontogenetic state (McElroy et al., 2017). Additionally, scorpions may select different prey according to the amount of venom in their venom glands. Scorpions from which the venom was extracted less than 24 hours before, avoided feeding on larger prey (Silva et al., 2019).

In other pincered taxa, feeding ecology is an important driver for the evolution of the weapons that first touches the food. In decapods (Crustacea) for example, diet seems to be the main factor determining differences in chela morphology and size (Elner & Campbell, 1981; Seed & Hughes, 1995; Taylor, 2001; Vermeij, 1977; Yamada & Boulding, 1998). In scorpions, no clear evidence of a similar correlation has been

provided yet. However, there may be some rationale to consider diet as a possible driver of scorpion chela evolution. Between different scorpion families, and sometimes even between species belonging to the same genus, chela shape can range from having a stout and robust manus and short fingers to a very slender manus and elongated fingers (see Figure 3.3.1). Such differences in shape are highly correlated with differences in performance. Pinch force in scorpion species with stouter chelae is much higher than that measured in species having slender chelae (van der Meijden et al., 2010). The strongest species also have thicker cuticle (Kellersztein et al., 2019), probably to withstand the higher stress generated during maximum biteforce (van der Meijden et al., 2012). Rupturing the exoskeleton of hard-bodied prey requires exerting a significant amount of force, a feat that scorpions with slender chelae may not be able to accomplish without risking breaking their fingers (van der Meijden et al., 2012). Lamoral (1971) reported *Opistophthalmus carinatus* (Scorpionidae), a very stout-pincered scorpion, sporadically feeding on terrestrial hard-shelled crustaceans when no other food source is available. Baerg (1954) reported that the fine-pincered *Centruroides insulanus* (Buthidae) feeds on scarab beetles only if these are deprived of the hard elytra. It therefore seems that chela morphology, via performance, may limit feeding on harder prey.

Whereas in scorpions robust and slender chelae occur in separate species or sexes (see below), in several members of the order Decapoda (Crustacea), a single individual can have one robust (the “crusher”) and one slender chela (the “cutter”) (Brown et al., 1979; Govind, 1989; Schenk & Wainwright, 2001). The crusher chela produces a larger pinch force than the cutter and is mainly used to crack and break the hard shells of the prey, while the cutter chela is mainly used for feeding and prey manipulation (Vermeij, 1977). Decapod species feeding on motile, soft-bodied prey, have more elongated and slender chelae (Seed & Hughes, 1995). A similar functional specialization may underlie the chela diversity seen in scorpions. Although scorpions do not have different chela morphologies within one individual as some decapods do, the shape of the chelae can differ between sexes and between species. As in decapods feeding on more fleeting and soft-bodied prey, long chelae may aid in prey prehension by allowing a larger gape at the same opening angle, and a higher closing speed of the tips of the fingers, all else being equal. From consideration of lever mechanics, longer fingers (i.e., a longer out-lever) provide a lower mechanical advantage, therefore less force is transmitted from chela muscles to the tip of the movable finger. We recently found a negative correlation between pinch force and chela closing speed (Simone & van der Meijden, 2018), which means that species with a stronger grip are also slower (Figure 3.3.4). Faster chelae may be a suitable weapon to hunt fast prey, but lever mechanics

limit the maximum pinch force, and thus restrict the bearer to soft rather than hard-bodied prey. The negative relationship between chela pinch force and closing speed may thus lead to a functional trade-off (Arnold et al., 2011) and allow, or may even be driven by, niche partitioning between different species of scorpions.

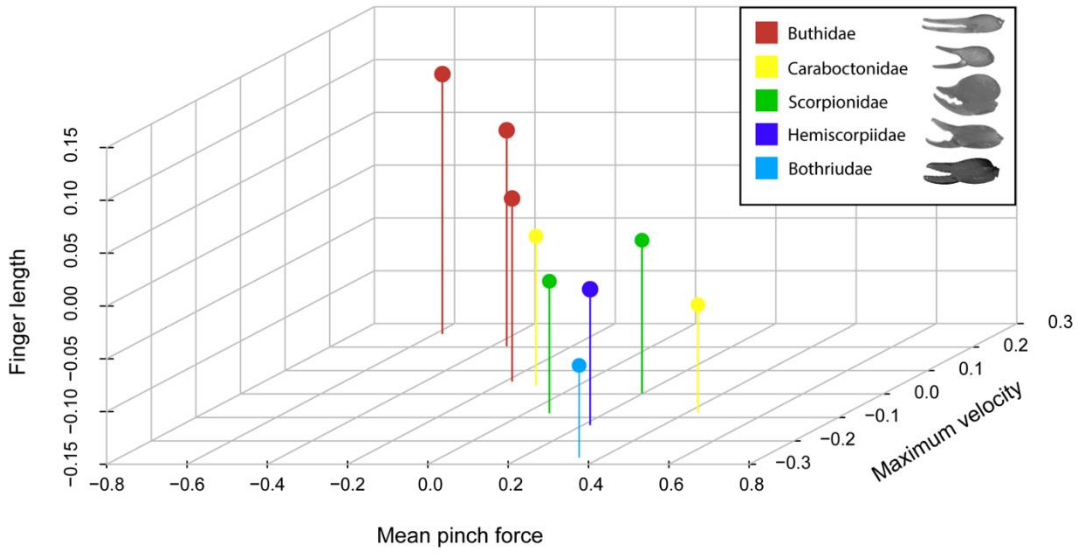


**Figure 3.3.1** 3D graph showing the relationships between chela morphology and performance. On the X-axis is the pinch force (corrected for overall body size), on the Y-axis the size-corrected finger length and on the Z-axis the size-corrected maximum closing speed of the chela. A representative member of each family is shown in the legend: *Hottentotta gentili* (Buthidae), *Caraboctonus keyserlingi* (Caraboctonidae), *Pandinoides cavimanus* (Scorpionidae), *Hadogenes paucidens* (Hemiscorpiidae), *Bothriurus chilensis* (Bothriuridae). Species with relatively longer fingers are faster but also weaker than species having shorter fingers. Since the variables have been corrected for overall body size, no units can be given with the axes.

### 3.3.2 Defensive behaviour

The chelae and the venom delivery system are not only useful weapons for apprehending and incapacitating prey but also very efficient weapons employed in active defence. Experimentally eliciting a defensive response in scorpions can be simple: disturbing a scorpion is usually enough to cause it to show defensive behaviour (Coelho et al., 2017; Miller et al., 2016; Palka & Babu, 1967; van der Meijden et al., 2013), and the most excitable species just need to feel a puff of air to elicit stinging behaviour (Nisani & Hayes, 2015). A more intense attack may be simulated by touching crucial body parts, such as the prosoma (Nisani & Hayes, 2011; Rasko et al., 2018; van der Meijden et al., 2015). Defensive behaviour has been shown to differ between species (Newlands, 1969; van der Meijden et al., 2013; Warburg, 1998), perceived threat level (Lira et al., 2017; Nelsen et al., 2020) and sex (Carlson et al., 2014; Lira et al., 2020; Miller et al., 2016).

It is important to point out that defensive stinging is very different from predatory stinging (Coelho et al., 2017; van der Meijden & Kleinteich, 2017). Defensive strikes are

much faster than predatory ones, and do not include the exploratory touching described above. Depending on metasoma morphology, a defensive strike can have an open or folded trajectory (Coelho et al., 2017). Species with a muscular or elongated metasoma tend to be faster and have a more open trajectory. Closed trajectories have been observed in species having shorter pedipalps. However, comparative motion shape analysis is in its infancy, and the reasons for these differences in trajectory shape between species are not yet understood.

Likewise, defensive pinching behaviour seems to be different from the predatory grasping of prey. While all scorpions always use their chelae in prey prehension, not all species use their chelae in every defensive case, but sometimes limit their defensive response to stinging only (van der Meijden et al., 2013). Warburg (1998) recorded scorpions from different families fighting each other to observe the different strategies used in intra-guild competition and predation. He observed that scorpion species that have strong chelae rarely used their stinger and were less prone to sting, while species with more slender chelae controlled the opponent with the chelae but were always searching for a suitable spot to sting. This may indicate some functional trade-off or compensation in defensive use. Some scorpion species use their stout chelae as protective shields, placing them in front of the chelicerae to protect the head from frontal attacks (Harington, 1978; Newlands, 1969). Some of these species are burrowers and rock crevice-dwellers and use their large chelae to prevent unwelcome visitors to access their burrow (Newlands, 1969).

The use of weapons in defensive behaviour is correlated with the perceived threat level and may be different between the two sexes. A more intense threat tends to increase the frequency of defensive stinging (Lira et al., 2017; 2020; Nelsen et al., 2020; Nisani & Curiel, 2019) and, in some cases, also the volume of venom delivered ((Nisani & Hayes, 2011). However, the opposite trend has also been recorded (Rasko et al., 2018). In this study, the authors tested how the scorpion, *Hadrurus arizonensis* (Caraboctonidae), applied its venom defensively during a simulated repeated attack, consisting of 10 consecutive challenges. They found that, surprisingly, the tested specimens invested more venom in the early phases of the threat and that the average volume delivered after 10 consecutive stings was only the 8% of the total yield obtained prior to the beginning of the experiments. Inter-sexual differences have been reported in scorpion defensive behaviour. In the sexually dimorphic species *Centruroides vittatus* (Buthidae), females show higher stinging frequency than males when the metasoma was grabbed to elicit a defensive response (Carlson et al., 2014). However, comparable experiments performed with the similarly sexually dimorphic species *Tityus pusillus*

(Buthidae) and *Vaejovis carolinianus* (Vaejovidae) showed no differences in stinging frequency between sexes (Lira et al., 2020; Nelsen et al., 2020).

The defensive sting is known to sometimes take place without any venom expenditure (a “dry sting”). Dry stings are reported in different venomous taxa and associated with defensive behaviour (Herzig, 2010; Naik, 2017; Pucca et al., 2020). Even if venom is an efficient weapon in defence, it comes with a high energetic cost and its expenditure has to be carefully metered (Morgenstern & King, 2013). Replenishment of the venom glands increases metabolic rate by 21% to 39% for a minimum of 72 hours (Nisani et al., 2007, 2012). This may temporarily make the scorpion a less-efficient predator and make it more vulnerable to potential attackers (Lira et al., 2017; Nisani et al., 2007; Silva et al., 2019). Dry stings, by inflicting pain through mechanical damage, may therefore save the energetically costly resource of venom, while still delivering a painful warning (Anderson, 2018).

Pain induction is one of the most common strategies applied by living organisms to deter a predator/attacker. Pain could be useful for defensive purposes but, when considering predation, fast pain induction could represent an evolutionary conflict. While on one hand pain induction could be a good strategy to deter predators from pursuing their attack (Eisner & Camazine, 1983; Schmidt, 2004), on the other hand pain could enhance prey struggling and make establishing a firm grip on the prey more difficult (Eisner & Camazine, 1983; Schmidt, 2019; Ward-Smith et al., 2020). A venom that is used both to incapacitate prey and deter predators should therefore induce pain in its main predators, and paralysis or death in prey. This requires specific toxins for each of these tasks to be present in the venom.

The pain response is mediated by receptors belonging to the nociception system. These sense and transmit environmental stimuli like changes in temperature, mechanical stress or chemical concentration to the central nervous system, allowing the organism to take action to avoid further damage. The transient receptor potential (TRP) channels are transducers of the nociception system and associated with the pain-inducing response (Basbaum et al., 2009; Cao et al., 2013; Yang et al., 2010). It is not thus surprising that several pain-generating toxins isolated from scorpion venom have a high affinity for TRP channels (Cid-Uribe et al., 2020; Hakim et al., 2015; Lin King et al., 2019; Yang et al., 2017). Some specialized scorpion predators like grasshopper mice and bats (Holderied et al., 2011; Hopp et al., 2017; Rowe & Rowe, 2006, 2008) possess an altered molecular configuration of other voltage-gated channels belonging to the nociception system which provide some immunity to the lethal and algogenic effects of scorpion venom (Hopp et al., 2017; Niermann et al., 2020; Rowe et al., 2013). The pain-inducing effects of scorpion venom are generally evaluated through injection of aliquots

of crude venom into the plantar region of mice hindlegs (Chen et al., 2001). In these tests, pain effects are evaluated by the time the mice spend licking their paws. By using this assay, it has been possible to show that buthid scorpion venoms are more painful than venoms of non-buthid scorpions (Rowe et al., 2011), and that, in *Centruroides vittatus* (Buthidae), males are more painful than females (Miller et al., 2016). Moreover, even if predators generally prefer to feed on scorpions that inflict less painful stings, significant consumption of more painful species has been reported (Niermann et al., 2020). When under a strong predatory pressure, however, scorpions can rely on the rapid phenotypic plasticity of their venom to develop an effective defence. When a scorpion is continuously exposed to the presence of a mammalian predator, the production of anti-mammalian toxins in its venom increases (Gangur et al., 2017).

Some species of the genus *Parabuthus* (Buthidae) apply their venom externally, as a toxungen rather than a venom (Nelsen et al., 2014). These species are able to spray their venom towards their attacker, similar to the “spitting” behaviour of some cobra species (Newlands, 1969, 1974; Nisani et al., 2011, 2015). The first symptom following contact of the venom with the human eye is immediate pain (Newlands, 1974). These “venom-spraying” events are unambiguously voluntary, and their use depends on the level of threat perceived by the scorpion. Nisani (2015) showed that spraying events occurred only if the scorpions were grabbed with tweezers and not when the defensive response was elicited by simply blowing puffs of air on the animals. Moreover, *Parabuthus* species have never been reported to spray venom on their prey during feeding trials (Rein, 1993, 2003). Spitting cobras (Ward-Smith et al., 2020; Westhoff et al., 2005; Westhoff et al., 2010), vinegarroons (Eisner et al., 1961; Schmidt et al., 2000), and other animals like bombardier beetles (Eisner, 1958) and earwigs (Eisner, 1960) likewise spray toxins to deter attackers/predators, but do not use them to incapacitate their prey.

Another very peculiar defensive use of the metasoma and telson has been observed in some species of the genus *Ananteris* (Buthidae). When grabbed with tweezers, members of this genus are able to cast off their metasoma which also contains their hindgut (Lira et al., 2014; Mattoni et al., 2015). Similarly to autotomised lizard tails, autotomised metasomas continue to move for a few seconds. Differently from lizard tails, no regeneration has been ever observed. Metasoma autotomy decreases predatory success (García-Hernandez & Machado, 2020) but it has been observed that acaudate males survived for several months and mated, thus clearly increasing fitness (Mattoni et al., 2015).

### 3.3.3 Mating behaviour and sexual dimorphism

While weapons are used for the same purpose by the two sexes in predatory and defensive behaviour (be it sometimes to different degrees (Carlson et al., 2014)), in intra-specific competition and mating behaviour, members of each sex may use their weapons to accomplish different tasks.

#### *3.3.3.1 Male-male antagonism*

Adult scorpions change their behaviour during the mating season. Males become more vagrant and actively look for a partner (Benton, 1992b; Polis & Farley, 1979; Tourtlotte, 1974), which also leads to a higher chance of intrasexual encounters. Literature accurately reporting intrasexual contests between male scorpions is practically non-existent, with the only formal description of one of these events reported for the species *Hadrurus arizonensis* (Caraboctonidae) (Tallarovic, 2000). In this species, intraspecific contests are divided into three phases: i) alert phase, ii) contact phase, and ii) contest phase. In the alert phase, the opponents face each other with both pedipalps and metasoma raised up. Differently than when performing defensive alert postures, during the intraspecific alert phase both opponents show unique behaviours like metasoma wagging (personal observation YS in *Tityus pachyurus*, Buthidae) and a fast shaking of the whole body without leg movements called “juddering”. These behavioural units have been extensively characterized in the literature on scorpion mating behaviour (Alexander, 1957; Briceño & Bonilla, 2009; Brownell & Van Leo Hemmen, 2001; Rosin & Shulow, 1963) but, due the lack of studies in this topic, are never reported in male-male competition. During the contact phase, scorpions grab each other with their pedipalps. In the contest phase, they try to grab the metasoma of their opponent or, alternatively, try to flip it on one side (Tallarovic, 2000). During these contests, no actual stings have ever been reported, and the whole behaviour seems to be ritualized. The contest ends when one of the competitors holds its position while the other one retreats (Benton, 1992a; Tallarovic, 2000).

#### *3.3.3.2 Courtship and mating*

When a mature male encounters a female, courtship generally happens. Courtship and mating behaviour have been extensively studied in several families of scorpions, and a few taxon-specific differences in the various phases of the courtship ritual have been reported (e.g. the presence/absence of cheliceral massage and sexual sting) (Alexander, 1957, 1959; Brownell & Polis., 2001; Polis, 1990). Courtship generally

starts with the male approaching the female. The juddering behaviour has been observed in different scorpion families in this phase of the mating ritual and is associated with the production of vibrations that communicate the position of the male to the female, or that help the male control the female's aggressiveness (Alexander, 1957; Briceño & Bonilla, 2009; Brownell & Van Leo Hemmen, 2001; Rosin & Shulow, 1963). The male then attempts to grasp the female's pedipalps. The initiating phase is very dynamic and involves males using both their weapons to manipulate the female. The chelae are used to establish a firm grip on the female's pedipalps to control her movements, which is essential for the next phases of the mating. At the same time, in several species, the male rubs, clubs, and even stings the female. After this first contact, the male starts to guide the female by performing specific movements in a dance-like ritual called "promenade à deux". During this phase, the male moves forward and backwards, dragging the female until he finds a suitable place to deposit the spermatophore. The spermatophore is a stalk-like structure extruded from the male's genital opening, that serves as a pedestal for the sperm. Once the spermatophore has been deposited, the male guides the female on it, and as soon as the female takes up the sperm package from the spermatophore (leaving only the pedicel anchored to the substrate), the pair separates, ending the mating. This is just a brief illustrative summary of the main phases of scorpion courtship. For more inclusive literature please refer to (Alexander, 1957, 1959; Benton, 1992b; Francke, 1979; Polis & Farley, 1979; Tallarovic et al., 2000).

The degree of sexual dimorphism in scorpions is highly variable. Simplifying the classes created by Koch (1977) on Australo-Papuan scorpions, and later by Polis (1990), it is possible to identify two main patterns of sexual dimorphism in scorpions:

I. Differences in body size but not in shape of secondary sexual characters.
II. Differences in shape and size of secondary sexual characters.

Sexual size dimorphism in scorpions has been reviewed by Mclean et al. (2018), with females being generally larger than males probably due to selection on fecundity and to the direct contribution of developing embryos to body size (Carlson et al., 2014). However, few exceptions of male-biased size dimorphism are present, like in the cases of *Liocheles australasiae* (Scorpiondae) (Koch, 1977) and *Tityus trinitatis* (Buthidae) (Alexander, 1959; Kjellesvig-Waering, 1966). In the scope of this review, the differences in shape and size of secondary sexual characters deserve particular focus.

The greatest differences in shape and size of sexually selected characters in scorpions mainly lie in their weapons. Sexual dimorphic species of scorpions generally present differences in chela shape and/or size between the two sexes. In these species, one of the sexes tends to have more robust chelae, while the other sex generally presents a slenderer chela morphology.

In the members of the families with males having the more robust chelae (e.g., some Buthidae, Scorpionidae), a potential advantage for males may be more space for muscles, and therefore a larger pinch force. This, together with a different distribution and shape of the denticles on the fingers, would provide a firmer grip on the female's pedipalps during the courtship, or aid to defeat weaker male competitors. A more bulbous chela may also provide less opportunity for male competitors or unwilling females to find purchase.

In some species, the males have more elongated and slender chelae than females (Kraepelin, 1907). This pattern is morest extreme in some members of the families Chactidae (González-Gómez et al., 2020), Scorpionidae (Leeming, 2019) and Buthidae (Lourenço & Otero Patiño, 1998; Sánchez-Quirós et al., 2012). In sexual dimorphic buthid species, the elongation of both pedipalps and metasoma occurs in the last or second-to-last moult (Farzanpay & Vachon, 1988). Slenderer chelae are associated with lower biteforce in males (González-Gómez et al., 2020), suggesting that in these cases, selection for higher biteforce is not the main driver determining chela morphology. Studies of the mating ritual of *Centruroides margaritatus* (Buthidae) and *Chactas reticulatus* (Chactidae) (Lourenço et al., 2003; Sánchez-Quirós et al., 2012) show that males of these species use their elongated chelae similarly to other scorpions. To date, no functional study has shown which of the potential functions and advantages is the driver for sexual dimorphism of chela size in a particular species. Having such elongated appendages may permit the two sexes of the same species to feed on different prey and exploit a different foraging niche, lowering inter-sexual competition (González-Gómez et al., 2020), although this cannot be the primary driver of sexual dimorphism.

In some species of Scorpionidae, Hemiscorpidae and Buthidae, males have a prominent tooth on the dorsal side of the movable finger, which is thought to enhance grip on the female's pedipalps. Once the female has been grasped, its fingers are generally placed in the space between the manus and the tooth, with the latter blocking the retreating of female's fingers, thus reducing the chances of them slipping out (Booncham & Sitthicharoenchai, 2007). In Bothriuridae, males present a spine-like apophysis close to a depression on the surface of their manus. This depression has the function to create a 'cul de sac' for the female's fingers, distally closed by the apophysis (Maury, 1975). A similar depression in the manus, most likely serving the same function, is also present in adult males of *Pandinoides cavimanus* (Scorpionidae).

The venom delivering system is also a character that may be highly variable in sexually dimorphic species. The general trend is that males have a slenderer, more elongated metasoma, and a more swollen telson than females. Having a longer metasoma has not been related with performance improvements in either locomotion

activity or frequency of stinging (Carlson et al., 2014). However, a longer metasoma allows faster strikes (Coelho et al., 2017) which can be useful in performing quick defensive responses, especially during the mating season, when the increased sexual vagrancy of males increases chances of predator encounters.

A longer metasoma in males may be advantageous during courtship; males may sting or club the female while keeping it at a greater distance, thus reducing the chances of being stung by an aggressive partner. This behaviour most likely has the function of reducing the aggressiveness of a reluctant female (Alexander, 1957, 1959; Benton, 1992b; Francke, 1979; Jiao & Zhu, 2010; Polis, 1990; Tallarovic et al., 2000). Whether venom injection occurs during sexual stings is still not clear. However, Jiao and Zhu (2010) hypothesized that males may deliver a “dry” or a “wet” sting according to the level of aggressiveness of the female. Moreover, differences in telson shape (Booncham & Sitthicharoenchai, 2007; Kraepelin, 1907; Sentenská et al., 2017) and venom between sexes have been found in several species of scorpions performing sexual stinging (D’Suze et al., 2015; De Sousa et al., 2010; Rodríguez-Ravelo et al., 2015; Yamaji et al., 2004), with male venoms possessing some unique venom components. In the species *Scorpio maurus* (Scorpionidae), however, females present a more complex venom profile than males (Abdel-Rahman et al., 2009). The role of sex-specific toxins in reproductive ecology has not been investigated yet.

Some authors have hypothesized that differences in the length of the metasoma may be useful in sex recognition in case of intra-specific encounters (Alexander, 1959; Ross, 2009). However, metasoma grabbing in the early stages of mating has also been reported in species where the sexual dimorphism is not very marked (Tallarovic et al., 2000).

In Bothriuridae, males possess accessory glands located on the ventral face of the telson, with the function of producing secretions when the male rubs the metasoma onto the female’s body (de la Serna de Esteban, 1978; Olivero et al., 2015; Peretti, 1995, 1997). According to Peretti (1997), these secretions have the function of increasing female’s receptivity. In *Bothriurus bonariensis* (Bothriuridae), the composition of these secretions has been found to change depending on the population analysed (Olivero et al., 2015). Another example of sexually dimorphism in telson shape is given by the genus *Anuroctonus* (Chactidae). Males of this genus have a secondary bulb of unknown function at the base of the aculeus, which is absent in females (Polis, 1990; Soleglad & Fet, 2004).

## 3.4 Conclusion

Scorpions use their chelae and venom delivery system in the most fundamental aspects of their ecology. The two weapon systems of scorpions perform in different contexts of usage (predation, self-defence and intra-sexual competition), and in some cases interact. How these interactions evolve in different species, or whether there is a trade-off between the weapon systems, is not yet resolved. Despite much recent progress in functional studies, several topics still remain underexplored. Of course, a disproportionate fraction of the literature is devoted to venom research. The importance thereof is unambiguous for advances in human health as potential new medicine, as well as in the treatment of scorpion stings as a neglected health risk. Currently basic information on pedipalp anatomy, diet, functional studies of weapons, and intra-sexual interactions are sparse or even absent. With the current increased interest in the functional aspects of scorpion weapons, we hope that also these areas will soon reveal new insights in the fascinating ecology of the scorpion weapon constellation.

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# Chapter 4

## Biomechanics of chela closing performance

## 4.1 Crushers versus graspers: Biomechanical model of finger adduction reveals high integration in the musculoskeletal system of scorpion chelae

### Introduction

Correlation among traits (integration) but also their independence from other traits (modularity) are fundamental determinants of morphological diversity (Klingenberg, 2008; Zelditch & Goswami, 2021). While strong integration has been generally considered as a limiting factor for morphological diversity (but see Felice et al., 2018), modularity can counteract this effect by decomposing correlated traits into smaller and more independent modules, increasing the overall rate of morphological evolution (Goswami et al., 2014).

Physical laws and functional trade-offs can also constrain the occupation of all areas of the theoretical trait morphospace (Alexander, 1985; Higham et al., 2021). Functional trade-offs occur when the benefits in fitness obtained from one functional performance comes at the cost of another (Garland et al., 2022; Stearns, 1989). In traded-off systems, physiological and structural elements can show a high degree of integration towards a single functional optimum (Hedrick et al., 2020; Klingenberg, 2014). Highly integrated systems facing functional trade-offs have thus more limited adaptive options compared to less integrated ones. Nevertheless, even groups with high integrated systems and showing trade-offs may present high rates of diversification, especially at the extremes of the morphological gradient (Burress & Muñoz, 2022; Goswami et al., 2014).

Due to simple lever mechanics, an increase of the mechanical advantage leads to an increased output force and simultaneously to a decreased output speed resulting in a mechanical trade-off between force and velocity (Herrel et al., 2009; Simone & van der Meijden, 2018). Moreover, also muscle contraction is subject to the force/speed trade-off. Muscles generate maximum force when the speed contraction is zero (isometric contraction). On the other hand, when muscles contract at certain speed, the force generated is lower than that generated isometrically or during eccentric contraction (Hill, 1938).

Nevertheless, in many muscle-lever systems the force-speed trade-off is circumvented (McHenry, 2011; Osgood et al., 2021). The pincer of pistol shrimps, the

hind leg of grasshoppers, the mandible of trap-jaw of ants are all examples where extremely fast and powerful movements are achieved through the release of stored elastic energy (Blanco & Patek, 2014; Burrows & Sutton, 2012; Patek et al., 2006). In other cases, structural asymmetry is the solution to overcome the constraint posed by the force-speed trade-off (Govind, 1989; Schenk & Wainwright, 2001). In many species of decapods, one of the pincers (chelae) has a greater mechanical advantage and more robust design while the other has more slender design and lower mechanical advantage (Govind et al., 1987). Morphological asymmetry is thus an evolutionary strategy to perform functions subjected to trade-offs by bearing different sets of differently designed morphological traits.

Similarly to some groups of decapods, scorpions present a striking diversity in the morphology of their chelae. Scorpion chelae are formed of the tibia (or manus) and tarsus (or movable finger), which are the last two segments of the pedipalps. The tibia is the immovable portion and contains most of the muscles responsible for the closing of the chelae. An additional closing muscle is located in the patella, the segment immediately posterior to the tibia (Gilai & Parnas, 1970). Scorpions lack an antagonist set of muscles to open their chelae (Alexander, 1967; Snodgrass, 1952). Scorpion chelae can vary from slender and long-fingered to more robust and short-fingered (van der Meijden et al., 2010; 2012). Differently from crabs, scorpions do not exhibit chela asymmetry and the factors driving their chela morphological diversity remain largely unexplored. Recently, Coelho et al. (2022) found a correlation between the morphology of chelae and microhabitat properties. Their results highlight a high degree of trait integration associated with habitat use. However, adaptation to different microhabitats may be one of the multiple drivers of chela diversity, as chelae have several other functions, including sensing, mating and intraspecific competition (Simone & van der Meijden, 2021).

Scorpions are exceptionally suited to study the interactions between functional traits, their performance, and their ecological roles because of their unique compartmentalization of functions into different modules. In a predatory context, many venomous animals like spiders or snakes rely on a unique functional trait, the fangs, to grasp, inject venom and chew their prey. Scorpions on the other hand perform each one of these three tasks by using three different body parts: they grasp the prey by using the chelae, inject venom through the stinger and chew the victim through the chelicerae.

Chelae are the first structures getting in touch with prey, therefore chela closing force and speed are two key performance traits that can have an impact on scorpion foraging performance. Chela closing faces a force speed trade-off where species with

fine and long-fingered chelae are faster but significantly weaker compared to species bearing more robust and short-fingered chelae (Simone & van der Meijden, 2018). While the observed variance in closing performance is partially explained by the length of the movable finger, the remaining variance was ascribed to potential variation in the physiological properties and architecture of the closing muscles.

In this work, volumetric scans of the architecture of the chela closing muscles of two scorpion species at the extremes of the gradient of morphological diversity are presented. Based on this data, we created a landmark-based biomechanical model of movable finger abduction/adduction from which closing force and muscle contractile speed at each degree of rotation can be estimated. The output data of the muscle architecture and closing force variation are analysed within- and between the focal species to identify patterns of kinematic integration. We aim to test whether muscle and skeletal architecture are tightly coordinated to achieve a specific performance outcome, or alternatively, evolved independently towards different performance optima in a relatively simple and tractable mechanical system.

**Material and methods**

*Performance measurements*

Chela closing forces of *Scorpio palmatus* and *Hottentotta gentili* were obtained following Van der Meijden et al (2010) using a Kistler force transducer (Kistler Inc., Winterthur, Switzerland) mounted on a purpose-built holder connected to a charge amplifier as described in Herrel et al. (1999). To measure chela closing speed, the protocol described in Simone et al (2018) was followed. Closing force, closing duration, angular velocity, and maximum opening angle were used to inform the biomechanical model of finger abduction/adduction built for the two focal species positioned at the extremes of the morphological continuum observed in scorpions.

Closing force, maximum angular velocity and morphological measurements were also gathered in ten other species of scorpions (see **Table_suppl_4.1.1**). These variables were used in phylogenetic comparative analysis to investigate whether sarcomere length drives variation in kinematics across the phylogeny.

***3D morphological analysis***

*Sample preparation for Synchrotron scan*

Scorpions were fixed in 3.7% formaldehyde solution and placed in a small polypropylene tube for X-ray phase contrast synchrotron microtomography (Sr-µCT) as

described in Betz et al (2007). 900 radiographic images (CCD 2048×2048 pixels, with binning at 1024×1024 pixels) were acquired using a FReLoN CCD Camera with an exposure time of 0.15s with an effective pixel resolution of 14.8µm. Beam energy was set at 25 keV and the specimens were scanned at beamline ID19 at the ESRF in Grenoble.

*3D volume rendering and landmark definition*

All scan data were imported as Tiff-files into Amira (version 5, Mercury Computer Systems Inc., Chelmsford, MA, USA) to generate 3D surface of the internal anatomy (ligaments, apodemes, and muscle bundles) and the cuticular elements of both manus and patella.

Per muscle bundle, volume was estimated using the module "*Material Statistics*" in Amira. Amira was also used to place specific landmarks (shown in panel A of **Figure 4.1.1**) and to retrieve their 3D coordinates. The landmarks placed at the tip of the movable and fixed finger (Mt and Ft respectively) and on the medial and lateral joints (Mj and Lj respectively) are landmarks in common. Additionally, per muscle bundle, specific landmarks were placed: the insertion point of the apodeme on the movable finger (Ai), the proximal end of the apodeme (Ao), the origin and the insertion point on the apodeme of 10 different fascicles ($Fo_j$ and $Fi_j$ respectively). The muscle fascicles are modelled as a line between Fo and Fi while the apodeme is modelled as a line passing between Ao and Ai.

In this study, due to the impossibility to segment single fibres in all bundles, fascicle length was used as proxy for fibre length.

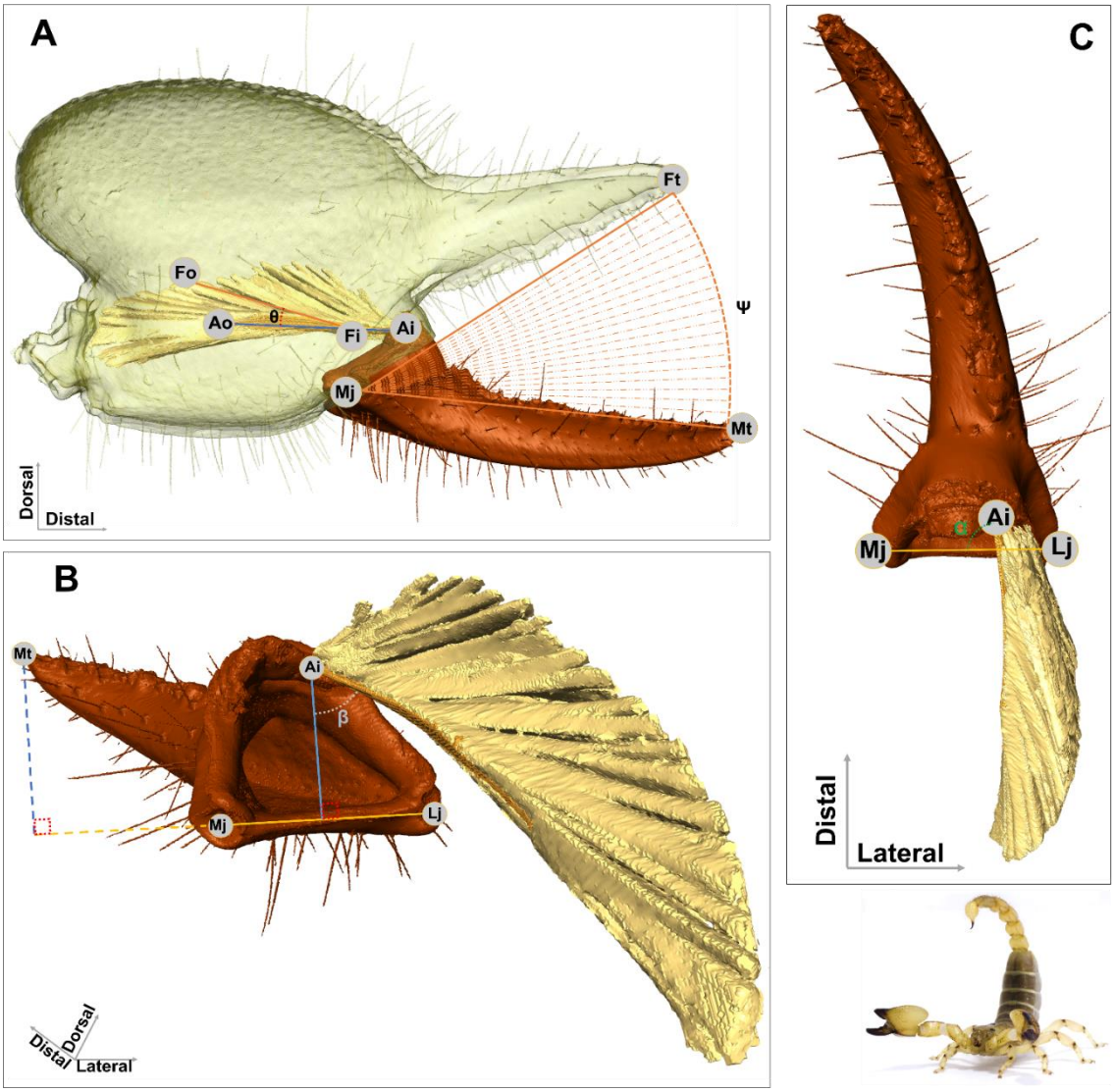


**Figure 4.1.1** Medial view of the whole *Scorpio palmatus* chela (A) shows the location of the landmarks used to build the biomechanical model. Posterior (B) and dorsal (C) view of the movable finger, to highlight the two insertion angles of the apodeme used to correct bite force estimations. The movable finger is shown in bold brown color, the manus cuticle in transparent grey, the muscle apodeme in orange, and the muscle bundle in yellow. The landmarks applied are the following: Ai, apodeme insertion; Ao, Apodeme origin; Fi, fascicle insertion; Fo, fascicle origin; Ft, fixed finger; Lj, lateral joint; Mj, lateral joint; Mt, movable finger; α, latero-median apodeme insertion angle; β, dorso-ventral apodeme insertion angle; θ, pennation angle; Ψ, opening angle.

### *Musculoskeletal model*

The 3D coordinates of the landmarks were imported in Matlab (The MathWorks Inc., Natick, MA, USA). Mj was chosen as the new origin of the coordinate system and the coordinates of all landmarks were translated to the new reference axis. After this first translation, in order to place the Lj on the X-axis, the coordinates were rotated around the Z and Y axis using the *rotationmat3d* function (Stanley, 2022). After these two rotations, the axis of rotation of movable finger lies on the X axis.

The chelae scanned were fixed at different opening angles; *Scorpio* was almost fully open while *Hottentotta* was almost totally closed. To compare the outputs of the two chela models the movable finger of the two focal species needs to be rotated to achieve the same functional opening state. To fully open the chela, the movable finger was rotated around the X axis until the reaching the maximum opening angle. This angle is obtained from the kinematic output of fastest chela-closing performance of the specimen having the closest prosoma length to the scanned ones (Sc3331 for *Scorpio palmatus* and Sc3271 for *Hottentotta gentili*). By selecting the fastest closing performance it should be possible to estimate the maximum contraction speed of muscles.

After the last rotation, the apodeme rotated with the movable finger. One of the assumptions of the model is that the apodeme has constant length and the angle relative to the coordinate system is constant along the whole abduction/adduction event. A further rotation of Ao around the axis of rotation is needed while keeping unaltered the rotated coordinates to make the rotated apodeme parallel to the unrotated one. The position of the Ao has to be corrected per degree of rotation. The angle at which rotation takes place is calculated using the following formula:

$$\varepsilon_{(\Psi)} = acos \frac{\vec{\mu} \cdot \overrightarrow{v_{(\Psi)}}}{\|\vec{\mu}\|\|\vec{v}\|} \qquad (1)$$

Where $\varepsilon_{\Psi}$ is the angle between the unrotated apodeme and the rotated one per Ψ degree of rotation and $\vec{\mu}$ and $\vec{v}$ are respectively the direction vectors of the unrotated apodeme and the rotated one, $\|\vec{\mu}\|$ and $\|\vec{v}\|$ are the magnitude of unrotated and rotated apodeme (which are identical by definition). Once the angle is calculated, the coordinates of Ao and $Fi_j$ are rotated around the X axis and the rotated apodeme will be parallel to the unrotated one and $Fi_j$ is corrected accordingly.

*Fibre length*

The calculation of the fibre length per degree of rotation is obtained as the 3D distance between the Fo (corresponding to those at the widest opening angle) and the Fi on the apodeme per degree of rotation.

*Pinnation angle*

The pinnation angle (θ) is defined as the angle described by the fibre vector and the apodeme vector. The pinnation angle is calculated per degree of rotation of the movable

finger using the formula (1). In this case $\vec{\mu}$ and $\vec{v}$ are the directions of the fibres and the apodeme and $\|\vec{\mu}\|$ and $\|\vec{v}\|$ their magnitudes.

*Mechanical advantage*

Mechanical advantage (MA) is the ratio between the effective in-lever and the effective out-lever (calculated per degree of rotation). The effective in-lever was calculated as the minimum perpendicular distance between the line of action of the apodeme and the axis of rotation using the Matlab function *line to line distance* (Douillet, 2022). This variable changes its magnitude due to the change of the position of Ai and in the dorso-ventral apodeme insertion angle (β) (see below). The effective out-lever was instead calculated as the minimum distance between the projected line passing through Ft, perpendicular to the axis of rotation using the function *point to line distance* (Rik, 2022). Differently from the speed, which is always calculated at the tip of the movable finger, force is generally measured more proximally. To take this into account, the effective out-lever was shortened by one third and the MA corrected consequently.

Effective in-lever corresponds to muscle moment arm. The latter was used to calculate the fascicle/moment arm ratio per bundle (Lieber & Ward, 2011; Williams et al., 2008). This ratio is largely used in comparative biomechanics to infer function across muscles with different designs. Large fascicle/moment arm ratios are indicative of muscles with longer fibres and relatively short moment arms while small ratios are indicative of muscles with short fibre length and large moment arms (Lieber & Ward, 2011; Maganaris et al., 2006).

*Physiological cross-sectional area*

The calculation of the Physiological cross-sectional area (PCSA) is given by the formula:

$$PCSA_{(\Psi)} = \frac{Volume}{fiber\ length_{(\Psi)}} \cos\left(\theta_{(\Psi)}\right) \quad (2)$$

The model assumes that the muscle contraction is isovolumetric therefore this quantity is constant per Ψ degree of rotation. PCSA is a proxy of the number of fibres arranged in parallel within a muscle. However, because of the formula used, while contracting, fibre length gets shorter and pinnation angle increases, leading to an increasing value of PCSA while movable finger is closing. For closing force estimation, we used a fixed value of PCSA retrieved at 15º.

*Dorso-ventral apodeme insertion angle*

The apodeme's orientation relative to the effective in-lever requires attention due to a force component that remains untransmitted to the out-lever. This component, directed towards the axis of rotation, does not contribute to the torque for movable finger abduction. In the B panel of **Figure 4.1.1** the dorso-ventral apodeme insertion angle (β) is the angle defined by the apodeme vector and the effective in-lever vector. In our model, β is calculated per degree of rotation using the formula (1) where $\vec{\mu}$ and $\vec{v}$ are the directions of the effective in-lever vector and the apodeme vectors and $\|\vec{\mu}\|$ and $\|\vec{v}\|$ are their magnitudes. The component that is transmitted to the out-lever is the sin(β).

*Medio-lateral apodeme insertion angle*

The medio-lateral orientation of the apodeme vector and the axis of rotation (α) is another source of force loss (see panel C **Fig 4.1.1**). Differently from β, this angle remains constant and is calculated by using the formula (1) where $\vec{\mu}$ and $\vec{v}$ are the directions of the axis of rotation and the apodeme vectors and $\|\vec{\mu}\|$ and $\|\vec{v}\|$ are their magnitudes. Only the sin(α) is transmitted to the out-lever.

*Muscle stress*

Muscle stress (σ) is the force per unit of cross-sectional area that a muscle can exert. It depends on the contractile state of the sarcomeres (Rospars & Meyer-Vernet, 2016). The assumption in the current model is that muscle stress remains constant during muscle contraction. To estimate muscle stress, two different approaches were followed. The first approach consists in estimating muscle stress by using its definition of ratio between force and PCSA. Closing forces of the scanned specimens were estimated using the method described in Bicknell et al. (2022). We assumed that all the closing forces used to infer the one of the scanned specimens were recorded at an opening angle of 15º.

To obtain the stress, muscle force at the muscle insertion point has to be calculated. To do so, we divided the estimated force at the out-lever by the MA of the biggest muscle when the chela has an opening angle of 15º. The choice of the MA is also arbitrary; we decided to use the MA of the muscle having the larger PCSA due to the larger contribution to the total closing force. To finally calculate the stress, the force at the insertion point of the movable finger was then divided by the sum of the PCSAs of all the muscle bundles.

$$\sigma = \frac{\frac{F_{(15)}}{MA}}{\sum PCSA} \quad (3)$$

For the second approach to estimate muscle stress, we performed a reduced major axis regression (RMA) using as response variable the log-transformed muscle stress and as independent variable the log-transformed sarcomere length. A regression equation in the form Y=a + bX was created using the muscle stresses and the sarcomere lengths from (Taylor, 2000), with a and b being respectively the RMA intercept and slope. To obtain the stress of the scanned species, X was substituted with the log-transformed mean sarcomere length measured for both focal species. To transform the value of the stress from log-scale to the original scale (kPa), we calculated $10^Y$. This analysis was inspired by Püffel et al. (2023).

*Closing force estimation*

Once the muscle stress is calculated, it is possible to estimate the closing force at the out-lever generated by each bundle muscle per degree of rotation following formula:

$$F_{(\Psi)} = PCSA\, x\, MA_{(\Psi)} x\, \sin(\alpha) x\, \sin(\beta)_{(\Psi)} x\, \sigma \quad (4)$$

In order to compare the two approaches, the closing force was calculated using the two different values of stress estimated.

*Muscle contraction speed*

To measure muscle speed contraction, two variables need to be known: muscle displacement per degree of rotation and the time passed to rotate each degree during the whole closing event. The muscle displacement was calculated as the distance travelled in the Y plane by the Ai per degree of rotation. To predict the time at which each degree of rotation occurs, the opening angle of the movable finger per frame was retrieved from the fastest closing event recorded from the specimen having the closest prosoma length. Each frame corresponds to 2 milliseconds, and it also reports the opening angle. The cumulative sum of frame rates and the opening angle at each frame were used to feed a polynomial curve fit and time per degree of rotation was then predicted. Muscle contraction speed was calculated as the derivative of displacement versus time per degree of rotation.

We assume in the model that muscles are composed of the same fibre type and show the same force-speed curve concavity.

**Sarcomere length measurements**

For all twelve species for which performance data were measured, manus and patellar closing muscles were dissected with the help of a Leica M80 stereomicroscope. Manus closing muscles were divided into four different groups: dorsal, lateral, medial, and ventral aiming to isolate muscle bundles with long, intermediate, and short moment arms. Each group of dissected muscles was placed in an aqueous solution of $HNO_3$ 30% for 48 hours at 10°C to dissolve the connective tissue surrounding the muscle fascicles. After 48 hours, individual fibres were separated with a dissection probe. To block chemical digestion and preserve the muscle fibres, nitric acid was removed and substituted with 50% aqueous solution of glycerol.

Microscopic pictures of individual fibres were taken using an Axio zoom V16 microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) mounted with an Axio cam MRc5 at the Centre de Microscopie de fluorescence et d' IMagerie numérique (CeMIM, Paris). Images of individual fibres were magnified up to 270 times, magnification at which the Z-disks of sarcomeres were distinctly observed (see panel A of **suppl_fig_4.1.1**). To reduce mistakes in focusing, a stack of 100 planes (total stack height = 100 µm) was compiled through the ZEN pro software (blue edition). Stacks were loaded in Fiji (Schindelin et al., 2012) and a straight line perpendicular to the Z-disks was drawn to obtain a pixel intensity plot along the length of the drawn line. The pixel intensity plot has on the Y axis the value in gray scale of the pixel and on the X axis the position in micrometers along the line. This set of 2D coordinates was extracted and loaded into a custom script in R. The script applies a low-pass $4^{th}$ order Butterworth filter with a frequency of 15Hz. By using the function *findpeaks* from the package "gsignal" (Van Boxtel et al., 2021) the coordinates of the top peaks were retrieved and the distance on the X axis between two consecutive peaks was calculated as individual sarcomere length (a visual example is provided in **suppl_fig_4.1.1** of supplementary materials).

*Statistical analysis*

All statistical analysis were performed in R 4.2.2 version (R Development Core Team, 2022).

*Within species analysis*

Within species, we performed T-student tests to assess whether significant differences in sarcomere length exist across manus and patellar closing muscles. Moreover, we also performed linear models within and across the two focal species to

test for differences in sarcomere length across the four different muscle groups (dorsal, lateral, medial, and ventral).

*Analysis across focal species*

We used student t-tests to test for differences in sarcomere length, pinnation angles, mechanical advantage, and fascicle moment arm ratio between the two focal species. Since not all the variables had normal distribution of residuals and homoscedasticity, before running the T-test, all the variables were normalized through $\log_{10}$ transformation. Moreover, we were also interested in detecting patterns of pairwise co-variation of the four most representative muscle architecture variables: fibre length, pinnation angle, PCSA, and moment arm. To quantify and visualize patterns of covariation, a pairwise correlation matrix of the four mentioned variables was built. The correlation coefficients were calculated and graphically compared among the two species analysed. The pairwise correlation coefficients and their significance were retrieved through the function *ggpairs* of the package "GGally" (Schloerke et al., 2021). The same package was also used to plot the correlation matrix. We also run Student t-tests to test for differences in sarcomeres length of manus closing muscles and across species.

*Phylogenetically informed analysis*

For the comparative analysis a recent transcriptome-based phylogeny of scorpions was used (Santibáñez-López et al., 2019). The topology of the phylogenetic tree was adapted from Coelho et al (2022) with subsequent pruning to encompass only the twelve species analysed in this study. To run phylogenetic comparative methods, sarcomere length, maximum angular velocity, and size-corrected closing force were averaged at species level. Sarcomere length and maximum angular velocity are size-independent variables. Conversely, to enable meaningful comparisons, closing force needs to be corrected for scorpion body size. Since an universally accepted single measurement for overall scorpion body size is lacking (Fox et al., 2015), the study relies on a set of various morphometric measurements. Ten distinct measurements were employed to ascertain body size (**Fig_supp_4.1.2**). All measurements were taken using digital callipers (Absolute IP67, Mitutoyo Inc., Kawasaki, Japan) following Stahnke (1970). These ten measurements were selected due to their robust association with scorpion body size (Coelho et al., 2022). An isometric body size (IsoSize) was calculated by projecting $\log_{10}$-transformed morphometric measurements onto an isometric vector. The size corrected closing force was calculated as the residuals of linear model having $\log_{10}$-transformed closing force regressed against the isometric body size vector (Coelho et al., 2017; Kaliontzopoulou et al., 2010).

Size corrected closing force and angular velocity were regressed against mean sarcomere length in a PGLS model using the *pgls* function in the package “caper” (Orme et al., 2018). Because the limited number of species analysed in the current study might not provide enough support, we ran two PGLS models for each response variable. In the first model we impose λ = 1, while in the second, the analysis runs using the estimated λ with the maximum likelihood calculated. The fitting of the two models was then compared using likelihood-ratio-test through the function *lrtest* from the package “lmtest” (Zeileis & Hothorn, 2002). In the final analysis, the sarcomere length was compared within a phylogenetic framework. The objective of this analysis was to evaluate whether the spatial distribution of sarcomere length across muscles with varying moment arm lengths maintains consistency across both fast and strong species that were examined. To do so, we normalized sarcomere lengths using Z-scores. Positive or negative Z-scores indicate, respectively, longer or shorter sarcomeres in comparison to the mean sarcomere length of all closing muscles. The Z-scores of sarcomere length were introduced as independent variables in the phylogenetic generalized least squares (PGLS) models, wherein the response variables were either maximum angular velocity or size-corrected closing force. As previously described, different lambda values were assessed to account for differing evolutionary scenarios.

## Results

### *Performance data*

For this study, ten closing events, five per chela, were analysed in 113 specimens from twelve different species for a total of 1135 chela closing events. The highest angular velocity was recorded in a chela closing event of *Tityus pachyurus* (Pocock, 1897), corresponding to 55.5 rad/s while the lowest was recoded in an event of a *Scorpio palmatus* with a value of 17.6 rad/s. The largest closing force of 12.1 N was recorded in *Scorpio palmatus* while the lowest was recorded in *Tityus stigmurus* (Thorell, 1876) with a value of 0.35N.

Angular velocity was on average 26.6 ± 5.48 rad/s in *Scorpio palmatus* and 38.5 ± 10.5 rad/s in *Hottentotta gentili* while closing force was on average 8.00 ± 1.76 N in *Scorpio palmatus* and 0.58 ± 0.06 N in *Hottentotta gentili* (see **Table_suppl_4.1.1**).

### *Muscle architecture in focal species*

The muscle architecture across the two focal species shows remarkable differences. One of the more striking differences is the size of patellar muscle in the two

species, representing the 50% of the volume of all closing muscles in *Hottentotta* while only 1.5% in *Scorpio* (see **Table 4.1.1**).

**Table 4.1.1** Summary table of kinematic and physiological variables measured for the two focal species. Muscle bundle numeration does not imply any homology across the two focal species. Numbers from 1-12 are relative to the manus bundles while pat stays for the patellar closing muscle.

| Species | Muscle | Fiber length (m) | PA (˚) | MA | Moment arm (m) | β (º) | α (˚) | ACSA ($m^2$) | PCSA ($m^2$) | Volume ($m^3$) | % Vol. total | Fascicle/ moment arm |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Hottentotta gentili* | 1 | 1.56E-03 | 22.9 | 0.078 | 7.20E-04 | 97.0 | 96.5 | 8.67E-07 | 7.98E-07 | 1.35E-09 | 5.40 | 1.94 |
| | 2 | 2.03E-03 | 12.8 | 0.095 | 8.70E-04 | 114 | 92.7 | 8.20E-07 | 8.05E-07 | 1.70E-09 | 6.78 | 2.34 |
| | 3 | 1.94E-03 | 11.8 | 0.092 | 8.48E-04 | 103 | 79.9 | 5.13E-07 | 5.02E-07 | 8.72E-10 | 3.48 | 2.29 |
| | 4 | 2.18E-03 | 26.6 | 0.087 | 8.00E-04 | 108 | 88.2 | 7.19E-07 | 6.42E-07 | 1.58E-09 | 6.29 | 2.72 |
|  | 5 | 1.89E-03 | 24.3 | 0.078 | 7.21E-04 | 93.8 | 91.1 | 1.35E-06 | 1.23E-06 | 2.55E-09 | 10.2 | 2.62 |
| | 6 | 1.86E-03 | 18.8 | 0.093 | 8.51E-04 | 107 | 108 | 7.43E-07 | 7.03E-07 | 1.40E-09 | 5.56 | 2.19 |
| | 7 | 1.43E-03 | 19.2 | 0.091 | 8.33E-04 | 125 | 105 | 6.70E-07 | 6.33E-07 | 9.96E-10 | 3.97 | 1.71 |
| | 8 | 1.58E-03 | 11.6 | 0.105 | 9.68E-04 | 112 | 86.7 | 3.55E-07 | 3.48E-07 | 5.75E-10 | 2.29 | 1.63 |
| | 9 | 1.99E-03 | 13.9 | 0.090 | 8.29E-04 | 120 | 106 | 4.68E-07 | 4.55E-07 | 9.56E-10 | 3.81 | 2.40 |
| | 10 | 1.71E-03 | 22.2 | 0.105 | 9.65E-04 | 75.8 | 122 | 1.48E-07 | 1.37E-07 | 2.52E-10 | 1.01 | 1.77 |
| | 11 | 1.60E-03 | 17.6 | 0.114 | 1.05E-03 | 85.5 | 74.7 | 2.07E-07 | 1.97E-07 | 3.31E-10 | 1.32 | 1.53 |
| | 12 | 1.79E-03 | 22.0 | 0.146 | 1.34E-03 | 110 | 83.4 | 1.56E-07 | 1.45E-07 | 2.20E-10 | 0.878 | 1.34 |
| | pat | 7.62E-03 | 0.0 | 0.110 | 1.01E-03 | 104 | 96.6 | 1.54E-06 | 1.46E-06 | 1.23E-08 | 49.0 | 7.53 |
| *Scorpio palmatus* | 1 | 2.60E-03 | 31.3 | 0.361 | 2.07E-03 | 98.9 | 98.3 | 7.17E-06 | 6.13E-06 | 1.84E-08 | 29.4 | 1.25 |
| | 2 | 3.05E-03 | 34.2 | 0.341 | 1.95E-03 | 85.0 | 89.9 | 2.13E-06 | 1.76E-06 | 6.45E-09 | 10.3 | 1.56 |
| | 3 | 2.34E-03 | 20.5 | 0.247 | 1.41E-03 | 80.2 | 91.6 | 1.74E-06 | 1.63E-06 | 4.01E-09 | 6.41 | 1.66 |
| | 4 | 2.95E-03 | 21.5 | 0.306 | 1.75E-03 | 108 | 88.2 | 2.04E-06 | 1.90E-06 | 5.96E-09 | 9.52 | 1.68 |
|  | 5 | 2.08E-03 | 25.3 | 0.216 | 1.24E-03 | 69.2 | 81.8 | 1.23E-06 | 1.11E-06 | 2.53E-09 | 4.04 | 1.68 |
| | 6 | 1.96E-03 | 26.8 | 0.283 | 1.62E-03 | 75.0 | 79.5 | 1.81E-06 | 1.61E-06 | 3.50E-09 | 5.59 | 1.21 |
| | 7 | 2.17E-03 | 25.7 | 0.321 | 1.84E-03 | 83.7 | 79.1 | 1.24E-06 | 1.12E-06 | 2.65E-09 | 4.23 | 1.18 |
| | 8 | 1.56E-03 | 30.6 | 0.250 | 1.43E-03 | 67.9 | 77.2 | 1.10E-06 | 9.51E-07 | 1.71E-09 | 2.73 | 1.09 |
| | 9 | 2.22E-03 | 24.9 | 0.294 | 1.68E-03 | 98.3 | 97.3 | 1.48E-06 | 1.34E-06 | 3.24E-09 | 5.18 | 1.32 |
| | 10 | 3.57E-03 | 15.5 | 0.242 | 1.39E-03 | 62.0 | 85.8 | 9.96E-07 | 9.63E-07 | 4.08E-09 | 6.51 | 2.58 |
| | 11 | 1.20E-03 | 35.0 | 0.221 | 1.27E-03 | 57.8 | 76.9 | 7.17E-07 | 5.87E-07 | 5.23E-09 | 8.36 | 0.95 |
| | 12 | 2.37E-03 | 18.7 | 0.288 | 1.65E-03 | 117 | 114 | 4.51E-07 | 4.27E-07 | 8.54E-10 | 1.36 | 1.44 |
| | 13 | 5.45E-03 | 5.29 | 0.224 | 1.28E-03 | 67.6 | 79.2 | 3.74E-07 | 3.71E-07 | 1.06E-09 | 1.69 | 4.25 |
| | 14 | 4.91E-03 | 16.3 | 0.240 | 1.37E-03 | 62.0 | 85.8 | 1.07E-06 | 1.03E-06 | 2.03E-09 | 3.24 | 3.58 |
| | pat | 4.50E-03 | 0.0 | 0.295 | 1.69E-03 | 80.6 | 88.6 | 1.98E-07 | 1.98E-07 | 8.86E-10 | 1.41 | 2.66 |

The number of muscle bundles within the manus differs between the two scanned species. In *Scorpio* a total of fourteen distinct bundles were identified whereas in *Hottentotta*, only twelve. Analysing the pairwise correlation matrix of the four analysed muscle architecture variables, an overall absence of significant patterns among them was noted. However, the most remarkable finding is the distinct correlation pattern between moment arm and PCSA in the two species. In *Hottentotta*, this pattern is negative and highly significant (α= -0.857) meaning that in this species the strongest muscles typically have shorter moment arms. Conversely, in *Scorpio* moment arm and PCSA are positively correlated (α= 0.651) indicating that strongest muscles in this species are inserted with longer moment arms (see **Fig. 4.1.2**).

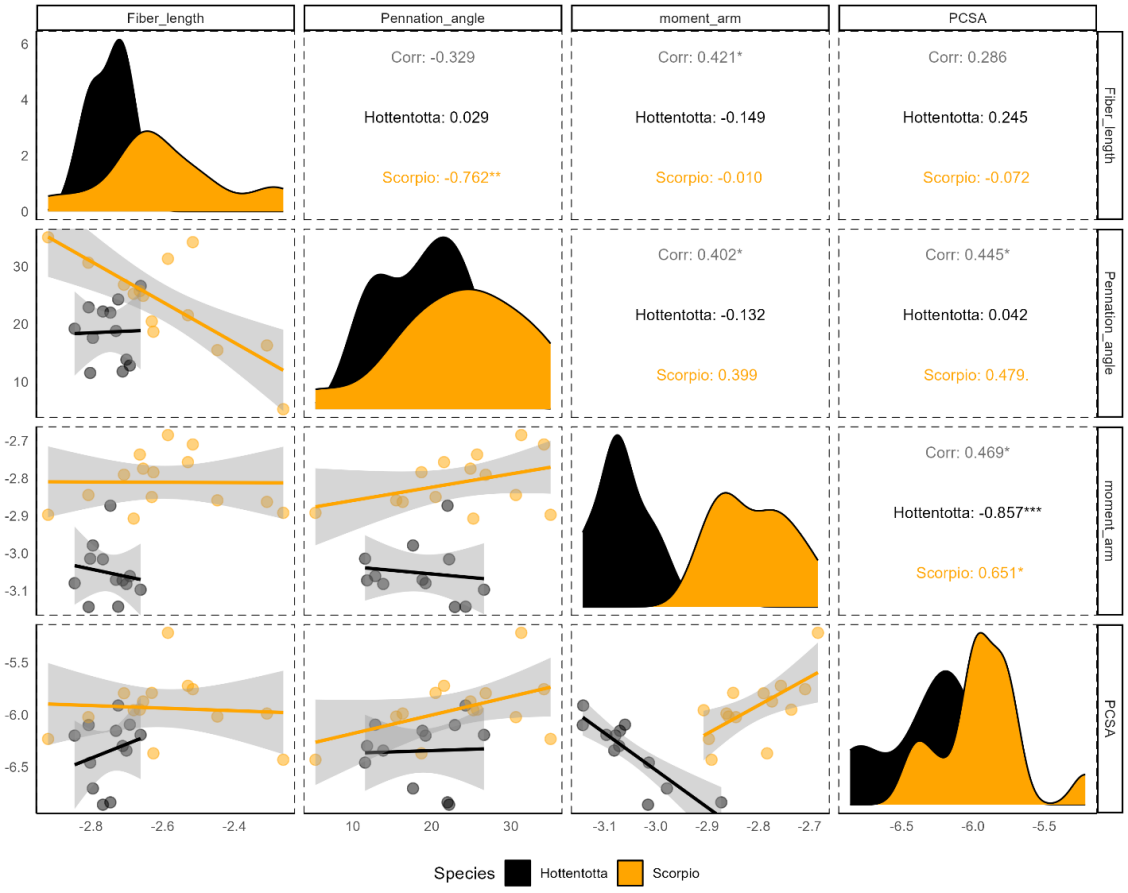


**Figure 4.1.2** Correlation plot across four determinants of architectural muscle. Asterisks show the level of significance.

Average pinnation angles in manus closing muscles in *Hottentotta* are smaller than those in *Scorpio* (see **Table 4.1.1**), although marginally not significant (*P* = 0.062). There is no statistical difference between the two species in the fascicle length/moment arm ratio (*P* = 0.45). However, **Fig_supp_4.1.5** shows that within manus closing muscles, this index is less heterogeneous in *Hottentotta* compared to *Scorpio*. In the latter species,

three muscle bundles present particularly larger fascicle length/moment arm ratios compared to the rest (see **Table 4.1.1**).

Sarcomeres were significantly longer in Scorpio than in *Hottentotta* (see **Table_suppl_4.1.2**) ($P < 0.001$). In both species the sarcomeres of the manus closing muscles were longer than those measured in the patellar muscle (see **Fig_suppl_4.1.6**). Moreover, sarcomere length was not evenly distributed across the four distinct groups of manus closing muscle bundles (see **Fig_suppl_4.1.7**, **Table_suppl_4.1.3**, and **Table_suppl_4.1.4**). In *Hottentotta*, sarcomeres from muscle bundles with a longer moment arm (dorsal) were shorter than sarcomeres measured from muscle bundles with shorter moment arm (ventral). The opposite was observed in *Scorpio*.

*Model output*

The muscle stress estimated from the biomechanical model is 841KPa in *Scorpio* whereas it is 897KPa in *Hottentotta*. The muscle stress estimated from the RMA model is 339KPa in *Scorpio* while it is 257KPa in *Hottentotta*.

The effect of the opening angle on closing force is remarkably different between the two species. The peak value of closing force is reached at larger opening angles in *Hottentotta* (44°), while in *Scorpio* the peak closing force is reached at medium opening angles (18°). The profile in **Fig. 4.1.3** also shows how, following the peak force, a substantial decline in bite force values takes place in *Hottentotta*. Conversely, in *Scorpio*, the decrease in force after the peak is not as pronounced.

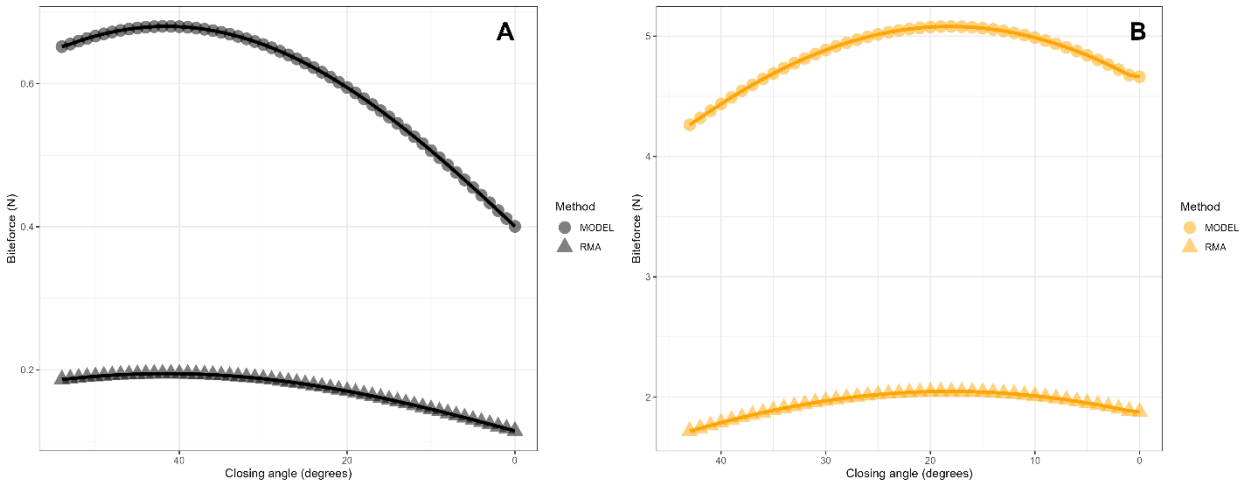


**Figure 4.1.3** Force generated per closing degree in *Hottentotta* (black) and *Scorpio* (orange). The different dot shapes represent the estimation of the force with stress calculated from the RMA method (triangles) and from the model (circles).

Muscle contraction speed does not display a significant difference between the two species (**Fig_suppl_4.1.8**). Moreover, muscle bundles having shorter moment arms must contract slower while muscles with longer moment arms must contract faster. The biphasic shape of the speed/opening angle curve has no biological meaning, and it is an artifact due to the values predicted from the polynomial fit.

Muscle bundles in *Scorpio* are inserted with a better mechanical advantage than the muscles in *Hottentotta*. Moreover, the peak in maximum mechanical advantage is generally reached at the beginning of the closing event in *Hottentotta* while in *Scorpio* peak mechanical advantage is reached when the chela is almost fully closed.

*Phylogenetic comparative analysis*

Sarcomere length has a marginal significant phylogenetic signal (λ = 0.71 P = 0.05). Although not significantly, the PGLS models show that sarcomere length tends to be negatively correlated with maximum angular velocity (β = -4.75, se = ± 2.75; P = 0.114) and positively correlated with size-corrected closing force (β = 2.39, se = ± 1.61; P = 0. 168) across the twelve species analysed. In both models the most likely value of λ is 0. When λ=1 is imposed, the overall results do not change, rather the fit of the model with the data gets worse.

The results of the PGLS with maximum angular velocity and size-corrected speed regressed against the Z-scores of the sarcomere length of ventral and dorsal bundles are shown in **Suppl_Table_4.1.5**. Positive Z-scores are significantly associated with larger angular velocity only in the models where λ is imposed to be 1. However, this significant result is lost when the model is run with the most likely value of lambda (in this case 0). Likelihood ratio test confirms a significantly better fit of the data when λ=0. In the case of the size-corrected closing force, irrespective of the value of lambda imposed, no significant effect of the Z-scores of sarcomere length in both ventral and dorsal bundles is observed.

**Discussion**

In this study the biomechanical determinants of chela closing performance were analysed in two species of scorpions with contrasting chela morphologies. We generated 3D models of the chelae of both focal species founding contrasting patterns in the organization, architecture, and mechanical advantage of both closing muscle bundles and structural elements. Across all these traits there is a strong integration towards two functional optima of performance: closing force in short-fingered species and closing speed in long-fingered ones. The optimization of closing force is obtained through larger

mechanical advantage of levers and muscles and longer sarcomere length while in long-fingered species closing speed is optimized by through the production of large angular acceleration in the first phases of the closing event.

*Determinants of fast closing in long-fingered species*

The architecture of the chela closing muscles in *Hottentotta gentili* shares common features with the muscle architecture of specialized sprinters. The most important similarity observed is the inverse relationship between PCSA and muscle moment arm. In this species, the strongest muscles are the ones with shorter moment arms. These muscles are attached in the ventral part of a sclerotized bulge located in the proximal portion of the movable finger which is absent in *Scorpio* (see **Fig. 4.1.4**).

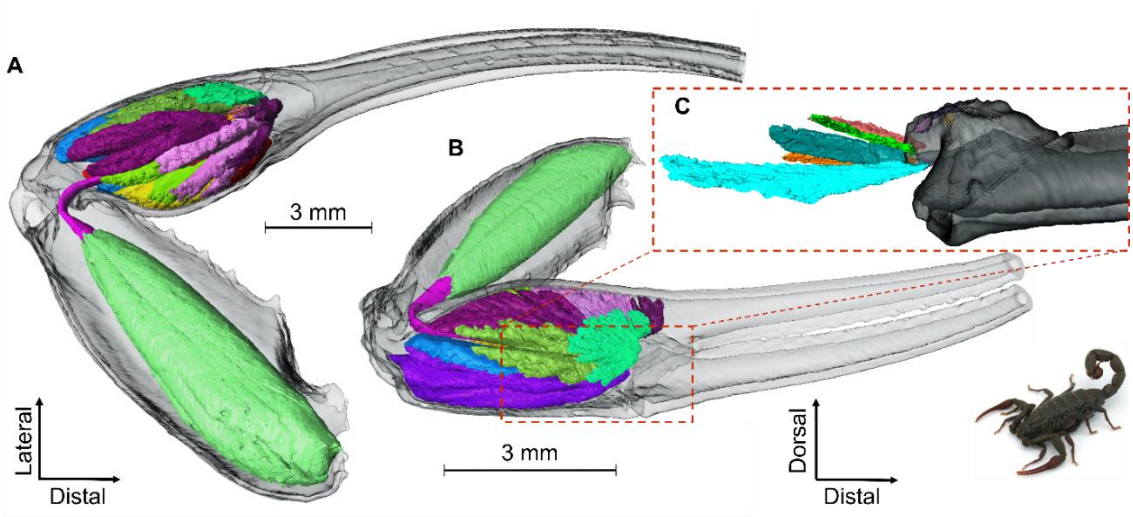


**Figure 4.1.4** Dorsal (A) and lateral (B) view of *Hottentotta gentili* closing muscles. In C a close-up of the proximal portion of the movable finger in which the sclerotized bulge is visible and allows muscles to insert with very small moment arms.

In fast rotations, muscles with shorter moment arms may still generate large torques about a joint due to their reduced shortening rates (Nagano & Komura, 2003). Due to the force-velocity relationship, the muscles with lower shortening rates generate relatively more tension than the muscles with higher shortening rates (Lieber & Ward, 2011). The low contraction speed is a consequence of a reduced sarcomere shortening, making them operate primarily on the plateau and at the beginning of the descending limb of their length-tension curve (Lieber & Ward, 2011; Lieber et al., 1997). Muscles with short moment arms may also have a higher proportion of fast-twitch fibres as already reported in humans (Gandevia & Mahutte, 1980). Keeping the PCSA the same, muscles having higher proportion of fast-twitch fibres develop more tension than muscles with higher proportion of slow twitched fibres and consequently provide larger torque about the joint.

The slow contraction coupled with larger PCSAs, relatively longer sarcomeres, great fascicle-to-moment arm ratios and the highest mechanical advantage reached at large opening angles, make these muscles better designed to generate more force, and therefore more angular acceleration, in the very first phases of the chela closing. The gastrocnemius of professional runners (Lee & Piazza, 2009; Nagano & Komura, 2003), the muscles of the thoracic limbs in greyhound dogs (Williams et al., 2008) and the pelvic muscles in ostrich (Smith et al., 2006) are similarly designed to the chela closing muscles of *Hottentotta gentili*. In these species, a large joint torque is needed to counterbalance the remarkable ground reaction force and the inertia due to the limb mass generated during explosive acceleration (Alexander, 2006). In scorpions, the moment of inertia of the movable finger and the resistance exerted by the air likely have negligible effects on finger rotation. In *Hottentotta gentili,* this large force generated at the beginning of the rotation is likely used to accelerate the movable finger. In high-load contractions, this muscle design is not as effective. In this condition, muscles generally contract almost isometrically. Therefore, to generate greater tension, muscles with larger PCSAs and longer moment arm are necessary. In *Hottentotta gentili*, the muscle bundles with longer moment arm are the ones having the smallest PCSA and relatively shorter sarcomeres to the average sarcomere length. An important contribution in chela closing performance is provided by the big patellar closing muscle. This muscle might have a crucial role in both low-and high load contraction. The patellar closing muscle is a parallel fibred muscle with very short sarcomeres which recalls an optimal design to allow fast displacement. However, this muscle in *Hottentotta* comprises 50% of the volume of all closing muscles and it is inserted to the movable finger with a relatively long moment arm. Increasing the size of a muscle rather than the length of its sarcomeres, allows to generate a discrete force input without any cost of contraction speed. The lack of spatial constrain in the patella segment allowed this strategy and might explain the need for fast closing species to evolve such remarkable big muscle to optimize speed but not at cost of force.

*Determinants of strong closing in short-fingered species*

The chela closing muscles in *Scorpio palmatus* appear remarkably suitable for generating high forces under high-load conditions, particularly when the chela is partially or nearly fully closed. This is primarily attributed to the positive correlation between PCSA and muscle moment arm. These muscles not only possess the theoretical capability to yield the highest force, but they are also anchored with longer moment arms, consequently resulting in augmented torque and thus an amplified force at the pincer finger (Lieber & Ward, 2011; Powell et al., 1984). Furthermore, output can also be attributed to these muscles' relatively longer sarcomeres. Longer sarcomeres generate

more action-myosin cross bridges and consequently can generate a higher tension (Huxley, 1957). Under low-load conditions, muscles with longer moment arms must contract faster than those having a shorter moment arm, thereby generating smaller torques about the joint (Lee & Piazza, 2009; Osgood et al., 2021). Due to the force-speed curve, a fast contraction results in low force generation and the muscles contracting slower are the ones providing most of the torque to close the chela. However, the muscles with shorter moment arm in this species are the ones with the smallest PCSA and with the relatively shortest sarcomeres generating low peak force and consequently a low angular acceleration.

In *Scorpio palmatus*, the fascicle to moment arm ratio of the chela closing muscles shows an interesting pattern absent in *Hottentotta gentili*. While all muscles in *S. palmatus* have small fascicle to moment arm ratios, three of the muscle bundles (10, 13 and 14) having the shortest moment arm also have the largest ratio values. They present a typical design of fast contracting muscles, specifically long fibred, almost parallel and with relatively shorter sarcomeres (Gans, 1982). It seems thus that in *Scorpio,* but not in *Hottentotta*, two distinct families of muscles exist: the first is designed to optimize force generation dorsally and a second one more suited to produce fast displacement ventrally. Synergistic muscles with different architectures are for instance the human wrist extensors, the extensor carpi radialis brevis (ECRB) and extensor carpi radialis longus (ECRL). These muscles differ in the sarcomere shortening rate and in muscle moment arm length. Lieber et al (1997) proposed that the muscles with different architecture but synergist action might provide more force generation over wider range of velocities or alternatively they may generate the same amount of force and excursion but with less muscle mass expenditure. In *S. palmatus* however, the muscles designed to produce higher closing forces take up about 88% of the volume of all closing muscles. This disparity between the two muscle families makes both scenarios unlikely.

*Sarcomere length is an important determinant of closing performance*

The distribution of sarcomere length across the dorsal and ventral chela closing bundles does not exhibit consistency across species characterized by varying speeds. For example, across the fast-closing scorpions, the spatial pattern of distribution of sarcomere length described in *Hottentotta* is not replicated in the sexually dimorphic species (*Tityus pachyurus* and *Parabuthus transvaalicus*). In these species, males possess rounded and more robust chelae and the sarcomeres in the dorsal muscle bundles are longer than their ventral counterparts (see **Fig_suppl_4.1.2**). The same scenario is observed in the strong closing scorpions where sexual dimorphic species

(*Chactas* sp.) do not mirror the sarcomere distribution pattern reported for *Scorpio.* Another divergence to the pattern is observed in *Chaerilus stockmannorum* (see **Fig_suppl_4.1.3**). This species belongs to an early divergent family in the scorpion phylogeny being one of the sister groups of Buthidae (Sharma et al., 2015). Hence, some phylogenetic constraints might be playing a role in shaping the architecture of the chela closing muscles in this family. In conclusion, there is a clue that fast species may consistently present a different type of sarcomere distribution than the strong and slow species. However, this hypothesis is not fully supported by our comparative analysis of sarcomere length distribution across fast and slow species probably due to the inclusion of sexual dimorphic species.

The comparative analysis of sarcomere length across multiple species of scorpions with different chela morphology shows positive correlation between sarcomere length and chela closing force. This pattern has been observed in many groups of crustaceans. Specifically, in the chelae of crabs (Taylor, 2000), in the raptorial appendages of mantis shrimps (Blanco & Patek, 2014) and, within the same individual, across the crusher and the cutter chelae of adult lobsters (Govind & Lang, 1978). The sarcomere length variation found in scorpions ranges from 3 to 6 µm indicating that from the shortest to the longest sarcomere length there is 100% variation towards increasing force generation and contraction speed. This variation is quantified to the 50% across the spearers and smashers mantis shrimps (Blanco & Patek, 2014) and 60% across the crabs . This result fortifies the idea that chela closing muscles in fast and strong species evolved towards an optimisation or either speed or force. Contrarily to manus muscle bundles, there is a striking uniformity in the sarcomere length of the patellar muscle across species. This suggests that this muscle initially evolved to facilitate rapid finger abduction. However, in species with rapid closure, the necessity for force has led to the enlargement of this muscle. Conversely, in species with robust closures, this muscle plays a marginal role, as speed is not a priority and there may be spatial constraints in developing a large muscle, as seen in the fast-closing species.

*Biomechanical consequence on feeding ecology*

The distinct chela designs and markedly diverse closing muscle architectures observed in the two studied species suggest that chelae are differently optimized for distinct feeding tasks. Specifically, the force distribution across the entire closing event in *Hottentotta* shows a force peak during the initial phases, followed by a significant decline during the central phase of the event. This suggests that in *Hottentotta*, muscle architecture of closing muscle bundles and the elongated chela fingers are designed to

optimize the grasping of the prey rather than the holding or crushing. In contrast, *Scorpio* presents a different scenario. The peak force is generated in the middle of the closing event, and the decrease in force toward the final phases is minimal. Consequently, *Hottentotta* relies not on force for holding prey, but rather on the efficacy of its venom. The potent and rapid-acting venom plays a pivotal role in immobilizing the prey and diminishing the chances of escape. Additionally, to venom, friction can have an important role in prey holding. The numerous rows of small denticles are likely to enhance the friction coefficient, thereby increasing the grip on the prey's body. Conversely, *Scorpio* might not excel in grasping prey, but once captured, the grip on the prey is guaranteed by an extreme force crushing the body of the prey. Although congeneric frequently employ their venom to paralyse prey (Sarhan et al., 2013), in other scorpionids, prey is often consumed without venom utilization (Casper, 1985). Our findings imply the existence of a functional trade-off between grasping and holding mechanisms, which can profoundly impact predatory behaviour and possibly influenced scorpion venom evolution.

**Conclusions**

Our study on the nature of the biomechanical trade-off between force and speed in the chela closing performance in scorpions revealed strong integration of chela musculoskeletal components towards either fast or strong closing performance. Moreover, our results uncovered the existence of a functional trade-off between grasping and holding/crushing mechanisms which can have profoundly influenced scorpion predatory behaviour and venom evolution. Different predatory performances might be also mirrored by different prey preferences. The short-fingered species may prey on slow hard-bodied prey more frequently than the long-fingered species, which may instead be more efficient in preying on quick and soft-bodied prey. Unfortunately, diet in scorpions is still a challenging yet unexplored field due to its technical difficulties. Advances in the development of molecular techniques to retrieve prey DNA from scorpion digestive tract may soon allow testing whether species with contrasting chela morphology also have a different foraging niche.

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# Chapter 5
# Molecular analysis of scorpion diet

## 5.1 Metabarcoding analysis of different portions of the digestive tract of scorpions (Scorpiones, Arachnida) following a controlled diet regime shows long prey DNA half-time

**Introduction**

For many years, direct observations of predation events and the analysis of the remains of consumed prey were the most used approaches to investigate diet composition in arthropods (reviewed in Sunderland, 1988). These methods require years of investigation to gather a comprehensive number of records/samples, as well as taxonomic expertise. Due the quality of the samples, species-level identification is not always possible (Nielsen et al., 2018). Moreover, these methods insufficiently provide information about diet in liquid feeders (Symondson, 2002) and in species living underwater (Hyslop, 1980) or beneath the soil (Pompanon et al., 2012).

Several molecular techniques have been developed to study diet (reviewed in Nielsen et al., 2018). Recently, DNA-based diet analysis of stomach or scat content has been the most widely used tool to analyse diet and map trophic interactions (Cristescu, 2014; King et al., 2008; Liu et al., 2020; Nielsen et al., 2018; Pompanon et al., 2012; Sousa et al., 2019).

One of the main challenges of DNA-based diet analysis is the undesired amplification of the DNA of the consumer taxon. The co-amplification of the consumer DNA can be limited using a combination of exclusion and universal primers (Cuff et al., 2021; Lafage et al., 2020). When the consumer and the ingested item are phylogenetically distant, species-specific primers are used to detect a specific targeted consumed item. This approach may be useful for conservation purposes (Egeter et al., 2015, 2019) or to investigate herbivore diets (De Barba et al., 2014).

Consumer DNA amplification can also be reduced by using consumer-specific blocking primers (Deagle et al., 2009; Toju & Baba, 2018; Vestheim & Jarman, 2008). However, the use of blocking primers can prevent the amplification of prey species that are phylogenetically close to the consumer (Piñol et al., 2014; 2015). Other studies have suggested extraction strategies based on the retention of short DNA fragments, presumably belonging to digested item, and the exclusion of longer fragments most likely belonging to the consumer (Krehenwinkel et al., 2017).

The detectability of DNA of the ingested items also depends on how long the ingested DNA resides in the digestive tract of the consumer. One of parameters to assess the detectability of ingested DNA is its half-life ($D_{50}$) (Greenstone et al., 2014; Uiterwaal & DeLong,

2020). The $D_{50}$ is the time at which sequences belonging to a consumed meal are detected in half of the consumers tested. However, the $D_{50}$ parameter is sensitive to the type and size of the ingested meal (Schattanek et al., 2021), the digestion rates (Uiterwaal & DeLong, 2020) and the analysed portion of the digestive tract (Macías-Hernández et al., 2018).

Molecular sequencing of stomach contents has been used to assess the diet composition of many arthropods, and has proven to be a successful tool to investigate the diet of liquid-feeding arachnids like spiders (Krehenwinkel et al., 2017; Lafage et al., 2020; Macías-Hernández et al., 2018; Sierra Ramírez et al., 2021), mites (Heidemann et al., 2014; Pérez-Sayas et al., 2015) and ticks (Kirstein & Gray, 1996; Pichon et al., 2003). The diet of scorpions has to date not been studied using this method.

Scorpions are important predators in many different biomes, ranging from deserts to humid rainforests. In arid environments in particular, scorpions can represent the highest fraction of the animal biomass, second only to termites (Polis & Yamashita, 1991). Consequently, in these environments their high densities and their predatory habits may have a great impact on arthropod population dynamics (Polis & McCormick, 1986; Shachak, 1980).

Studying scorpion diet is particularly challenging because, besides being liquid feeders, scorpions have a particularly slow metabolic rate when compared to similar sized arthropods (Lighton et al., 2001). As a consequence, these animals feed infrequently and are able to survive several months without eating (Pimenta et al., 2019; Stahnke, 1966; Vachon, 1957). Because of these factors, investigating scorpion diet using traditional methods is difficult and time consuming. Information about their natural diet is known only for a few of the approximately 2,600 scorpion species currently recognized. Moreover, works on this topic report only a small subset of the potential prey items these species may consume (for reviews, refer to Polis, 1990; Simone & van der Meijden, 2021). The most complete scorpion diet assessment was done for *Paruroctonus mesaensis* (Stahnke) (Polis, 1979). It was conducted using direct observation and recognition of the remains of the prey left in the scorpion burrows and took 5 years to complete.

Like many other arachnids, scorpions have an external pre-digestion of the prey. Specifically, prey is chewed through chelicerae, while digestive enzymes secreted from coxal glands of the legs I and II act on prey tissues (Alexander, 1972; Polis, 1990; Venkateswararao, 1967). Pre-digested prey fluids are sucked into the oral cavity through the pumping action of the muscles of the pharyngeal chamber wall (Pavlovsky & Zarin, 1926). Once the prey fluids are pumped into the pharyngeal chamber, they are then conveyed to the central digestive tube (the mid-gut) running almost the whole length of the scorpion body. The mid-gut is strongly branched, and diverticula extend through the whole body of the animal, and even reach the legs (Pavlovsky & Zarin, 1926; Snodgrass, 1952). The diverticula end in different parts of the hepatopancreas, an organ responsible for the internal digestion, absorption and storage of the

ingested food (Fuzita et al., 2015; Warburg, 2012). The last part of the digestive tract leads the food to its expulsion through the anus, which is located in the metasoma or “tail”, just before the venom vesicle.

In scorpions, particles of food are found both in the mid-gut lumen and in the cells composing the epithelium of the peripheral branches running in the interstitial tissue of the hepatopancreas (Goyffon & Martoja, 1983; Zouari et al., 2006). The digestion process happens in two different types of cells covering the epithelium of the mid-gut diverticula: the basophilic cells (or zymogen cells) and the digestive cells. The basophilic cells perform the extracellular digestion while the digestive cells absorb the fluid containing food from the lumen of the diverticula. The latter, through intracellular digestion, stores the nutrients in characteristic vacuoles (Goyffon & Martoja, 1983; Zouari et al., 2006). The hepatopancreas is composed of interstitial cells that surround the mid-gut diverticula and apparently its function is limited mainly to food storage. The waste products of both extra- and intracellular digestion are expelled into the lumen of the caudal portion of the mid-gut, conveyed into the hindgut, and then expelled through the anus.

In this work, we test a DNA-based approach to assess scorpion diet for the first time, using scorpions fed under a laboratory-controlled diet regime. The two main objectives of this work are i) identifying the best portion of the digestive tract to select to gather the highest amount of prey DNA, ii) estimating the $D_{50}$ of prey-DNA across different portions of the digestive tract. Secondarily, different extraction methods have been compared to assess whether a bead-based method should be preferred to a salt-out one.

## Methods

*Feeding Experiment*

14 subadult specimens of the Vietnamese forest scorpion, *Heterometrus laoticus* (Couzijn, 1981) were subjected to a controlled diet regime for nine weeks. All the tested scorpions were bred in our laboratory and kept in captivity for several years with humid substrate, a photoperiod of 12:12 light/dark at 24-26°C and fed only with fresh crickets (*Acheta* sp.) every 3 weeks prior to the study commencing. The experiment consisted of three feeding sessions. During each session one prey type was provided to each scorpion. The first prey type offered was an adult cricket, *Acheta* sp. (week 0) followed by larvae of mealworms, *Tenebrio molitor* (week 3), and adult Argentinian cockroaches *Blaptica dubia* (week 6). The total mass of each prey type offered was controlled to standardize the food intake per feeding session (see Appendix 5.1.S1). After each feeding session, a period of starvation of three weeks followed. This is the same starvation time used in the only available published work about food detection in the scorpion digestive tract (Quinlan et al., 1993). Three weeks after the last feeding session (week 9), scorpions were anesthetized using isoflurane, and

subsequently euthanised by freezing (-20°C). Only six scorpions out of the 14 completed the feeding trials successfully. The other scorpions that refused to eat at least one of the offered prey were discarded from the experiment.

*Dissection protocol*

Euthanized scorpions were labelled and defrosted before the dissection procedure. First, scorpions were rinsed in distilled water and then placed into a container filled with 500 ml bleach at 5% for 40 minutes to remove external contaminant DNA (Greenstone et al., 2012; Linville & Wells, 2002). Scorpions were then rinsed in distilled water to remove any excess bleach and placed on a sterilized dissection plate. To minimise cross-contamination, a new set of gloves and new scalpel blade were used for each specimen. All reusable tools used during the dissection were rinsed in 60% bleach, then in 70% ethanol and finally flamed between specimens. The dissection was performed under a stereomicroscope (Motic SMZ-168) and started with carefully removing the dorsal portion of the exoskeleton from prosoma to the end of the metasoma. The heart was carefully removed and the hepatopancreas underneath was moved laterally until it was possible to observe the mid-gut. The latter was then carefully removed and stored in a 2 ml cryotube and labelled as mid-gut (MG). The whole hepatopancreatic mass was stored in a 15 ml tube and labelled as HP. To extract the last part of the digestive tract, all the muscles in the five metasomal segments were removed and the last part of the digestive tube stored in a 2 ml cryotube and labelled as hindgut (HG). **Figure 5.1.1** shows three different stages of the dissection process. The content of the dissected gut portions was homogenized and the whole volume divided in half. The DNA from each half was subsequentially extracted using two different methods.


**Figure 5.1.1** A) Scorpion prepared for dissection. (B) The hepatopancreas is exposed once the mesosomal and prosomal cuticle of scorpion is removed. (C) The mid-gut is exposed after removal of the hepatopancreatic mass, ovaries, book lungs, and the ventral nervous chord. The removal of the dorsal cuticle and the muscles of the metasoma exposes the hindgut. (D) Detail of the hepatopancreatic mass with its typical granular texture. (E) Close-up of the hindgut

*DNA Extraction*

Two different extraction methods were used. The first is a salt-out method and the second one uses Agencourt AMPure XP® beads (Beckman Coulter, Ma, USA) and Qiagen® (Qiagen, Germany) buffers (for detailed protocols, see **Appendix 5.1.S2**). DNA extracted through the salt-out method was stored in 100 µl of pure demineralized water while DNA extracted through the bead-based method was stored in 70 µl of the AE buffer (Qiagen®).

*Blocking primer design and PCR optimization*

Sequences of COI of *Heterometrus laoticus*, along with representative sequences of different species of scorpions and other arthropods possibly included in scorpion diet were retrieved from the NCBI website (see **Appendix 5.1.S3**).

Universal primers for terrestrial arthropods fwhF2 (forward) and fwhR2n (reverse) (Vamos et al., 2017) were chosen for this study for their high efficiency in targeting arthropod DNA in environmental studies (Elbrecht et al., 2019). This set of primers amplifies a 205 bp portion of the Folmer fragment (Folmer et al., 1994).

The gathered sequences were aligned in Geneious using the MUSCLE algorithm (Edgar, 2004). The blocking primer we designed, Simone_2021_HlBlk, 5'-CCYCCTTTGTCTTCTAGTATATTTC 3C – 3', shares the first five bases with the last five of

primer fwhF2 and matches the next 21 bases of the sequence of *H. laoticus* (AY156573) without ambiguities.

To assess the specificity of the blocking primer, we tested the pair Simone_2021_HlBlk / fwhR2n in the NCBI Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and retrieved all the possible targets within the invertebrate dataset having up to three mismatches with at the least one of the aforementioned pair of primers.

Subsequently, blocking primer influence on non-targeted taxa was tested by performing PCRs using DNA extracted from tissue of fresh specimens of *H. laoticus*, crickets, mealworm larvae, and Argentinian cockroaches as templates. DNA extracted from these samples was first quantified using a Nanodrop 1000 (Thermo Scientific). To avoid having different DNA concentrations among samples biasing the result of the amplification process, we diluted all samples to 1 ng/µl concentration. The PCRs were carried out with and without blocking primers in volumes of 10 µl, comprising 5 µl Qiagen Multiplex PCR Master Mix (Qiagen, Germany), 0.4 µl of each primer (10 pM) and 1 µl extracted DNA. PCRs done with the blocking primer also included 0.4 µl of Simone_2021_HlBlk at 100 pM. Cycling conditions used initial denaturing at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s, with a final extension at 60°C for 10 min.

Increasing concentrations of blocking primer were also tested. Using the DNA extracted from fresh leg tissue of *H. laoticus*, blocking primer efficiency was tested at 10x, 15x and 20x the concentration of the amplification primers. PCR conditions were the same as indicated above. Optimization of annealing temperature was performed. The same template DNA was used to perform PCRs with and without blocking primers using a temperature gradient in eight steps from 42°C to 60°C. The other PCR conditions were kept unchanged. All PCR results were checked by visually inspecting 2 µl of each PCR product on a 2% agarose gel stained with GelRed (Biotium, USA).

*Dual step Illumina library preparation*

Based on the optimisation tests mentioned above, we selected an annealing temperature of 56°C and a blocking primer concentration of 10x. Three PCR replicates per sample were prepared using 5 µl of Qiagen Multiplex PCR Master Mix, with 0.4 µl each of primers fwhF2 and fwhR2n at 10 pM, 0.4 µl of primer Simone_2021_HlBlk at 100pM and 1 µl of extracted DNA for a total volume of 10 µl. To test the efficiency of the blocking primers, PCRs of six arbitrary selected samples, amplified without the addition of blocking primers, were also sequenced. A negative PCR was included to control for possible contaminants. For all PCRs, the universal primers already had the adaptors for the Illumina sequencing. Library preparation, including indexing PCR (second PCR), fragment purification, normalization and

final pooling followed the steps indicated in chapter 3.3 from (Paupério et al., 2018) with some minor modifications, as follows: before the second PCR all 3 replicates of the first PCR were pooled together per sample; total volume for the second PCR consisted of 14 µl, including 7 µl KAPA HiFi PCR Kit (KAPA Biosystems, USA), 0.7 µl of each "index" and 2.8 µl of diluted PCR product (only strong PCR products were diluted). Lastly, bead purification was carried out using Agencourt AMPure XP® beads (Beckman Coulter, Ma, USA) for an amount of 0.75% of the final library volume. The final library was then sequenced on a MiSeq machine using an Illumina Reagent Kit v3 600-cycle (Illumina, California, USA). The run, aiming for a coverage of 30,000 reads per sample, was shared with other projects on freshwater macro invertebrates and bat samples.

*Bioinformatic pipeline*

Sequence data were processed using the MBC pipelines package (Galhardo et al., 2018, commands used are provided in Appendix 5.1.S4) following Peixoto et al. (2020). Shortly, reads produced on the MiSeq platform were demultiplexed according to the sample-specific indexes using BASESPACE (basespace.illumina.com). Paired-end reads were aligned using FLASH2 (Magoč & Salzberg, 2011) and primers were removed with cutadapt (Martin, 2011). Using VSEARCH (Rognes et al., 2016), sequences were dereplicated, singletons were removed, and sequences outside the expected amplicon lengths of 70–130 bp (without including primers) were removed. Default settings were used for all of commands. The amplicons were mapped against the NCBI nucleotide database using the BLAST algorithm. Taxonomy was assigned to each query using a lowest common ancestor (LCA) approach. For each query relatively stringent percentage identity thresholds were used: 99% for species level, 97% for genus level, 95% for family level and 93% for higher-than-family level. Data were filtered to reduce false positives, tag-jumping and other contaminants. In order to have a reliable minimum sequence copy threshold, we further filtered the data following Drake et al. (2022). We applied the "Max contamination" filter together with different "Sample %" thresholds (ranging from 0.08% to 1%). The optimal "Sample %" threshold of 0.25% was selected in order to maximize the removal of contaminants while preserving most of the reads of the target prey items.

*Statistical analysis*

We use three methods to analyse the effect of gut portion and extraction method on each prey type separately and then on all prey types pooled. The first method converts all the filtered reads into a binary presence/absence type of data. This dataset was analysed through Generalized Linear Mixed Models (GLMMs) with the prey presence/absence data as the

response variable and the gut portion and the extraction method as fixed variables. Specimens were included as random factors. The data were modelled following a logistic distribution.

In the second and third method, in order to account for the large variance in total number of reads among samples, we modelled the response variable as a ratio of the taxon reads to the total number of reads per sample.

The second method consists of a GLMM model where gut portion and extraction method are the fixed variables and the ratio of prey read count/total number of reads per sample is the response variable. In this method the response variable follows a quasibinomial distribution.

The third method differs from the second in using a zero-inflated beta regression.

We used the “lme4” package (Bates et al., 2015) for binary and binomial GLMMs and the package “glmmTMB” (Brooks et al., 2017) for the zero inflated beta-regression.

Likelihood ratio tests (LRT) were used to evaluate all the models using a nested approach (from null to full model, adding each variable sequentially to a previous model) through the function lrtest of the package “lmtest” (Zeileis & Hothorn, 2002).

The same three methods were also applied to the samples sequenced with and without the blocking primer. The response variable and the random factor are the same described for each method while the fixed variable in these models is the presence of the blocking primer.

For all the models generated, the corrected Akaike Criterion Index (AICc) was used to select the best fitting model using the function AICc from the package “MuMIn” (Burnham & Anderson, 2002).

To fit the detection against the time from ingestion of the three targeted prey types for each gut portion type we used probit regression. From these models we calculated the probability of detection for each day and the day at which the probability of detection is 50% ($D_{50}$).

All analyses were performed in R 4.1.2 (R Development Core Team, 2021).

**Results**

After removing the singletons, sequencing generated a total of 852,788 reads for which 45,943 (5.4%) belong to the three targeted prey. Of the total reads, 805,068 (94.4%) belong to *Heterometrus laoticus*, while only 1,777 (0.2%) reads came from contaminants. Five PCRs out of 41, namely 2-HP-SALT, 3-HG-SALT, 10-HG-SALT 8-HP-SALT, and 10-HP-SALT, were excluded from the statistical analysis. The first three because they accounted for a total of zero reads, while the last two had a read count below that of the negative. The “Max contamination” filter alone cleaned all the negatives. The “Sample %” threshold of 0.25% removed the 90% of all the artifacts. **Fig. 5.1.2** shows the great variance in the detection of

the three target prey, and that the majority of the reads come from just two specimens out of the six sequenced.

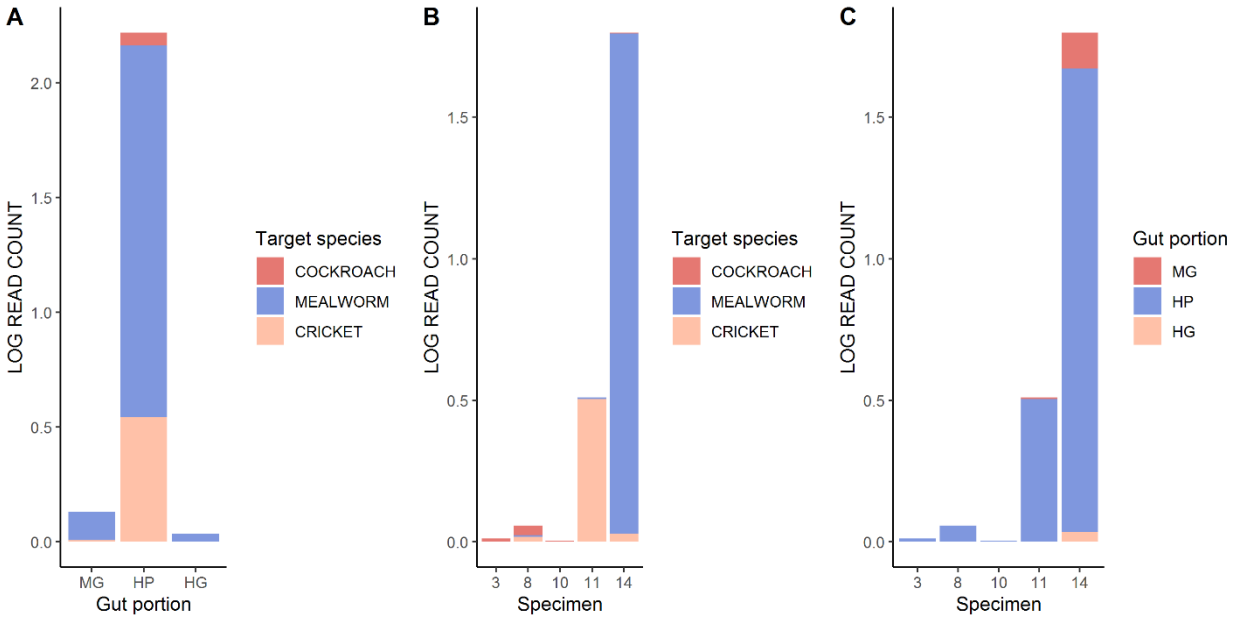


**Figure 5.1.2** A) Abundance of the three prey species per extraction method. (B) Abundance of the prey species per specimen. (C) Abundance of reads of the target prey per gut portion for all the specimens

The presence or absence of the blocking primer for the six samples tested did not significantly affect the amplification of the target prey (Binary; Primer_Chisq1 = 9.07E-02, p-value = 0.732), (Quasibinomial; Primer_Chisq1 = 4.91E-02, p-value = 0.825) (Beta-regression; Primer_Chisq1 = 0.157, p-value = 0.693).

**Table 5.1.1** Likelihood Ratio Test of nested models using different response variables and statistical analysis. Significant p-values (<0.05) are highlighted in bold.

| **Binary (0,1)** | **Model description** | **AICc** | **#Df** | **LogLik** | **Df** | **Chisq** | **Pr(>Chisq)** |
|---|---|---|---|---|---|---|---|
| null model | detection ~ 1 + (1 \| Specimen) | 94.8 | 2 | -45.3 | | | |
| model 1 | detection ~ Gut_portion + (1 \| Specimen) | 76.7 | 4 | -34.1 | 2 | 22.4 | **1.36E-05** |
| full model | detection ~ Gut_portion + Extr_method + (1 \| Specimen) | 78.6 | 5 | -33.9 | 1 | 0.293 | 0.588 |
| **Binomial (0≤X<1)** | | | | | | | |
| null model | Prey_reads/Total_sample_reads ~ 1 + (1 \| Specimen) | 23.4 | 2 | -9.63 | | | |
| model 1 | Prey_reads/Total_sample_reads ~ Gut_portion + (1 \| Specimen) | 22.9 | 4 | -7.25 | 2 | 4.74 | *0.0932* |
| full model | Prey_reads/Total_sample_reads ~ Gut_portion + Extr_method + (1 \| Specimen) | 25.0 | 5 | -7.18 | 1 | 0.14 | 0.708 |
| **ZINF-Beta Regression (0≤X<1)** | | | | | | | |
| null model | Prey_reads/Total_sample_reads ~ 1 + (1 \| Specimen) | 43.6 | 4 | -17.6 | | | |
| model 1 | Prey_reads/Total_sample_reads ~ Gut_portion + (1 \| Specimen) | 47.9 | 6 | -17.5 | 2 | 0.2303 | 0.891 |
| full model | Prey_reads/Total_sample_reads ~ Gut_portion + Extr_method + (1 \| Specimen) | 50.1 | 7 | -17.4 | 1 | 0.1866 | 0.666 |

The likelihood ratio tests on the nested logistic and the quasibinomial models respectively provided a significant and a nearly significant effect of the gut portion on read count of the three pooled target species. The same analysis found no significant effect of the extraction methods. The effects of both gut portion and extraction method were not significant in the zero-inflated beta regression model. In **Table 5.1.1** we report only the results of the methods applied to the pooled target prey because their higher combined numbers provide more statistical power.

The half-life estimated from the Probit regressions is 21.8 days for mid-gut, 51.5 days for the hepatopancreas and 15.8 days for the hindgut (see **Fig. 5.1.3**).

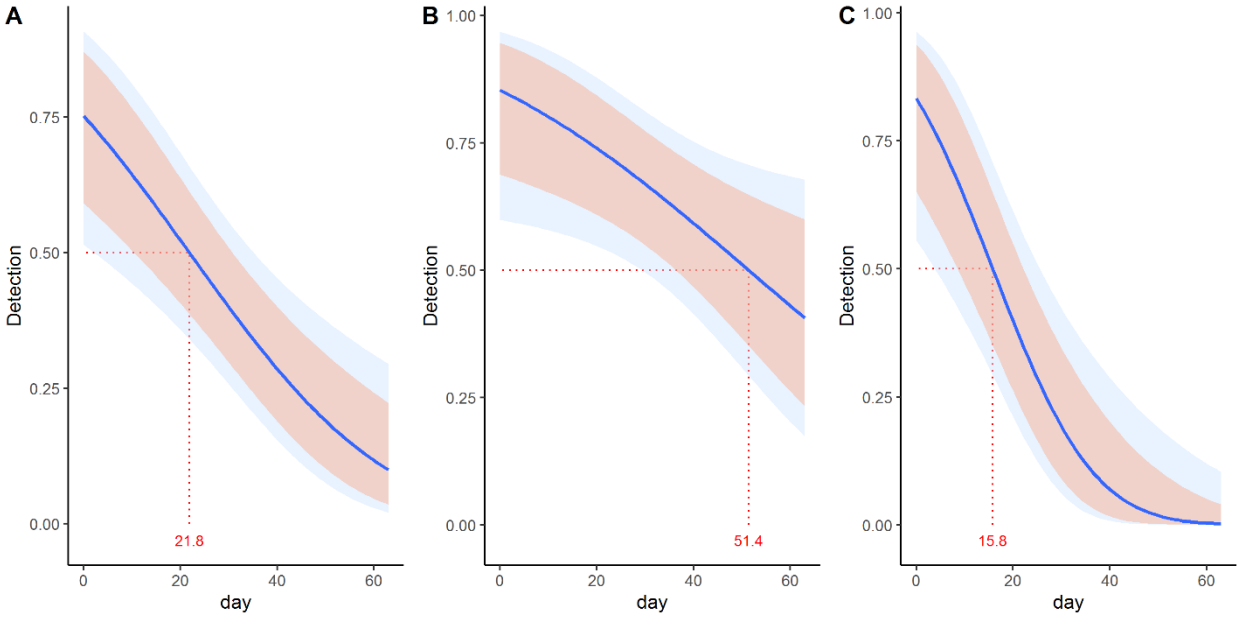


**Figure 5.1.3** Probit regressions of prey DNA decay over time per section of the digestive tract (blue line) in (A) mid-gut and (B) hepatopancreas (C) hindgut. The dotted red lines indicate the $D_{50}$ of prey DNA (in days) and the light blue band indicates the 95% confidence interval, while the 83% confidence interval is shown in salmon.

## Discussion

*Gut portion selection*

This study reports the results of a metabarcoding-based approach to identify how DNA sequences of partially digested prey are distributed across the three main sections of the scorpion digestive tract. The hepatopancreas retained prey DNA sequences the longest, followed by the mid-gut. This result finds support in similar studies conducted in spiders, where the opisthosoma (the tagma containing the mid-gut and the hepatopancreas), was the best body part to detect DNA of consumed prey (Krehenwinkel et al., 2017; Macías-Hernández et al., 2018; Miller-ter Kuile et al., 2021).

Presumably due to the highly degraded nature of the waste products of digestion (Goyffon & Martoja, 1983; Kanugo et al., 1962; Yokota, 1984), the hindgut was the gut portion from which the lowest number of prey DNA reads were detected and is therefore less suitable for metabarcoding diet analysis.

It is interesting that the last provided item (cockroach) can only be detected in the mid-gut and the hepatopancreas, suggesting that perhaps three weeks are not sufficient for the food to reach the last portion of the intestine (hindgut) in well-nourished scorpions. Digestion rate is affected by the feeding state of the consumer (Secor, 2001). Studies conducted in carabid larvae show that food detectability in the digestive tract decreases with an increased starvation period (Lövei et al., 1985). Similar results have been also found in other species of insects (Agustí et al., 1999). However, since our scorpions were fed regularly and the experimental regime was similar to the prior normal feeding regime, we expect that they were

not starving or overly satiated. Another unexpected result was the high number of reads of the first prey item detected in the mid-gut, contrasted with an almost total absence in the hindgut. Since our tested scorpions have been maintained exclusively on a diet of crickets prior to the beginning of the treatment, cricket permanence in the mid-gut and its consequent detection may be a result of prolonged exposure to that prey type in the past. Alternatively, as already observed in mites, the mid-gut is readily filled with food along its length, but the distribution of nutrients in the absorbing and storing tissues is not totally synchronous, and the most caudal portions of the mid-gut may be the last to be emptied (Bowman, 2017, 2019). This possibility is corroborated by the detection of asynchronous digestive processes across diverticula in scorpions fasting for one month (Goyffon & Martoja, 1983). Although we cannot draw robust conclusions due to our limited sample size, the lack of DNA of the oldest prey type in the hindgut may be explained by repeated scorpion defecations within the nine weeks of treatment. In other Heterometrus species, the first defecation event is within three days after the last meal (Kanugo et al., 1962).

*Food permanence in digestive tract*

While cytophysiological aspects of scorpion digestion have been well explored, studies on food permanence in scorpions' digestive tracts are lacking. The only data available on this topic can be found in Quinlan et al. (1993). Here, using an antibody-based technique, the authors detected prey antigens in the hepatopancreas of two species of scorpions that had undergone up to 32 days of food deprivation. Similar studies have been conducted in spiders, obtaining much lower detection times (up to 13 days) (Sopp, & Sunderland, 1989). In ticks, a PCR-based analysis of the composition of remnants of larval blood meals obtained from mid-gut of nymphs allowed the detection of host-DNA up to 270 days after the molt (Kirstein & Gray, 1996; Pichon et al., 2003). In recent years, due to improvement of high-throughput sequencing techniques, consumed meal detectability has been estimated through the calculation of its $D_{50}$. This parameter was used to show differences in prey detectability time across different families of spiders. The highest half-life value for spiders (16 days) was obtained in the family Lycosidae (Uiterwaal & DeLong, 2020). Our data suggests a high value for $D_{50}$ of at least 51 days for the hepatopancreas, showing that it stores food in an undigested or partly digested state for a long time. However, these results have to be considered cautiously because, even if the mass of the prey items ingested was roughly the same, we could not take into account the detectability half-life of each prey type because of our experimental design.

*Primer and tissue biases on target prey amplification*

None of the three prey species was present in the list of taxa that could be blocked by the blocking primer. The blocking primer has high specificity for scorpions, spiders, and wasps (see Appendix 5.1.S5). Additionally, when the blocking performance of the blocking primer was tested on different arthropod species, the amplification of the three prey species was unaffected or no evident attenuation was observed.

The performance of the universal primer Fwh2 in amplifying the orders Blattoidea, Ortopthera and Coleoptera was evaluated by Tournayre et al. (2020). In that study, the power of the amplification of the primer is very similar across the targeted taxa of our work.

A possible tissue-specific inhibition cannot be excluded to explain lack of detection of prey DNA in the hindgut and in the mid-gut. Tissue-specific inhibition has been observed in some portions of the digestive tract of the ants of the genus *Tetramorium*, specifically when the crop is not removed from the whole gaster (Penn et al., 2016). More experiments are needed to better address this possible methodological issue.

*Extraction method*

Our results show no significant difference between the two extraction methods. However, considering that the only PCRs that have failed were associated with DNA obtained using the salt extraction method, we do not recommend this extraction method. If salt extraction is considered during the design of a project including metabarcoding-based diet analysis, we suggest increasing the quality of the extracted DNA by implementing post-extraction cleaning procedures to reduce the amount of PCR inhibitors (Schrader et al., 2012). However, kit extraction protocols are high yielding and provide quality DNA and are therefore preferable to salt extraction methods (Dell'Anno et al., 2015).

## Conclusions

The main objective of the present work was to develop and validate a protocol for a DNA-barcoding approach using scorpions fed under controlled conditions, and to study the detectability of DNA in the gut content. This work represents the starting point for a more comprehensive characterization of the diet of scorpions sampled in the field and for which diet is mainly unknown. Deeper knowledge on the dietary composition of scorpions is an important step to understand how these predators affect arthropod communities. Differently from deserts, where the remains of consumed prey are better preserved and observational methods have thus been possible, in tropical or more humid biomes there is a total lack of knowledge about the roles of scorpions in the food network. Alternatively, thanks to the long $D_{50}$ of scorpions, the composition of their gut content may be used to obtain information to assess the composition of local prey species communities.

## 5.2 Metabarcoding analysis and chela closing performance in a sexually dimorphic species of scorpions

### Introduction

Sexual dimorphic traits are widely common in animals. Male and females may differ in body size, behaviour, coloration and in the size or shape of specific body parts (Andersson, 1994). While it is undisputed that the evolution of dimorphic traits linked to the interactions and competition within and between sexes is driven by sexual selection, ecological drivers to sexually dimorphic traits used beyond sexual contexts is often overlooked (De Lisle & Rowe, 2015; Shine, 1989). Feeding structures can morphologically differ between sexes leading often to the partitioning of the foraging niche to reduce intersexual competition (Selander, 1966;1972;) . In scorpions the most dimorphic traits are body size, the metasoma carrying the telson which can also differ between sexes, and the pedipalps (McLean et al., 2018). Moreover, besides morphology, also behaviour and venom composition can be sexually dimorphic traits (Carlson et al., 2014; González-Gómez et al., 2020; Miller et al., 2016).

*Chactas* is a genus of Neotropical scorpions including species presenting a significant sexual dimorphism of their pedipalps. Males have elongated and slender pedipalps while females have shorter and more robust ones. This morphological difference is translated in a different chela closing performance and venom toxicity. Females have larger pinch force but lower toxicity toward invertebrate target (González-Gómez et al., 2020). These differences can lead to partitioning of the foraging niche as already observed in other arachnids with morphologically contrasting feeding structures (Pekár et al., 2011). To test for intersexual trophic niche partitioning, we applied the same protocol described in section 5.1 to gather diet information in a population of *Chactas* sp. from Colombia. This model study is perfect to address this question because males and females are sympatric therefore accessing to the same prey items reducing bias due to intersexual spatial segregation.

**Figure 5.2.1** Female and male individuals of *Chactas* sp. It is noticeable from the figure the extreme dimorphism in pedipalp shape between the two sexes.

### Material and methods

In February 2020, few weeks before the first outbreak of COVID-19 two different populations of *Chactas* sp. were collected.

The first population was collected in San Jorge Botanical Garden, Ibagué, Colombia (4°27′3.08″N, 75°13′17.54″W). We collected and measured the chela closing speed and force performance in ten males and ten females following the protocol described in Simone et al. (2018). Morphological measurements of scorpion body parts including chela mechanical advantage were taken at nearest 0.01 mm using digital callipers (Absolute IP67, Mitutoyo Inc., Kawasaki, Japan) following Stahnke (1970).

The second population was sampled at Parque del Centenario, Ibagué, Colombia (4°26′52.53″N 75°14′34.67″W) and given its higher density than the one at San Jorge Botanical Garden, was instead used to perform diet analysis. 49 females and 26 males of *Chactas* sp. were collected at night using UV lamps and taken to the University of Ibagué where they were euthanized and stored at -80ºC until dissection. For this experiment, only the hepatopancreas was dissected and only the bead-based method was used to extract DNA as described in section 5.1. Since no sequences of Colombian *Chactas* were available in NCBI, we amplified the Folmer fragment of the COI (Folmer et al., 1994) and used it as reference to build the blocking primer following the same principles described in section 5.1 and the same pair of universal primers: fwhF2 (forward) and fwhR2n (reverse). The modification at the 3' was the same as in the described protocol in section 5.1. However, we extended the length of the blocking primers aiming for an increased specificity of the primers with the DNA template. The blocking primer used in these experiments has the following sequence:

Simone_2022_CHBlk 5'-CCYCCTCTTTCTTYTAATATGTTTCATTCTGGTGGATCGG-3'.

The efficiency of the blocking primer was tested at different concentrations (25X and 50x the concentration of the universal primers) and using different annealing temperatures in a gradient ranging from 50ºC to 60ºC. The selected annealing temperature after tests was of 57ºC. The PCRs were carried out with and without blocking primers in volumes of 10 µl, comprising 5 µl Qiagen Multiplex PCR Master Mix (Qiagen, Germany), 0.4 µl of each primer (10 pM) and 1 µl extracted DNA. PCRs done with the blocking primer also included 1 µl and 2 µl of the blocking primer Simone_2022_CHBlk at 100 pM. Cycling conditions used initial denaturing at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s, with a final extension at 60°C for 10 min. Library preparation followed the same steps described in section 5.1. The final library was

then sequenced on a MiSeq machine using an Illumina Reagent Kit v3 600-cycle (Illumina, California, USA). The run, aimed for a coverage of 150,000 reads per sample.

## Results and Discussion

Males and females of *Chactas* show significantly different chela closing performance. Males are weaker but faster than females although having larger mechanical advantage and same movable finger length. The analysis of the architecture of the chela closing muscles might solve this intriguing result. The molecular data presented here are only preliminary findings. Unfortunately, due to suboptimal results from the exploratory sequencing, we could not conclude this part of the thesis. This is evident in Figure 5.2.3, where we observed no significant impact of the blocking primer on predator DNA amplification across the three specimens, regardless of its concentration. Although there was a slight decrease in the total number of predator reads with a 50-fold higher concentration of the blocking primer compared to universal primers, it did not lead to an improvement in the total number of non-scorpion reads

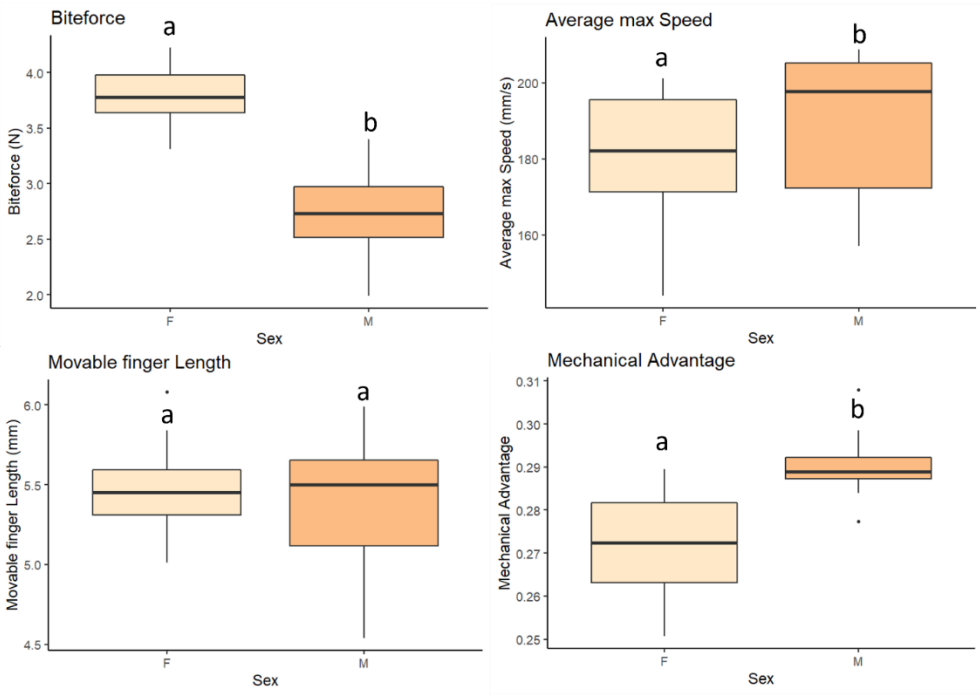


**Figure 5.2.2** Boxplot showing the differences in chela closing performance between males and females of *Chactas* sp. Although no significant difference was found in movable finger length and larger mechanical advantage in males, the latter were weaker but faster than females. Different letters above the boxplots represent significant statistical differences ($\alpha < 0.05$).

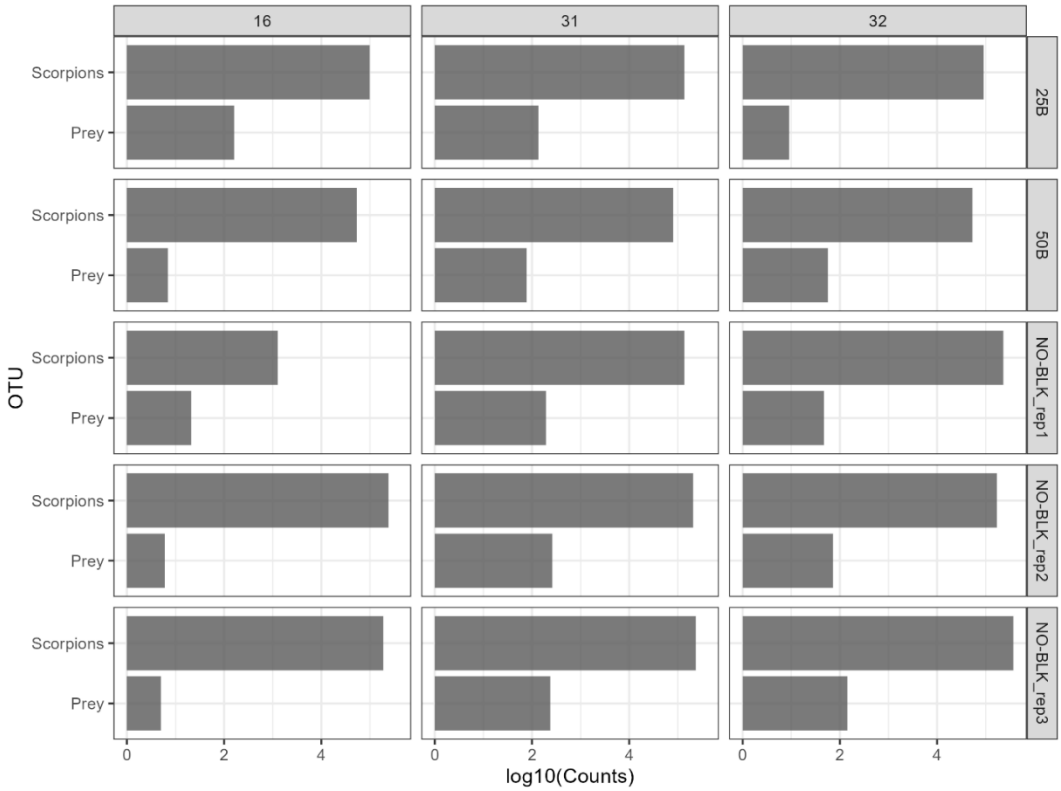


**Figure 5.2.3** Barplots showing predator and prey read count in the three specimens tested (16, 31, 32) with different concentration of blocking primer (25B, 50B) and without blocking primer (NO-BLK) and three replicates per specimen (rep1, rep2, rep3). The counts are in logarithmic scale.

Additionally, the number of putative prey reads obtained was exceptionally low, and these would likely be eliminated after applying standard post-sequencing filters for metabarcoding data. Table 5.2.1 reveals another unexpected result. When filtering out reads from putative prey from the total, it is striking that many occurrences, except for the Dermoptera data in specimen 31, do not exhibit consistency, making it challenging to categorize them as true positives. This inconsistency is particularly noticeable in the results of the three replicates conducted without blocking primers. In numerous cases, there is only a single observation across the three replicates, which is typically treated as a false positive. Alternatively, when multiple observations of the same prey item are reported across the three replicates, the values are so low that they could be attributed to sequencing errors or again, false positives.

Given these unsatisfactory outcomes, along with budget and time constraints, we were compelled to limit this part of the project to preliminary observations while exploring more effective protocol enhancements.

**Table 5.2.1** Read count of the possible prey items after predator's sequence were filtered out. In green the only confident results obtained from this preliminary sequencing are highlighted.

| CLASS | ORDER | FAMILY | COUNT | 16 B25 | 16 B50 | 16 rep1 | 16 rep2 | 16 rep3 | 31 B25 | 31 B50 | 31 rep1 | 31 rep2 | 31 rep3 | 32 B25 | 32 B50 | 32 rep1 | 32 rep2 | 32 rep3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Insecta | Dermaptera | | 667 | 0 | 0 | 0 | 1 | 0 | 127 | 60 | 112 | 164 | 203 | 0 | 0 | 0 | 0 | 0 |
| Insecta | Psocodea | Liposcelididae | 224 | 108 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 83 | 0 | 1 | 0 | 0 | 0 | 24 |
| Insecta | Hymenoptera | Formicidae | 83 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 78 |
| Insecta | Hymenoptera | | 65 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 47 | 18 | 0 | 0 |
| Insecta | Lepidoptera | Noctuidae | 59 | 8 | 0 | 0 | 0 | 0 | 0 | 6 | 23 | 0 | 0 | 0 | 5 | 10 | 0 | 0 |
| Insecta | Lepidoptera | Erebidae | 54 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 8 | 16 | 26 |
| Insecta | Hymenoptera | | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50 | 0 |
| Insecta | Coleoptera | Cerambycidae | 41 | 3 | 1 | 0 | 0 | 5 | 4 | 2 | 6 | 3 | 4 | 3 | 1 | 3 | 3 | 0 |
| Insecta | Psocodea | Peripsocidae | 38 | 38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Insecta | Diptera | Limoniidae | 36 | 0 | 2 | 10 | 0 | 0 | 0 | 3 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Insecta | Diptera | Psychodidae | 22 | 0 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 0 | 0 | 0 | 0 | 3 |
| Insecta | Hymenoptera | Formicidae | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 1 | 0 | 12 | 0 | 0 | 0 | 0 | 0 |
| Insecta | Siphonaptera | Ischnopsyllidae | 12 | 0 | 2 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Insecta | Coleoptera | Cerambycidae | 11 | 0 | 1 | 2 | 1 | 0 | 2 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| Insecta | Lepidoptera | Pyralidae | 11 | 0 | 0 | 0 | 4 | 0 | 2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 |
| Insecta | Hymenoptera | Formicidae | 11 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 0 | 0 |

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# Chapter 6

# General Discussion and Future Perspectives

## 6.1. General Discussion

*"Ecology is the least known aspect of scorpion biology."*

*-Gary Polis,* in *"The Biology of Scorpions (1990)"*

In the first sentence of the chapter on scorpion ecology in the book "The biology of Scorpions", Gary Polis stated:

*"Ecology is the least known aspect of scorpion biology."*

More than 33 years later, we still know very little about these intriguing and fascinating creatures. The main emphasis of this thesis is on the functional performance of chelae in feeding ecology. However, Chapter 3 shows the significant role played by chelae in defence against predators/attackers, as well as in contexts related to mating and male-male competitive behaviour. Remarkably, the use of chelae and venom in both foraging and self-defence exhibits striking similarities. In both contexts, an interesting dichotomy arises between species with slender and long-fingered chelae versus those with robust and short-fingered ones. The former species predominantly rely on their venom, rather than depending on their chelae, contrary to the latter. This differential use of chelae and stinger for both prey capture and defence might have had crucial role in venom evolution.

In feeding contexts, scorpions with different chela shapes cluster into two major groups: "graspers" and "crushers". This view is based on the results of the biomechanical analysis of the chela anatomy and closing performance. A trade-off between speed and force in chela closing was already shown in a previous study (Simone & van der Meijden, 2018). However, this study lacked data about the architecture of the chela closing muscles. Through the addition of new data, it was possible to gain new insights on chela evolution in scorpions. The main finding of Chapter 4 is that muscle architecture and structural elements of the chela co-varied differently in the two focal species, suggesting two distinct evolutionary trajectories: In the species with robust and short fingered chelae, evolutionary changes of chela morphology favoured closing force optimization, while speed optimization was favoured in the species with slender and long-fingered chelae. Our data show a significant degree of morphological and kinematic integration among the physiological and structural components of scorpion chelae leading to distinct ways to use the force generated during muscle

contraction. In species with stout and short-fingered chelae, force is maximum in the middle of the closing event, when likely the scorpion is already holding its prey. Conversely in the species having slender and long-fingered chelae, the peak force is reached during the initial phases of the closing event and both muscle architecture and sarcomere distribution, suggest that force is used to generate larger initial accelerations. In the latter species, after the peak of force at intermediate and low opening angles, there is a remarkable decline in the force produced. These results suggest that species with slender and long-fingered chelae are particularly efficient in grasping while being very poor holders. In feeding contexts, the "crushers" can rely on the force of their chelae to secure the prey from escaping, while "the graspers" need to rely on a quick acting venom to complement to their low closing force. "Graspers" may also rely on friction to compensate for their low holding force to get a firmer grip on their prey. These species have several rows of small sharp denticles on the contact surface of the movable and immovable finger. The function of these denticles has not been explored yet, but they possibly might act like sandpaper enhancing grip onto the prey body. Conversely, species with robust and short fingered chelae have few but bigger teeth. These structures, coupled with the larger closing force, can pierce in the prey body, preventing it from fleeing and causing deadly damage to the internal organs, without any need to expend venom.

The difference in speed and force among the two groups may also impact the types of prey consumed. Species with stout and short fingered chelae may likely include hard-bodied prey in their diet. However, capturing fast-moving prey might pose challenges, rendering such prey less likely to be included in their diet. On the other hand, the graspers can easily reach fast-moving prey, but hard-bodied prey can be more difficult to handle and hold. Although these consequences on diet of the speed force trade-off have been confirmed in crabs, lizards, turtles and mammals (Aguirre et al., 2003; Fabre et al., 2017; Herrel et al., 2002; Taverne et al., 2020), such conclusions are difficult to draw for scorpions due the lack of comprehensive dietary data. The study of scorpion diets is notably challenging due to their external pre-digestion process, slow metabolism relative to their body size, and for some species, secretive prey consumption occurring within the confines of their self-dug burrows.

As an innovative approach to circumvent the limits posed by the traditional techniques used to study scorpion diet, Chapter 5 introduces a metabarcoding-based approach to detect prey DNA from different sections of the digestive system of scorpions. Through this protocol we successfully traced the dietary items consumed by tested scorpions over nine weeks of a controlled diet regime. Furthermore, we identified the hepatopancreas as specific digestive portion with a higher likelihood of isolating prey DNA. Building upon these findings, we tried to extend the application of this approach to a natural population of *Chactas* sp. The goal of this study was to test whether the sexes consumed different prey items, potentially due to the constraints imposed by their different chela morphologies. This species is the perfect model

for this type of study because males and females present significant sexual dimorphism in their pedipalps and are sympatric, removing in this way the bias of different prey availability. Regrettably, the promising outcomes achieved with *Heterometrus laoticus* were not replicated. Our attempt to implement blocking primers, as shown in the original protocol, yielded no prey amplifications. Likewise, the increasing of sequencing depth, both with and without blocking primers, solely revealed predator reads. The scarcity of retrieved prey DNA in the limited samples analysed could be stochastic or attributed to the possibility that scorpions had their last meal over a timeframe exceeding the DNA half-life. Additionally, we cannot exclude some bias attributable to the primers designed in this study.

The results from this study could have been essential to understand the consequences of such extreme sexual dimorphism in a predatory context. The two sexes significantly differ in their chela closing performance and venom toxicity (González-Gómez et al., 2020). Specifically, females exhibit an average closing force that is 1.3N greater (3.8N) than that of males (2.5N), while males possess a more potent venom. These differences may already confer advantages in capturing either different prey types or individuals of the same prey type but varying in size or ontogenetic state. This potentially reduces intersexual competition, providing support for the intersexual foraging niche partition hypothesis. However, the metabarcoding approach only allows to recognize the prey to species level but provides no information about its ontogenetic state or size. In the case of intersexual foraging niche partitioning, an interesting question arising is: is intersexual foraging niche partition, the cause or the consequence of this extreme sexual dimorphism? To support the hypothesis that ecology drove the evolution of sexual dimorphic traits, De Lisle (2015) suggests the coexistence of four requirements. The sexes have diverged ecologically along a resource axis, with corresponding divergence in morphology that is associated with resource use (i), the sexual dimorphic trait is heritable (ii), resource competition between sexes limits adult resource acquisition affecting fitness (iii), the competition for the resources decreases as the morphological differences of the sexual dimorphic trait increase (iv). In my opinion, there should be a fifth requirement that is necessary to completely ascertain whether ecological factors are the main drivers of sexual dimorphic trait. The dimorphic trait should provide little to no benefits in sexual related contexts like courtship, mating, or male-male competition. In mosquitos, males and females have a morphologically distinct feeding apparatus that is used to forage on distinct food types and has no involvement in sexually related contexts. This example is the clearest example of a sexual dimorphic trait shaped by mainly ecological drivers. Additionally, In some birds it has also been proposed that sexual dimorphic bills lead to different diets or foraging strategies to reduce competition between sexes (Temeles et al., 2000). Nevertheless, bill shape might be related to, or even constraining, the bird song (Demery et al., 2021; Herrel et al., 2009) which can be a trait used in sexual related contexts, not meeting in some cases my fifth requirement.

However, unlike other causes of the evolution of sexual dimorphism such as sexual selection, resource competition as an ecological cause of sexual dimorphism remains controversial and lacks unambiguous evidence (Shine, 1989).

The other possible scenario involves no differences in diet, leading to the absence of an ecological cause as the driver of sexual dimorphism. In this case, the primary driver behind the differentiation of pedipalps in males may be linked to pure sexual selection, such as mate choice or intrasexual competition. Currently, there is no evidence of mate choice being performed by a specific sex in scorpions, and further research is needed to explore this aspect of scorpion sexual ecology. Conversely, it has been observed in other sexually dimorphic species that males having more elongated pedipalps compared to females, engage in a distinctive ritualized fighting style known as "arm-span competition" (Tang, 2023). In this behaviour, males size each other up by extending their pedipalps laterally, deciding whether to escalate to a real aggressive encounter or to retreat and avoid physical combat. This behaviour has already been observed in two distinct scorpion families, suggesting a convergent evolution of pedipalp elongation due to this peculiar fighting style (Tang, 2023; Wyman et al., in press). Scarce literature is available about male-male aggressive behaviour, however, the “arm span competition” contrasts sharply with the grappling and periodic head-to-tail alignment described in non-dimorphic species like *Hadrurus arizonensis* (Tallarovic, 2000) or other buthid species as described by Tang (2023).

In case of scorpions, chelae are involved in both male-male contexts and courtship behaviour, suggesting that, irrespective of the scenario, the sexually dimorphic pedipalps are more likely to have evolved under sexual selection or a combination of both sexual and natural selection rather than being driven by ecology only.

## 6.2. Further prospects and concluding remarks

This thesis project originated many years ago when we first documented the existence of the trade-off between speed and force in scorpion chela closing performance. Since then, during these past years developing my thesis, many steps forward have been taken. Specifically, we integrated the previous results with the first data about the architecture of chela closing muscles and show how these muscles operate during each degree of finger rotation. Thanks to this data we built a biomechanical model of finger adduction and discovered that force reaches its peak at different opening angles in species with contrasting chela morphology. Being aware of the lack of dietary data and how time and labour consuming the technical methodology is to gather these data, we proposed the first protocol to investigate

scorpion diet through NGS-based approach, revealing the extraordinary capacity of these animals to retain food for many weeks since their last meal. Unfortunately, we could not address whether species with contrasting chela shape eat different prey items. Nevertheless, we are already working on this topic especially in the prospect of improving the published protocol.

Further studies are needed to solve this intriguing but at the same time ambitious topic. Many of the assumptions made in the biomechanical model can be improved like investigating the fiber-type composition of closing muscles in species with different chela morphology. Additionally, electromyograms can help to understand whether muscles are activated simultaneously or recruited in a way to avoid wasting the force generated due to suboptimal orientation. However, much progress in understanding scorpion feeding ecology cannot be achieved without improving the knowledge about scorpion diet. In this direction, the protocol to gather prey DNA from scorpion gut content can be improved by using exclusion primers (as already successfully shown in spiders) or by refining the protocol for DNA extraction through the incorporation of DNA capture baits.

In conclusion, there is still a lot of work to do, many interesting species to study, experiments to perform and things to discover:

The best is yet to come.

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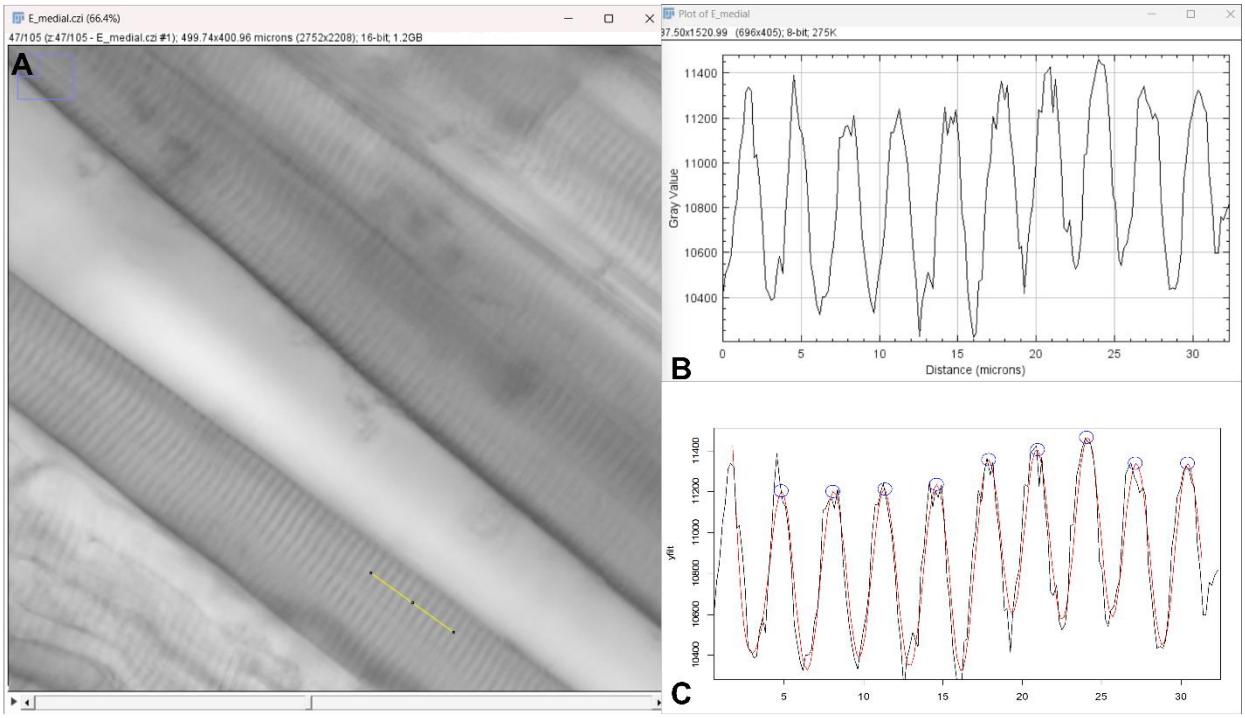
# Appendix A

## Supplementary Information

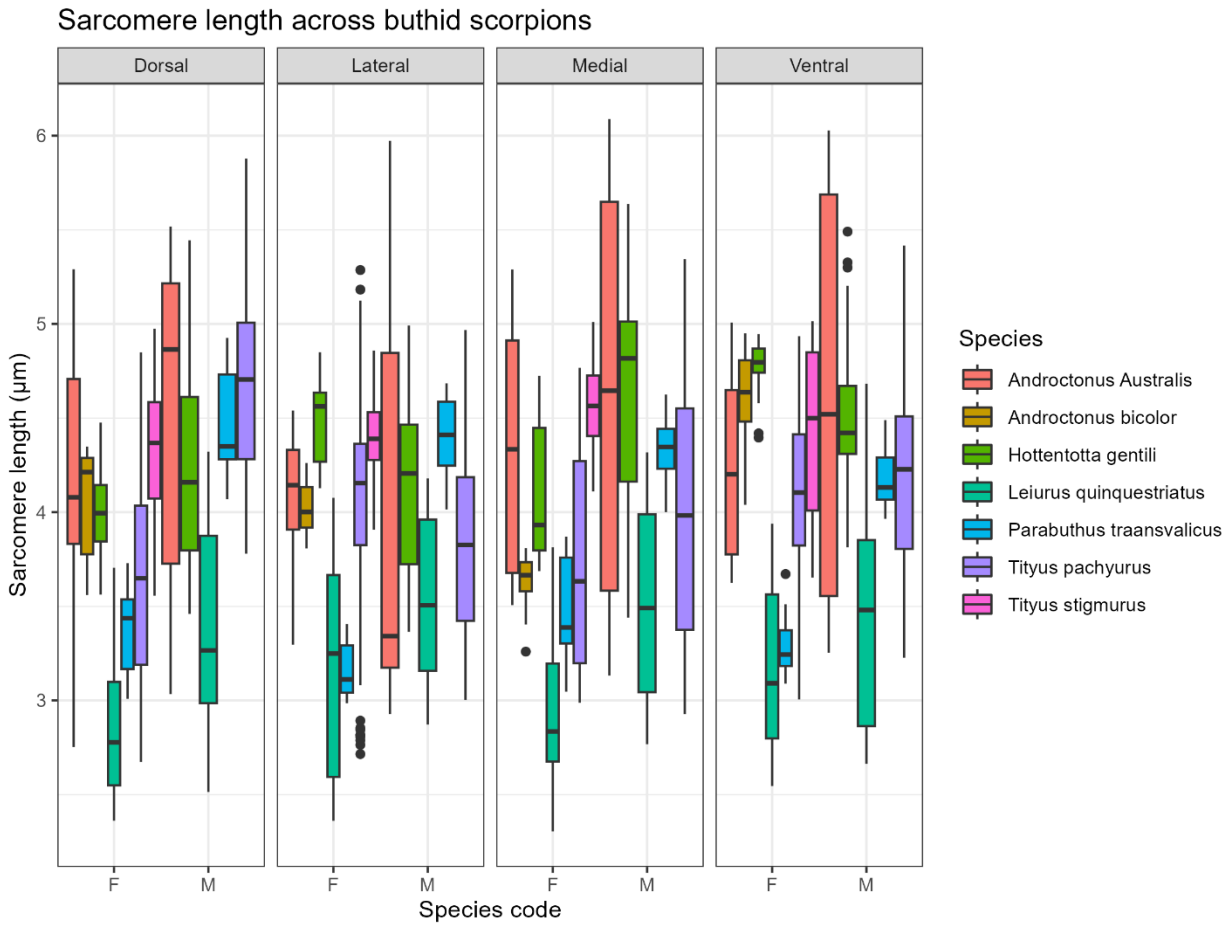
# Supplementary Information for Section 4.1.



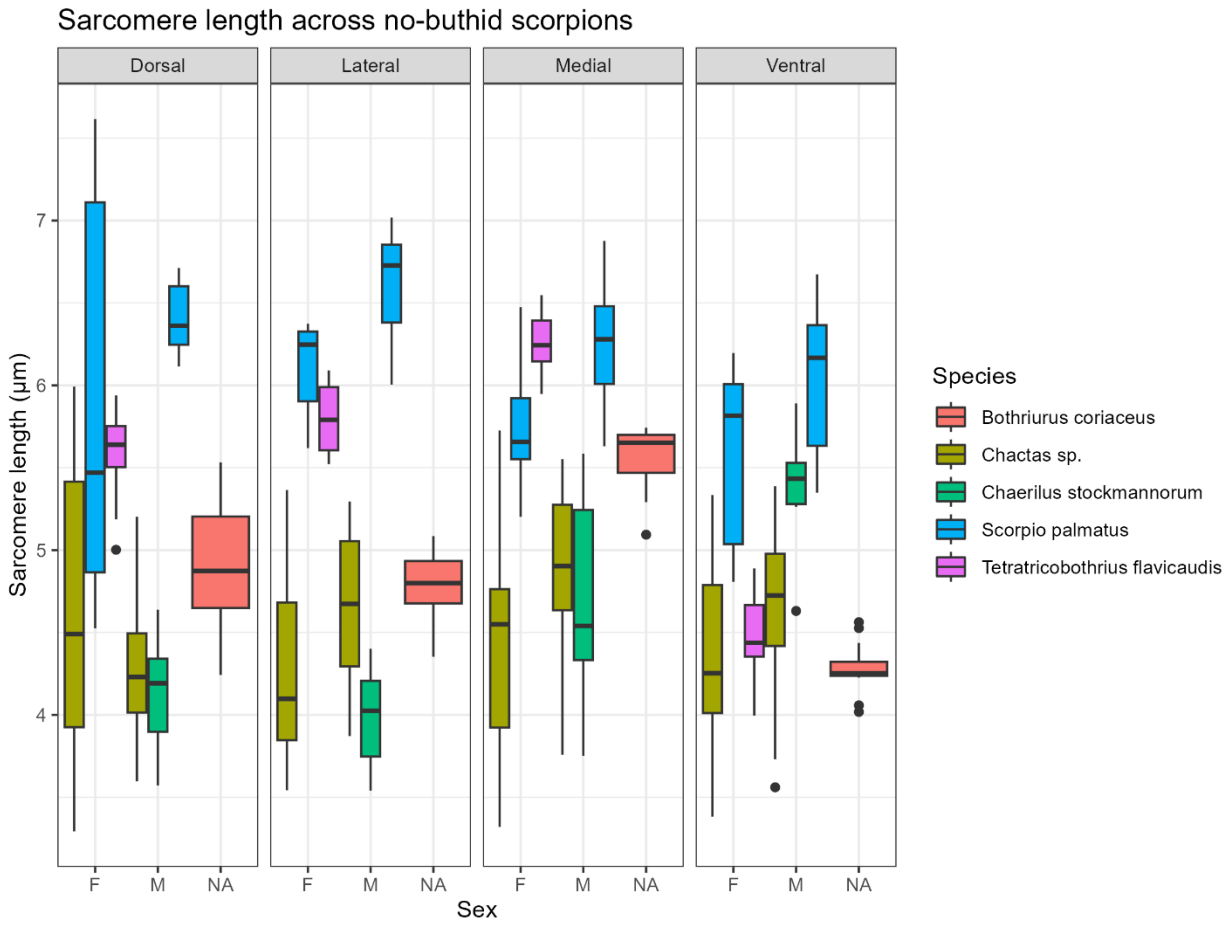

**Fig_suppl_4.1.1.** A) Close up of muscle fibers to highlight the striation pattern due to the sarcomere Z-disks. In Yellow a straight line crossing ten sarcomeres for which the intensity plot is generated (B). In (C) the output of the customized script showing with a red smooth line the filtered data and with the blue circles the peaks of the data distribution. The distance between two consecutives blue circles along the X axis is the sarcomere length in micrometers.


| | |
|---|---|
| 1 | PROSOMA LENGTH |
| 2 | PROSOMA WIDTH |
| 3 | 4TH MESOSOMAL TERGITE WIDTH |
| 4 | 1ST METASOMAL SEGMENT WIDTH |
| 5 | 1ST METASOMAL SEGMENT LENGTH |
| 6 | 2ND METASOMAL SEGMENT LENGTH |
| 7 | 3RD METASOMAL SEGMENT LENGTH |
| 8 | 4TH METASOMAL SEGMENT LENGTH |
| 9 | 5TH METASOMAL SEGMENT LENGTH |
| 10 | TELSON LENGTH |

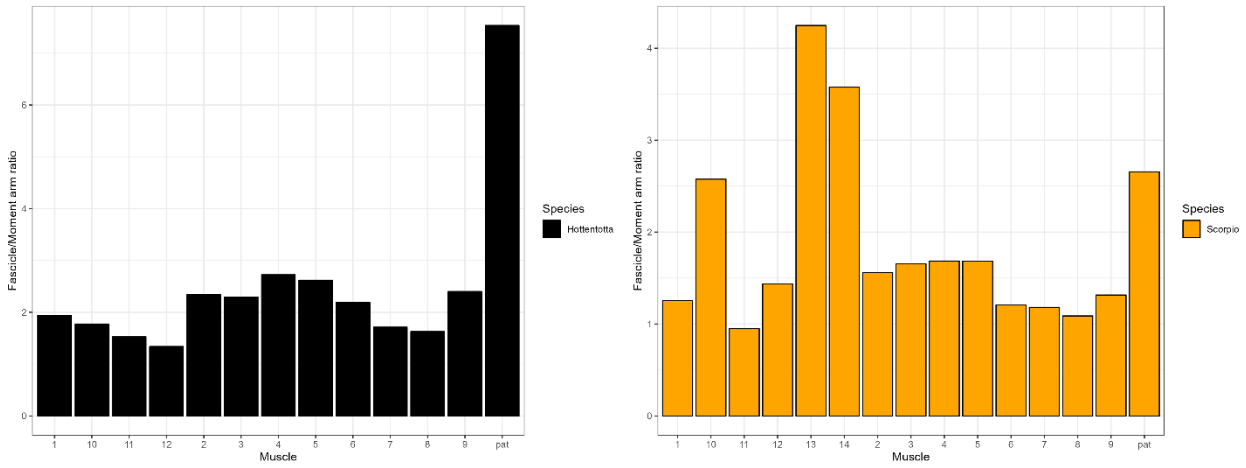
**Fig_suppl_4.1.2.** Dorsal view of a scorpion highlighting the ten morphological measurements used to build the IsoSize vector to correct variables for body size.



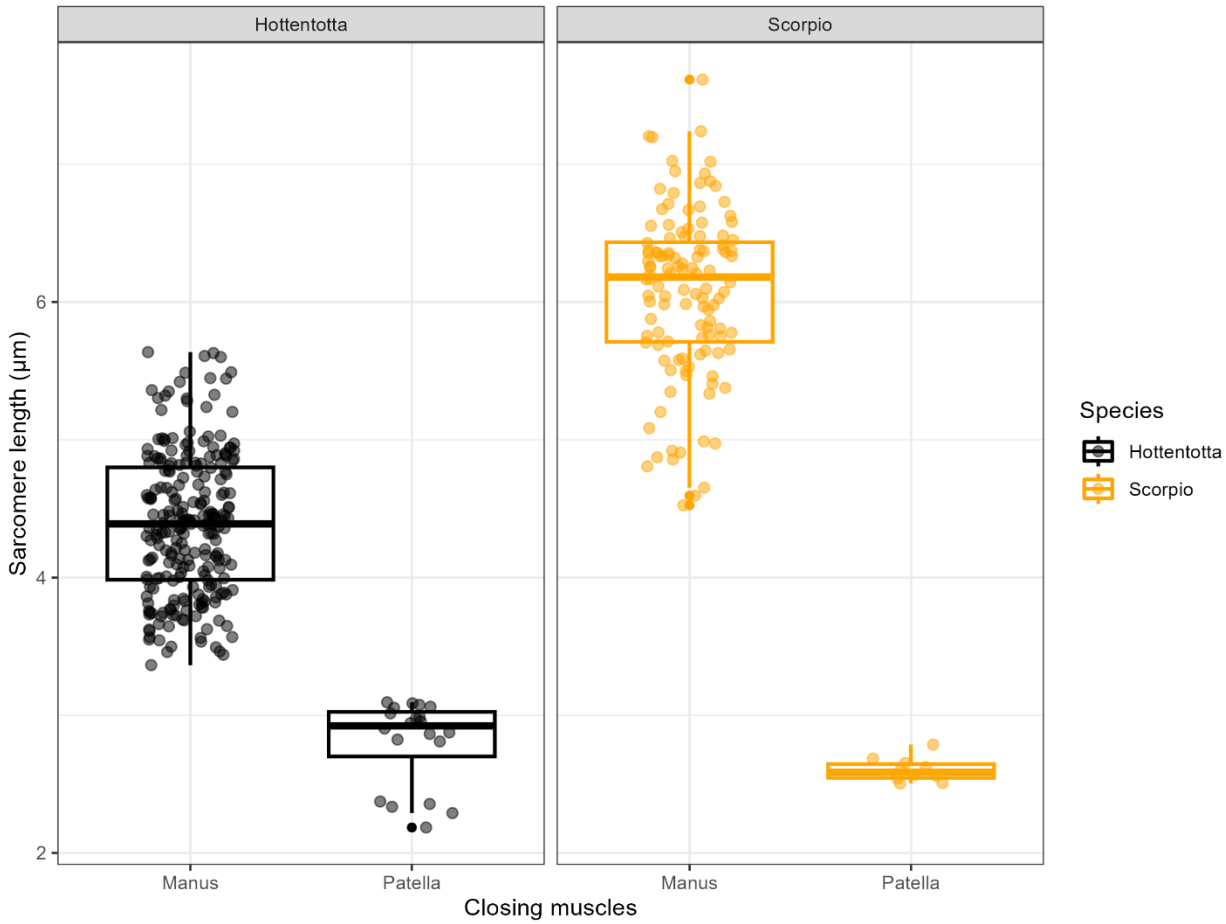

**Fig_suppl_4.1.3.** Boxplot showing the distribution of the sarcomere length in the four groups of manus closing muscles in buthids. It is interesting to note that the deviation from the pattern observed in *Hottentotta gentili* is observed in the species having strong sexual dimorphism in their chelae. More specifically, males in *Parabuthus traansvalicus* and *Tityus pachyurus* deviate from the pattern by having longer Dorsal sarcomeres and shorter Ventral sarcomeres. Both males of the two species have a more rounded and bulbous chelae than females.




**Fig_suppl_4.1.4**. Boxplot showing the distribution of the sarcomere length in the four groups of manus closing muscles in no-buthid scorpions. It is interesting to note that the deviation from the pattern observed in *Scorpio palmatus* is observed in *Chaerilus stockmannorum* and males of the sexual dimorphic species *Chactas*. Both deviate from the pattern observed in Scorpio by having shorter Dorsal sarcomeres and longer Ventral sarcomeres. Chaerilidae is an ancient family of scorpions, closer to Buthidae than the other no Buthid families while males in *Chactas* have more elongated and slender pediplaps than females.



**Fig_suppl_4.1.5.** Fascicle moment arm ratio of closing muscles in *Scorpio* and *Hottentotta*. Muscles names are not homologous, therefore the pairing across the two species has not functional meaning.




**Fig_suppl_4.1.6.** Manus and patella sarcomere length across *Hottentotta* (black boxblots and dots) and *Scorpio* (orange boxplots and dots).

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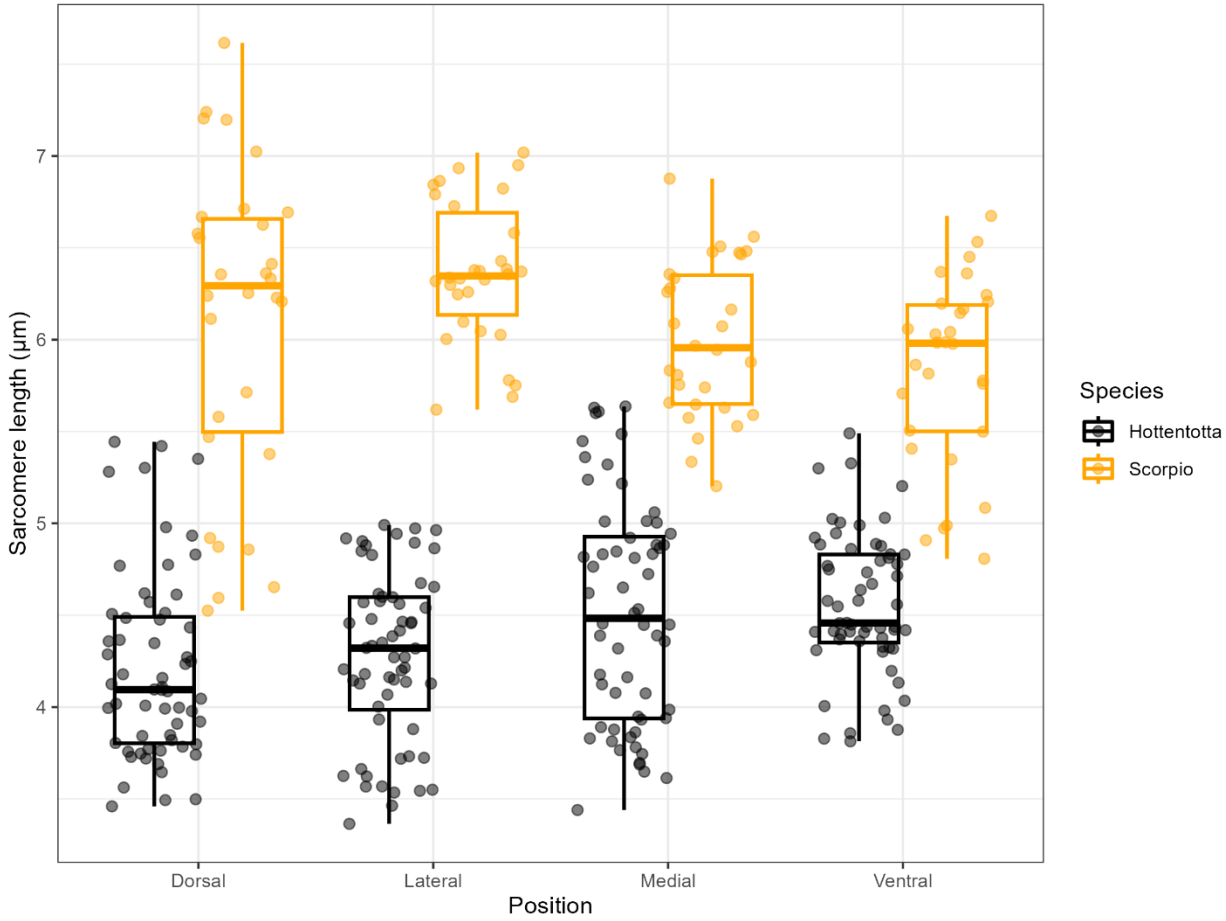
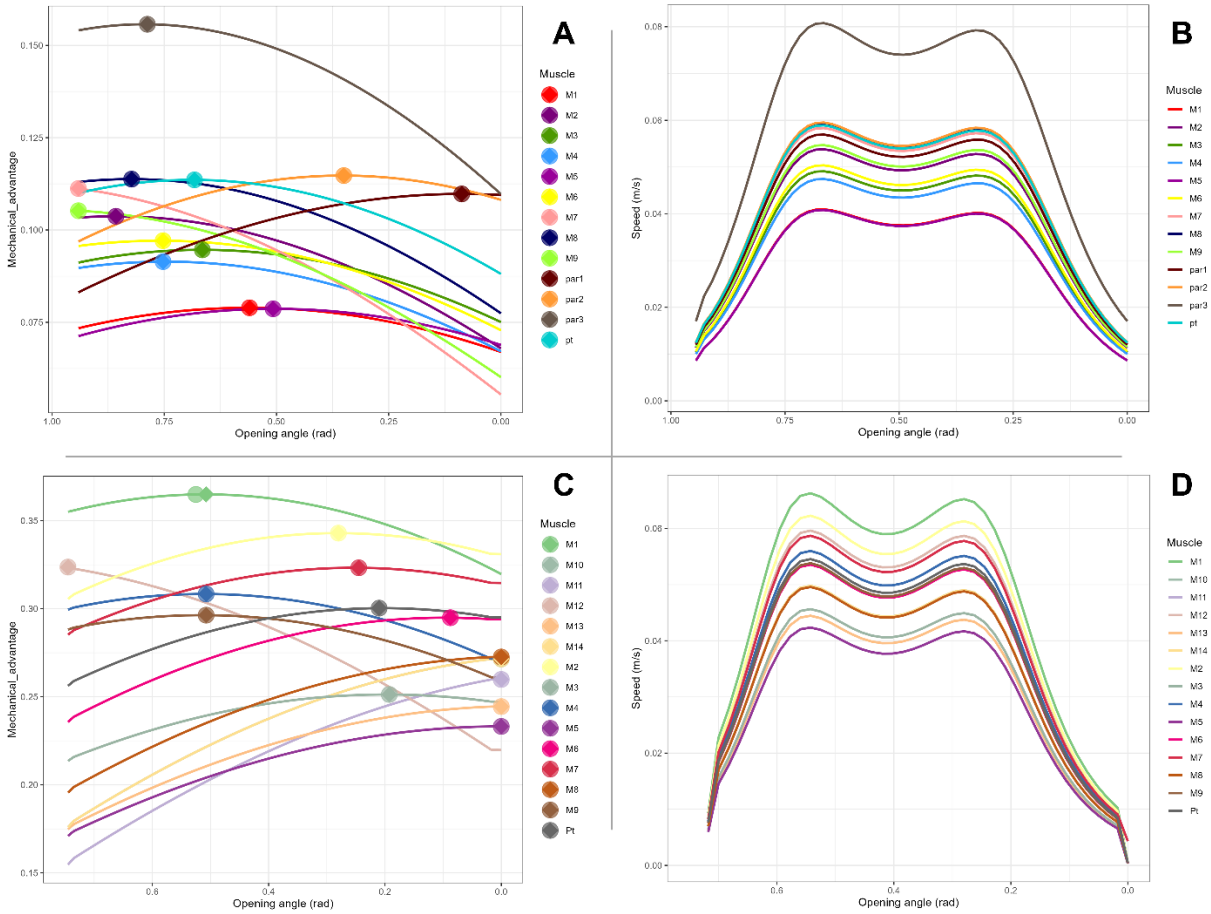
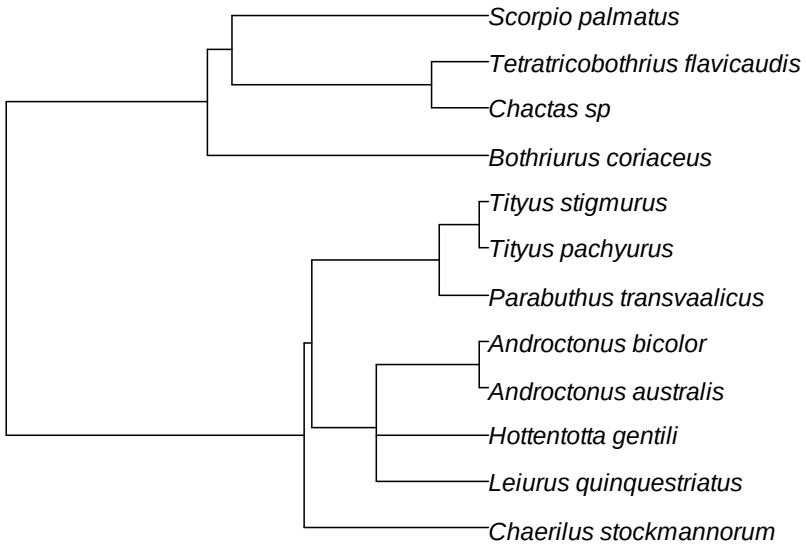



**Fig_suppl_4.1.7.** Sarcomere length across species grouped by different locations in the manus.




**Fig_suppl_4.1.8.** Mechanical advantage and muscle speed per degree for *Hottentotta* (A-B) and *Scorpio* (C-D). A-C Mechanical advantage (continuous colored line) in function of closing angle. Dots and diamonds respectively represent the maximum mechanical advantage and the opening angle at which the sin of the dorso-ventral angle is ~1 (i.e., dorso-ventral angle is 90 degrees). Note that muscle names have no functional meaning, i.e. they are not homologous.




**Fig_suppl_4.1.9.** Phylogenetic tree of the species analysed in the comparative models.

**Table_suppl_4.1.1**. Summary table of kinematic variables and sarcomere lengths across the twelve species analysed (Mean ± Standard deviation).

| Species | Maximum Velocity (mm/s) | Maximum Angular velocity (rad/s) | Bite force (N) | Manus sarcomere length (µm) | Patella sarcomere length (µm) | Mechanical advantage |
|---|---|---|---|---|---|---|
| *Chactas* sp. | 183 ± 19.3 | 32.3 ± 3.39 | 3.26 ± 0.642 | 4.52 ± 0.582 | 2.60 ± 0.482 | 0.281 ± 1.42 e-2 |
| *Tityus pachyurus* | 433 ± 53.4 | 42.7 ± 7.24 | 0.655 ± 0.197 | 4.02 ± 0.663 | 2.53 ± 0.196 | 0.141 ± 3.26 e-2 |
| *Bothriurus coriaceus* | 136 ± 8.37 | 32.7 ± 4.43 | 1.09 ± 0.378 | 4.88 ± 0.521 | 3.15 ± 0.217 | 0.294 ± 3.60 e-2 |
| *Androctonus bicolor* | 372 ± 97.2 | 46.1 ± 3.81 | 0.444 ± 0.0709 | 4.08 ± 0.401 | 2.87 ± 0.177 | 0.108 ± 1.28 e-2 |
| *Chaerilius stockmannorum* | 169 ± 19.0 | 27.6 ± 4.03 | 4.30 ± 0.466 | 4.56 ± 0.677 | 3.25 ± 0.120 | 0.316 ± 2.22 e-3 |
| *Androctonus australis* | 373 ± 54.3 | 29.5 ± 2.80 | 1.54 ± 0.227 | 4.32 ± 0.841 | 2.48 ± 0.382 | 0.167 ± 6.52 e-3 |
| *Parabuthus transvaalicus* | 339 ± 81.0 | 30.1 ± 6.09 | 1.16 ± 0.134 | 3.83 ± 0.568 | 2.60 ± 0.260 | 0.187 ± 2.60 e-2 |
| *Hottentotta gentili* | 470 ± 100 | 38.4 ± 10.5 | 0.581 ± 0.0567 | 4.39 ± 0.516 | 2.80 ± 0.307 | 0.144 ± 8.67 e-3 |
| *Tityus stigmurus* | 330 ± 35.2 | 37.6 ± 4.98 | 0.367 ± 0.0104 | 4.42 ± 0.343 | 2.72 ± 0.227 | 0.119 ± 4.38 e-3 |
| *Scorpio palmatus* | 189 ± 38.5 | 26.6 ± 5.48 | 8.00 ± 1.87 | 6.07 ± 0.602 | 2.60 ± 0.0878 | 0.298 ± 1.91 e-2 |
| *Tetratrichobothrius flavicaudis* | 171 ± 21.9 | 36.6 ± 4.50 | 1.76 ± 0.387 | 5.53 ± 0.686 | 2.40 ±0.0682 | 0.253 ± 1.24 e-2 |
| *Leiurus quinquestriatus* | 609 ± 58.2 | 45.2 ± 3.30 | 0.722 ± 0.0528 | 3.25 ± 0.535 | 2.47 ± 0.212 | 0.104 ± 2.00 e-2 |

**Table_suppl_4.1.2** This table reports the T-Student test results across muscle architecture variables. P-values below 0.05 are shown in bold while in light grey the P values slightly above 0.05.

| Variable | Hottentotta | Scorpio | P-value |
|---|---|---|---|
| Mean Pennation angle | 18.6445 | 23.82611 | 0.062293 |
| Mean MA | 0.09888599 | 0.27584881 | **3.35E-13** |
| Sin Dorso-Ventral angle | 0.9430838 | 0.9431648 | 0.9967 |
| Sin Latero-Medial angle | 0.9762615 | 0.9848634 | 0.4977 |
| Sarcomere length (Manus) | 4.388335 | 6.07462 | **2.20E-16** |
| Sarcomere length (Patella) | 2.803696 | 2.604069 | 0.05518 |

**Table_suppl_4.1.3**. Anova results of linear model with sarcomere length as the response variable and species and location in the manus (position) as independent variables. Results are shown across species and within the same species where only the predictor position was tested. Significant p-values (<0.05) are highlighted in bold.

| Scorpion | Fixed Factor | Sum.sq | Df | Pr(>Chisq) |
|---|---|---|---|---|
| Scorpio vs Hottentotta | Species | 227 | 1 | **2.00E-16** |
| | Position | 1.47 | 3 | 0.176 |
| Scorpio | Position | 18.2 | 3 | **5.66E-05** |

| | | | | |
|---|---|---|---|---|
| Hottentotta | Position | 4.41 | 3 | **2.00E-16** |

**Table_suppl_4.1.4**. Summary table showing how the distribution of sarcomere length is affected by each level of the predictor position. Significant p-values (<0.05) are highlighted in bold. Note that "Dorsal" is the reference level by the model.

| Scorpion | Levels | Estimate | Std.Error | t-value | Pr(>\|z\|) |
|---|---|---|---|---|---|
| Scorpio | (Intercept) | 6.11 | 0.124 | 49.1 | **2.00E-16** |
| | Lateral | 0.259 | 0.176 | 1.48 | 0.142 |
| | Medial | -0.303 | 0.169 | -1.79 | 7.55E-02 |
| | Ventral | -0.684 | 0.160 | -4.27 | **3.71E-5** |
| Hottentotta | (Intercept) | 4.21 | 6.62E-02 | 63.5 | **2.00E-16** |
| | Lateral | 6.66E-02 | 9.73-02 | 0.711 | 0.477 |
| | Medial | 0.301 | 9.54-02 | 3.16 | **1.80E-03** |
| | Ventral | 0.325 | 0.101 | 3.22 | **1.52E-03** |

**Table_suppl_4.1.5**. Results of the PGLS with angular velocity and size corrected bite force as response variables and the z scores of sarcomere lengths in dorsal and ventral muscles as dependent variables. The results of the two evolutionary models λ=1 (Brownian motion) and λ=0 (star phylogeny) are presented. Significant p-values (<0.05) are highlighted in bold.

| Response variable | Lambda | Levels | Estimate | Std.Error | t-value | Pr(>\|z\|) |
|---|---|---|---|---|---|---|
| Angular velocity | 1 | Intercept | 40.1 | 11.7 | 3.43 | **7.47E-03** |
| | | z-score Ventral | 6.40 | 2.74 | 2.33 | **4.44E-02** |
| | | z-score Dorsal | 6.18 | 5.98 | 1.03 | 0.328 |
| | ML(λ=0) | Intercept | 40.9 | 2.58 | 15.8 | **7.12E-8** |
| | | z-score Ventral | 0.648 | 3.52 | 0.198 | 0.847 |
| | | z-score Dorsal | 2.66 | 11.2 | 0.240 | 0.816 |
| Biteforce | 1 | Intercept | 0.127 | 0.657 | 0.193 | 0.851 |
| | | z-score Ventral | -0.0283 | 0.154 | -1.84 | 9.91E-02 |
| | | z-score Dorsal | 0.525 | 0.337 | 1.56 | 0.151 |
| | ML(λ=0) | Intercept | -9.67E-03 | 0.139 | -7.00E-02 | 0.946 |
| | | z-score Ventral | -2.73E-02 | 0.190 | -0.145 | 0.888 |
| | | z-score Dorsal | -0.101 | 0.601 | -0.168 | 0.870 |

# Supplementary Information for Section 5.1.

## Appendix 5.1 S1: Biomass of the ingested prey

Cricket biomass

| | | | | |
|---|---|---|---|---|
| **Scorpion 1** | **2**crickets | 0.69g | 10-05-19 | all consumed |
| **Scorpion 2** | **2**crickets | 0.73g | 10-05-19 | all consumed |
| **Scorpion 3** | **2**crickets | 0.65g | 10-05-19 | all consumed |
| **Scorpion 4** | **2**crickets | 0.68g | 10-05-19 | all consumed |
| **Scorpion 5** | **2**crickets | 0.82g | 10-05-19 | all consumed |
| **Scorpion 6** | **2**crickets | 0.71g | 10-05-19 | all consumed |
| **Scorpion 7** | **2**crickets | 0.66g | 10-05-19 | all consumed |
| **Scorpion 8** | **2**crickets | 0.77g | 10-05-19 | all consumed |
| **Scorpion 9** | **2**crickets | 0.82g | 10-05-19 | all consumed |
| **Scorpion 10** | **2**crickets | 0.81g | 10-05-19 | all consumed |
| **Scorpion 11** | **2**crickets | 0.69g | 10-05-19 | all consumed |
| **Scorpion 11** | **2**crickets | 0.59g | 10-05-19 | all consumed |
| **Scorpion 12** | **2**crickets | 0.63g | 10-05-19 | all consumed |
| **Scorpion 13** | **2**crickets | 0.77g | 10-05-19 | all consumed |
| **Scorpion 14** | **2**crickets | 0.83g | 10-05-19 | all consumed |

Tenebrio biomass

| | | | | |
|---|---|---|---|---|
| **Scorpion 1** | **6**tenebrios | 0.63g | 31-05-19 | 3 tenebrio consumed |
| **Scorpion 2** | **6**tenebrios | 0.64g | 31-05-19 | all consumed |
| **Scorpion 3** | **6**tenebrios | 0.57g | 31-05-19 | all consumed |
| **Scorpion 4** | **6**tenebrios | 0.58g | 31-05-19 | no prey consumed |
| **Scorpion 5** | **6**tenebrios | 0.65g | 31-05-19 | all consumed |
| **Scorpion 6** | **6**tenebrios | 0.64g | 31-05-19 | all consumed |
| **Scorpion 7** | **6**tenebrios | 0.59g | 31-05-19 | no prey consumed |
| **Scorpion 8** | **6**tenebrios | 0.57g | 31-05-19 | all consumed |
| **Scorpion 9** | **6**tenebrios | 0.66g | 31-05-19 | no prey consumed |
| **Scorpion 10** | **6**tenebrios | 0.65g | 31-05-19 | all consumed |
| **Scorpion 11** | **6**tenebrios | 0.64g | 31-05-19 | all consumed |

| | | | | |
|---|---|---|---|---|
| **Scorpion 12** | **6**tenebrios | 0.55g | 31-05-19 | no prey consumed |
| **Scorpion 13** | **6**tenebrios | 0.73g | 31-05-19 | no prey consumed |
| **Scorpion 14** | **6**tenebrios | 0.60g | 31-05-19 | all consumed |

Cockroach biomass

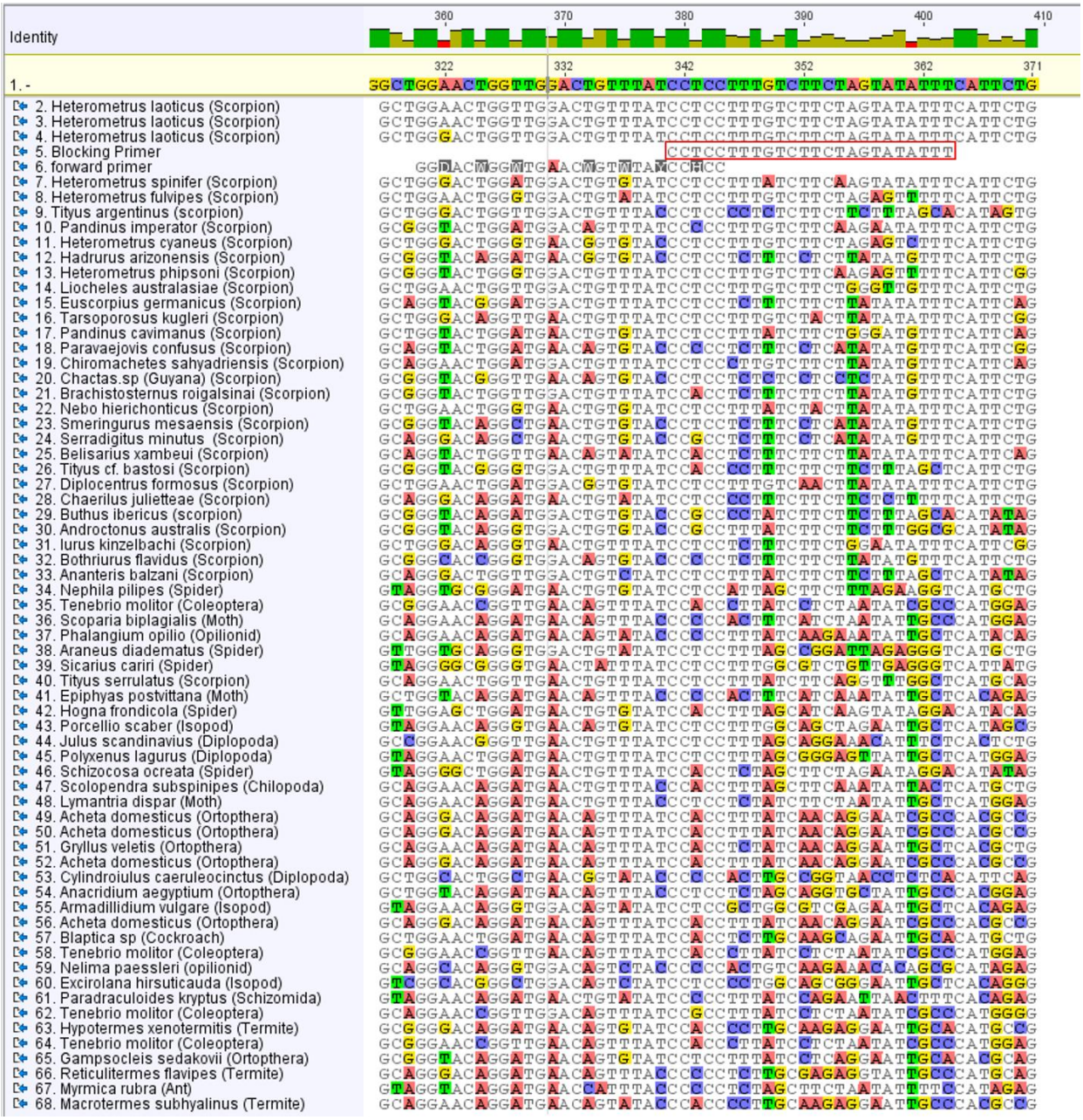
| | | | | |
|---|---|---|---|---|
| **Scorpion 1** | **1**cockroach | 0.64g | 21-06-19 | no prey consumed |
| **Scorpion2** | **1**cockroach | 0.74g | 21-06-19 | prey entirely consumed |
| **Scorpion3** | **1**cockroach | 0.76g | 21-06-19 | prey entirely consumed |
| **Scorpion5** | **1**cockroach | 0.74g | 21-06-19 | no prey consumed |
| **Scorpion6** | **1**cockroach | 0.81g | 21-06-19 | no prey consumed |
| **Scorpion 8** | **1**cockroach | 0.80g | 21-06-19 | prey entirely consumed |
| **Scorpion10** | **1**cockroach | 0.95g | 21-06-19 | prey entirely consumed |
| **Scorpion11** | **1**cockroach | 0.73g | 21-06-19 | prey entirely consumed |
| **Scorpion 14** | **1**cockroach | 1.02g | 21-06-19 | prey entirely consumed |

## Appendix 5.1 S2: Extraction protocols used

Proposed Beads + QIAGEN protocol for scorpions

1. Take small piece of sample (Usually for 200µL of buffer it's 20-30mg for 400µL of buffer it's 40-60 mg)
2. Add 300µl ATL solution, 20µL Proteinase K in each 2-mLtube.
3. Vortex for 1 min, short-spin and incubate at 56ºC for at least 3h in the oven, mix occasionally during incubation to disperse the sample.
4. Cool to room temperature. Vortex for 20s and short-spin. Transfer lysate (avoiding the pellet) to a new 2-ml tube.
5. Add 300µL AL solution, mix by inverting the tubes and incubate @RT for 2min, short-pin.
6. Transfer all lysate (avoiding the pellet) into a new 2-ml column prefilled with 300µL cold Isopropanol and 100µL magnetic beads suspension, re-suspended well by vortexing, short-spin.
7. Place the tube on the magnetic stand and let the magnetic beads collect at the magnet for 5min. Remove the supernatant using a pipette while the tube is still on the stand.
8. Remove the tube from the magnetic stand and add 500µL AW1 Buffer. Re-suspend the magnetic beads by vortexing, place the tube on the magnetic stand and let the magnetic beads collect at the magnet for 5min. Discard the supernatant using a pipette while the tube is still on the stand.
9. Remove the tube from the magnetic stand and add 500µL AW2 Buffer. Re-suspend the magnetic beads by vortexing, place the tube on the magnetic stand and let the magnetic beads collect at the magnet for 5min. Discard the supernatant using a pipette while the tube is still on the stand.
10. Without removing the tube from the magnetic stand add 350µL Ethanol 70% and keep for 30s. Remove the supernatant by using a pipette and air-dry for 5min, making sure that all the ethanol is completely evaporated, but do not over dry the beads.
11. Remove tubes from the magnetic stand and add 70µL Elution Buffer (AE) (warm). Re suspend the magnetic beads by vortexing, incubate tubes at 55°C for 5min, mixing occasionally.
12. Place the tube on the magnetic rack and let the magnetic beads collect at the magnet for 5min.
13. Transfer all supernatant (avoiding the beads) into a new microplate or clean tubes. For long-term storage of DNA, storing at -20°C is recommended.

## Appendix 5.1 S3: Sequence alignment used to create the blocking primer



## Appendix 5.1 S4. List of command used for the bioinformatic pipeline

**Commands used for each pair of paired-end fastq files:**

flash2 --max-overlap=100 -D -m 10 -t 2 -c file1.fastq file2.fastq.gz > file.flash2_merged.fastq

**Commands used for each resultant fastq file:**

vsearch --fastq_filter file.flash2_merged.fastq --fastq_maxee 1 --fastq_qmax 500 --notrunclabels --fasta_width 0 --qmask none --fastaout

file.flash2_merged.vsearch_qfilt.fasta

cutadapt -a "Primer_F...Primer_R" file.flash2_merged.vsearch_qfilt.fasta -f fasta --discard-untrimmed --no-indels --match-read-wildcards -j 2 -o
file.flash2_merged.vsearch_qfilt.cutadapt.fasta

vsearch --derep_fulllength file.flash2_merged.vsearch_qfilt.cutadapt.fasta --sizein --sizeout --minuniquesize 2 --notrunclabels --fasta_width 0 --qmask none --output
file.flash2_merged.vsearch_qfilt.cutadapt.vsearch_uniq.fasta

vsearch --fastx_filter file.flash2_merged.vsearch_qfilt.cutadapt.vsearch_uniq.fasta --fastq_minlen 70 --fastq_maxlen 130 --minsize 2 --sizein --sizeout --notrunclabels --fasta_width 0 --qmask none --fastaout file.flash2_merged.vsearch_qfilt.cutadapt.vsearch_uniq.vsearch_afilt.fasta

**Commands used for taxonomic assignment:**

blastn' -query file.flash2_merged.vsearch_qfilt.cutadapt.vsearch_uniq.vsearch_afilt.fasta -task megablast -db -outfmt '6 qseqid evalue staxid pident qcovs' -max_target_seqs 100 -max_hsps 1 -out blast.results.txt

## Appendix 5.1 S5. List of the extra-target blocked taxa

| Order | Total matches |
|---|---|
| Monstrilloida | 1 |
| Scorpiones | 28 |
| Araneae | 46 |
| Hymenoptera | 30 |
| Polydesmida | 9 |
| Diptera | 3 |

Products on potentially unintended templates

| Accession number | Taxa | Order | Mismatches Forward | Mismatches Reverse |
|---|---|---|---|---|
| KR049000.1 | Monstrillopsis sp. | Monstrilloida | 0 | 0 |
| KR190462.1 | Heterometrus longimanus | Scorpiones | 0 | 0 |
| KF548113.1 | Heterometrus laoticus | Scorpiones | 0 | 0 |
| JN018160.1 | Heterometrus laoticus | Scorpiones | 0 | 0 |
| AY156573.1 | Heterometrus laoticus | Scorpiones | 0 | 0 |
| KY017602.1 | Synothele arrakis | Araneae | 1 | 0 |
| HQ930135.1 | Chrysidinae sp. | Hymenoptera | 1 | 0 |
| MT491297.1 | Nannaria sp. | Polydesmida | 1 | 1 |
| MT491292.1 | Nannaria sp. | Polydesmida | 1 | 1 |
| MT491291.1 | Nannaria sp. | Polydesmida | 1 | 1 |
| MH119939.1 | Dryocosmus kuriphilus | Hymenoptera | 1 | 1 |
| MH144613.1 | Idiosoma intermedium | Araneae | 1 | 1 |
| MH801131.1 | Dryocosmus kuriphilus | Hymenoptera | 1 | 1 |
| KT756370.1 | Ummidia algarve | Araneae | 1 | 1 |
| KT756369.1 | Ummidia algarve | Araneae | 1 | 1 |
| KT756368.1 | Ummidia algarve | Araneae | 1 | 1 |
| KT756367.1 | Ummidia algarve | Araneae | 1 | 1 |
| KT756366.1 | Ummidia algarve | Araneae | 1 | 1 |
| KR417454.1 | Alysiinae sp. | Hymenoptera | 1 | 1 |
| KR415756.1 | Alysiinae sp. | Hymenoptera | 1 | 1 |
| KR413591.1 | Alysiinae sp. | Hymenoptera | 1 | 1 |
| KR411025.1 | Alysiinae sp. | Hymenoptera | 1 | 1 |
| KR406718.1 | Alysiinae sp. | Hymenoptera | 1 | 1 |
| KR406593.1 | Alysiinae sp. | Hymenoptera | 1 | 1 |
| KR818294.1 | Sellanucheza grandis | Polydesmida | 1 | 1 |
| KM996043.1 | Hymenoptera sp | Hymenoptera | 1 | 1 |
| KX096929.1 | Tonkinosoma flexipes | Polydesmida | 1 | 1 |
| KF308606.1 | Dryocosmus kuriphilus | Hymenoptera | 1 | 1 |
| JX174287.1 | Periegops suteri | Araneae | 1 | 1 |
| JX017357.1 | Periegops suteri | Araneae | 1 | 1 |

| | | | | |
|---|---|---|---|---|
| JF884317.1 | Thomisus onustus | Araneae | 1 | 1 |
| HQ929382.1 | Opiinae sp. | Hymenoptera | 1 | 1 |
| HQ552291.1 | Opiinae sp. | Hymenoptera | 1 | 1 |
| JF411598.1 | Dryocosmus kuriphilus | Hymenoptera | 1 | 1 |
| JF411595.1 | Dryocosmus kuriphilus | Hymenoptera | 1 | 1 |
| HQ979183.1 | Anyphaena sp. | Araneae | 1 | 1 |
| FJ198066.1 | Scorpio maurus | Scorpiones | 1 | 1 |
| FJ198065.1 | Scorpio maurus fuliginosus | Scorpiones | 1 | 1 |
| FJ198059.1 | Scorpio maurus | Scorpiones | 1 | 1 |
| AY156575.1 | Heterometrus swammerdami | Scorpiones | 1 | 1 |
| AY156572.1 | Heterometrus fulvipes | Scorpiones | 1 | 1 |
| DQ286810.1 | Dryocosmus kuriphilus | Hymenoptera | 1 | 1 |
| MN433260.1 | Synothele sp. | Araneae | 1 | 2 |
| MN433259.1 | Synothele sp. | Araneae | 1 | 2 |
| MN433222.1 | Synothele sp. | Araneae | 1 | 2 |
| MT491289.1 | Nannaria sp. | Polydesmida | 1 | 2 |
| MT462243.1 | Thomisus zyuzini | Araneae | 1 | 2 |
| MN151338.1 | Idiosoma sp. | Araneae | 1 | 2 |
| MN151337.1 | Idiosoma sp. | Araneae | 1 | 2 |
| MN151336.1 | Idiosoma sp. | Araneae | 1 | 2 |
| MT040948.1 | Idiosoma gutharuka | Araneae | 1 | 2 |
| MT636123.1 | Heteroonops vega | Araneae | 1 | 2 |
| MK454976.1 | Conothele baoting | Araneae | 1 | 2 |
| MK454975.1 | Conothele baoting | Araneae | 1 | 2 |
| MK454974.1 | Conothele baoting | Araneae | 1 | 2 |
| MK454971.1 | Conothele baoting | Araneae | 1 | 2 |
| MK454970.1 | Conothele baoting | Araneae | 1 | 2 |
| MK454967.1 | Conothele baoting | Araneae | 1 | 2 |
| MK454966.1 | Conothele baoting | Araneae | 1 | 2 |
| MK454965.1 | Conothele baoting | Araneae | 1 | 2 |
| MK454963.1 | Conothele baoting | Araneae | 1 | 2 |
| MK154243.1 | Xysticus sp. | Araneae | 1 | 2 |
| MF936424.1 | Alysiinae sp. | Hymenoptera | 1 | 2 |
| MF932583.1 | Alysiinae sp. | Hymenoptera | 1 | 2 |
| MF931924.1 | Alysiinae sp. | Hymenoptera | 1 | 2 |
| MF937582.1 | Alysiinae sp. | Hymenoptera | 1 | 2 |
| MF931355.1 | Alysiinae sp. | Hymenoptera | 1 | 2 |
| MG669365.1 | Oxidus riukiuria | Polydesmida | 1 | 2 |
| MH144662.1 | Idiosoma arenaceum | Araneae | 1 | 2 |
| MH144657.1 | Idiosoma kopejtkaorum | Araneae | 1 | 2 |
| MH144656.1 | Idiosoma kopejtkaorum | Araneae | 1 | 2 |
| MH144646.1 | Idiosoma clypeatum | Araneae | 1 | 2 |
| MH144645.1 | Idiosoma clypeatum | Araneae | 1 | 2 |
| MH144639.1 | Idiosoma schoknechtorum | Araneae | 1 | 2 |
| MH144638.1 | Idiosoma schoknechtorum | Araneae | 1 | 2 |
| MH144632.1 | Idiosoma mcnamarai | Araneae | 1 | 2 |
| MF983667.1 | Polydesmidae sp | Polydesmida | 1 | 2 |
| MF983591.1 | Diplopoda | Polydesmida | 1 | 2 |

| | | | | |
|---|---|---|---|---|
| KY295321.1 | Aganippe sp. | Araneae | 1 | 2 |
| KY295225.1 | Urodacus planimanus | Scorpiones | 1 | 2 |
| MG480910.1 | Eulophidae sp. | Hymenoptera | 1 | 2 |
| KX774369.1 | Megaselia tamilnaduensis | Diptera | 1 | 2 |
| KY703472.1 | Thomisus onustus | Araneae | 1 | 2 |
| KX537376.1 | Xysticus sabulosus | Araneae | 1 | 2 |
| KT794254.1 | Anahita aculeata | Araneae | 1 | 2 |

# Appendix B

## Other publications during the Ph.D.

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# The Terrific Skink bite force suggests insularity as a likely driver to exceptional resource use

Michael J. Jowers[1,2✉], Yuri Simone[1,2], Anthony Herrel[3,4], M. Pilar Cabezas[1,2,5], Raquel Xavier[1,2], Magaly Holden[6], Renaud Boistel[3], John C. Murphy[7], Mathieu Santin[8,9], Stephane Caut[10], Renoir J. Auguste[11], Arie van der Meijden[1,2], Franco Andreone[12] & Ivan Ineich[13]

**Natural history museum collections hold extremely rare, extinct species often described from a single known specimen. On occasions, rediscoveries open new opportunities to understand selective forces acting on phenotypic traits. Recent rediscovery of few individuals of Bocourt's Terrific Skink *Phoboscincus bocourti*, from a small and remote islet in New Caledonia allowed to genetically identify a species of land crab in its diet. To explore this further, we CT- and MRI-scanned the head of the holotype, the only preserved specimen dated to about 1870, segmented the adductor muscles of the jaw and bones, and estimated bite force through biomechanical models. These data were compared with those gathered for 332 specimens belonging to 44 other skink species. Thereafter we recorded the maximum force needed to generate mechanical failure of the exoskeleton of a crab specimen. The bite force is greater than the prey hardness, suggesting that predation on hard-shelled crabs may be an important driver of performance. The high bite force seems crucial to overcome low or seasonal variations in resource availability in these extreme insular environments. *Phoboscincus bocourti* appears to be an apex predator in a remote and harsh environment and the only skink known to predate on hard-shelled land crabs.**

The International Union for the Conservation of Nature (IUCN) declared 160 animal and plant species extinct in the last decade (2010–2019), but estimates suggest this may be as high as one thousand species per year[1–3]. The biological information that could have been gathered from now extinct species is irreplaceable and lost forever. Such species are often so rare that their only confirmed occurrence is from their initial description. Nevertheless, some species declared extinct are occasionally rediscovered many years later, and sometimes called Lazarus taxa. This happens because these species mostly occur in the understudied biomes[4,5] and many species descriptions are based solely on the holotype. Their restricted ranges and remote localities make biodiversity surveys challenging and remain the main reason for our lack of knowledge. On average, rediscoveries are made about 60 years after the Lazarus taxon has been described[6].

[1]CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, 4485-661 Vairão, Portugal. [2]BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal. [3]Département Adaptations du Vivant, UMR 7179 CNRS/MNHN, 57 Rue Cuvier, Case postale 55, 75231 Paris Cedex 5, France. [4]Evolutionary Morphology of Vertebrates, Ghent University, Campus Ledeganck, K.L. Ledeganckstraat 35, 9000 Gent, Belgium. [5]Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal. [6]Centre d'Écologie Fonctionnelle et Évolutive (UMR CNRS 5175), École Pratique des Hautes Études, Biogéographie et Écologie des Vertébrés, Campus CNRS, Montpellier, France. [7]Science and Education, Field Museum, 1400 Lake Shore Dr., Chicago, IL 60605, USA. [8]Inserm U 1127, CNRS UMR 7225, Centre for NeuroImaging Research, ICM (Brain and Spine Institute), Sorbonne University, Paris, France. [9]Institut du Cerveau – Paris Brain Institute – ICM, INSERM, CNRS, Sorbonne Université, 75013 Paris, France. [10]ANIMAVEG Conservation, 58 Avenue Allende, 94800 Villejuif, France. [11]Department of Life Science, The University of the West Indies, St. Augustine, Trinidad and Tobago. [12]Museo Regionale di Scienze Naturali, Via G. Giolitti, 36, 10123 Turin, Italy. [13]Institut de Systématique, Évolution, Biodiversité (ISYEB), Muséum National d'Histoire Naturelle, Sorbonne Université, École Pratique des Hautes Études, CNRS, Université des Antilles, CP 30, 57 Rue Cuvier, 75005 Paris, France. ✉email: michaeljowers@hotmail.com

PeerJ

# Biomechanical analyses of pterygotid sea scorpion chelicerae uncover predatory specialisation within eurypterids

Russell D. C. Bicknell[1,2], Yuri Simone[3], Arie van der Meijden[3], Stephen Wroe[1,2], Gregory D. Edgecombe[4] and John R. Paterson[1]

[1] Palaeoscience Research Centre, School of Environmental & Rural Science, University of New England, Armidale, NSW, Australia
[2] Function, Evolution and Anatomy Research Lab, School of Environmental and Rural Science, University of New England, Armidale, NSW, Australia
[3] CIBIO Research Centre in Biodiversity and Genetic Resources, Vila do Conde, Portugal
[4] Science Group, The Natural History Museum, London, UK

## ABSTRACT

Eurypterids (sea scorpions) are extinct aquatic chelicerates. Within this group, members of Pterygotidae represent some of the largest known marine arthropods. Representatives of this family all have hypertrophied, anteriorly-directed chelicerae and are commonly considered Silurian and Devonian apex predators. Despite a long history of research interest in these appendages, pterygotids have been subject to limited biomechanical investigation. Here, we present finite element analysis (FEA) models of four different pterygotid chelicerae—those of *Acutiramus bohemicus*, *Erettopterus bilobus*, *Jaekelopterus rhenaniae*, and *Pterygotus anglicus*—informed through muscle data and finite element models (FEMs) of chelae from 16 extant scorpion taxa. We find that *Er. bilobus* and *Pt. anglicus* have comparable stress patterns to modern scorpions, suggesting a generalised diet that probably included other eurypterids and, in the Devonian species, armoured fishes, as indicated by co-occurring fauna. *Acutiramus bohemicus* is markedly different, with the stress being concentrated in the proximal free ramus and the serrated denticles. This indicates a morphology better suited for targeting softer prey. *Jaekelopterus rhenaniae* exhibits much lower stress across the entire model. This, combined with an extremely large body size, suggests that the species likely fed on larger and harder prey, including heavily armoured fishes. The range of cheliceral morphologies and stress patterns within Pterygotidae demonstrate that members of this family had variable diets, with only the most derived species likely to feed on armoured prey, such as placoderms. Indeed, increased sizes of these forms throughout the mid-Palaeozoic may represent an 'arms race' between eurypterids and armoured fishes, with Devonian pterygotids adapting to the rapid diversification of placoderms.

**Subjects** Computational Biology, Evolutionary Studies, Paleontology, Zoology
**Keywords** Euarthropoda, Finite element analysis, Predation, Eurypterids, Sea scorpions

## INTRODUCTION

Feeding toolkits of proposed fossil predators are typically explored through functional morphology, often with comparison to modern analogues. In the last two decades, there

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Corresponding author
Russell D. C. Bicknell,
rdcbicknell@gmail.com

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*toxins*

MDPI

*Article*

# Intersexual Differences in the Gene Expression of *Phoneutria depilata* (Araneae, Ctenidae) Toxins Revealed by Venom Gland Transcriptome Analyses

**Diego Sierra Ramírez [1], Juan F. Alzate [2], Yuri Simone [3], Arie van der Meijden [3], Giovany Guevara [4], Lida Marcela Franco Pérez [5], Julio César González-Gómez [1] and Carlos F. Prada Quiroga [1,*]**

1 Grupo de Investigación Biología y Ecología de Artrópodos (BEA), Facultad de Ciencias, Universidad del Tolima, Altos de Santa Helena, Ibagué 730001, Colombia; dsierrar@ut.edu.co (D.S.R.); jcesargonzalez@ut.edu.co (J.C.G.-G.)
2 Centro Nacional de Secuenciación Genómica (CNSG), Facultad de Medicina, Universidad de Antioquia, Medellín 050010, Colombia
3 CIBIO/InBIO/Biopolis, Campus Agrário de Vairão, Rua Padre Armando Quintas 7, 4485-661 Vila do Conde, Portugal; yurisimone1@gmail.com (Y.S.); mail@arievandermeijden.nl (A.v.d.M.)
4 Grupo de Investigación en Zoología (GIZ), Facultad de Ciencias, Universidad del Tolima, Altos de Santa Helena, Ibagué 730001, Colombia; gguevara@ut.edu.co
5 Facultad de Ciencias Naturales y Matemáticas, Universidad de Ibagué, Carrera 22 Calle 67, Ibagué 730001, Colombia; lida.franco@unibague.edu.co
* Correspondence: cfpradaq@ut.edu.co

**Abstract:** The wandering spider, *Phoneutria depilata*, is one of Colombia's most active nocturnal arthropod predators of vertebrates and invertebrates. Its venom has been a relevant subject of study in the last two decades. However, the scarcity of transcriptomic data for the species limits our knowledge of the distinct components present in its venom for linking the mainly neurotoxic effects of the spider venom to a particular molecular target. The transcriptome of the *P. depilata* venom gland was analyzed to understand the effect of different diets or sex and the impact of these variables on the composition of the venom. We sequenced venom glands obtained from ten males and ten females from three diet treatments: (i) invertebrate: *Tenebrio molitor*, (ii) vertebrate: *Hemidactylus frenatus*, and (iii) mixed (*T. molitor* + *H. frenatus*). Of 17,354 assembled transcripts from all samples, 65 transcripts relating to venom production differed between males and females. Among them, 36 were classified as neurotoxins, 14 as serine endopeptidases, 11 as other proteins related to venom production, three as metalloprotease toxins, and one as a venom potentiator. There were no differences in transcripts across the analyzed diets, but when considering the effect of diets on differences between the sexes, 59 transcripts were differentially expressed. Our findings provide essential information on toxins differentially expressed that can be related to sex and the plasticity of the diet of *P. depilata* and thus can be used as a reference for venomics of other wandering spider species.

**Keywords:** next-generation sequencing; spider; transcriptomics; venomics; venom gland

**Key Contribution:** *Phoneutria depilata* is a spider with aggressive behavior and is the most widely distributed and has the highest impact on human health in Colombia. This is the first complete comparative study of the transcriptome of venom glands associated with the diet and sex of *P. depilata*. Studying the expression profiles of different toxin gene families, we detected a differential expression of certain genus-related toxins associated with sex and the plasticity of the diet.

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## 1. Introduction

Animal venoms are cocktails of bioactive compounds that alter the normal physiology of the envenomed animal, delivered into the victim through specialized body structures like stingers or fangs [1–3]. Venom is a multifunctional trait, mainly used in three different

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# First report of arm-span competition in buthid scorpions: male-male contest in Tityus cf. rosenbergi (Pocock, 1898)

--Manuscript Draft--

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| **Corresponding Author:** | Yuri Simone<br>CIBIO: Universidade do Porto Centro de Investigacao em Biodiversidade e Recursos Geneticos<br>Vairão, PORTUGAL |
| **Corresponding Author Secondary Information:** | |
| **Corresponding Author's Institution:** | CIBIO: Universidade do Porto Centro de Investigacao em Biodiversidade e Recursos Geneticos |
| **Corresponding Author's Secondary Institution:** | |
| **First Author:** | Jules Thornton Wyman |
| **First Author Secondary Information:** | |
| **Order of Authors:** | Jules Thornton Wyman |
| | Julien Wright-Ueda |
| | Quinn Agnew |
| | Ismael Castellano |
| | Yuri Simone |
| **Order of Authors Secondary Information:** | |
| **Abstract:** | Although courtship and mating behaviors have been described for nearly all scorpion lineages, intrasexual interactions in scorpions remain understudied. Recently, a novel ritualized behavioral unit, termed “arm-span competition,” in which individuals face off and extend their pedipalps laterally, was described from analyses of male-male contests in several scorpionid species. Here, we present the first documented observation of arm-span competition in a buthid scorpion, Tityus cf. rosenbergi (Pocock, 1898). Interestingly, both T. cf. rosenbergi and most scorpionid species known to engage in arm-span competition exhibit a similar sexual dimorphism: males have markedly longer and more slender pedipalps than females. We provide support for the hypothesis that sexually dimorphic pedipalps in these species arose as the result of selective pressure related to ritualized arm-span competition and suggest that this dimorphism may have evolved independently across multiple lineages. We also highlight the potential for citizen science to contribute rare observations to scientific literature. |