Novel Insights into Arachnid Venomics: Studies on Pseudoscorpion and Scorpion Venom

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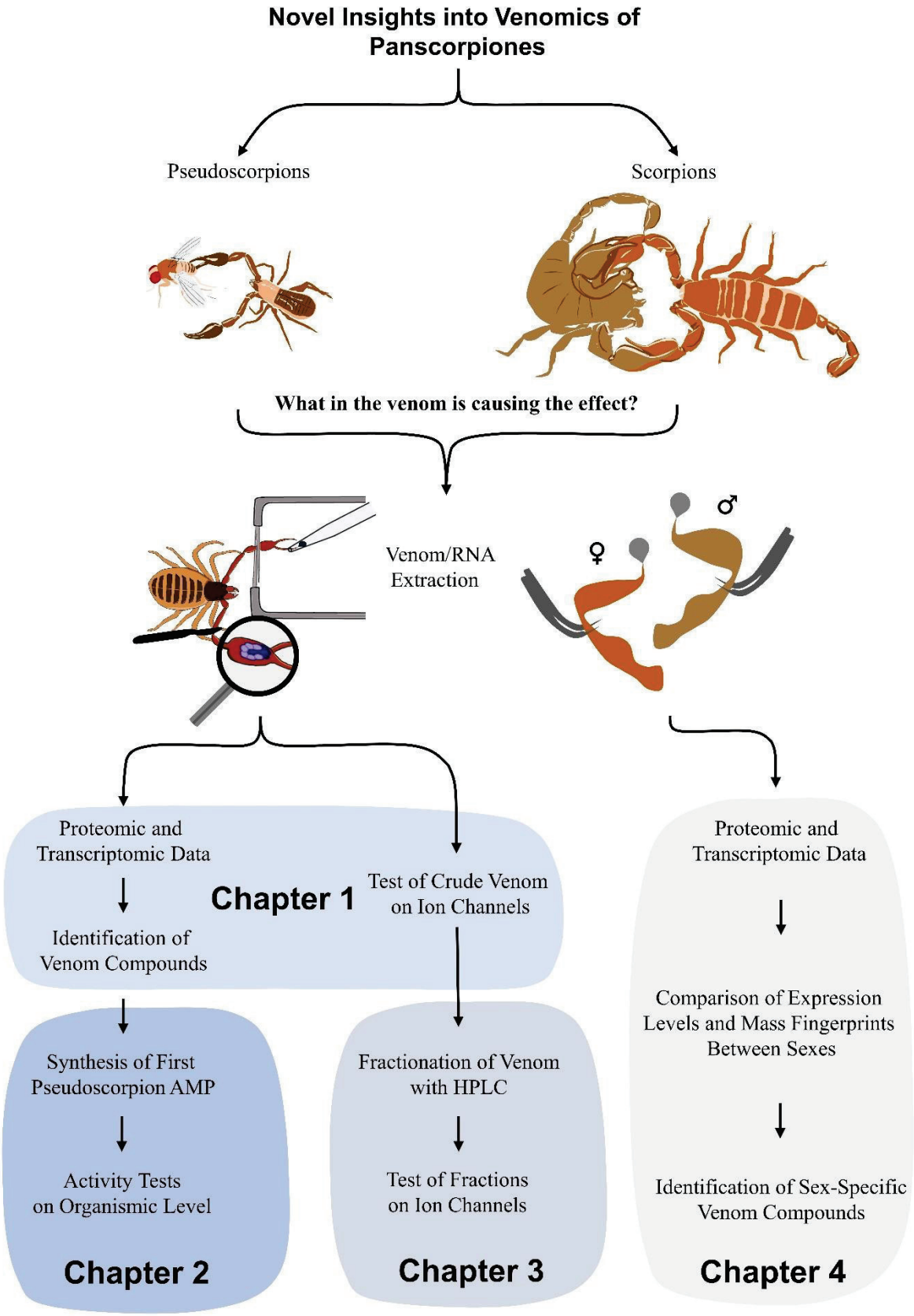
List of Abbreviations

AMP	Antimicrobial peptide
HPLC	High performance liquid chromatography
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MIC	Minimal inhibitory concentration
MS	Mass spectrometry
PTM	Post-translational modification

Graphical Abstract



Abstract

This dissertation addresses previously neglected aspects of venom research on Panscorpiones, which comprises scorpions and pseudoscorpions. Both have evolved unique venom delivery systems. Scorpions possess an elongated metasoma with a telson modified for venom production and injection, whereas pseudoscorpions produce venom in the chelal hands of the paired pedipalps and inject it via tiny teeth near the tip of the chelal fingers. Research on scorpion venom is relatively advanced for the medically relevant buthid scorpions. All non-buthid scorpion families are much less investigated in this regard, with some families having been totally ignored by venom research to date. An interesting and largely unexplored aspect of scorpion venom are intraspecific differences in venom composition. One cause of this variation might be mating-related as many scorpions perform a sexual sting during the promenade a deux. Sex-specific venom compositions have been described for some scorpion species, but the relevant venom compounds and respective genes were only rarely identified. Pseudoscorpions on the other hand were nearly completely neglected by venom research, with only a few studies reporting the putative venom composition by solely transcriptomic approaches or testing the activity of crude venom. In a study preceding this dissertation, we developed a sophisticated extraction protocol for obtaining venom from these tiny arachnids and identified the first venom compounds also supported by proteomic analyses.

Chapter 2.1 of this dissertation comprises a publication in which we provided the first comprehensive analysis of a pseudoscorpion's venom using a combined proteomic and transcriptomic workflow. Based on this, I identified the first genuine neurotoxins for a pseudoscorpion and a plethora of novel compounds. This is the foundation for selecting compounds for activity screenings to improve understanding the mode of action of pseudoscorpion venom and to identify compounds suitable as potential drug leads. We also demonstrated neurotoxic effects of pseudoscorpion crude venom on specific ion channels. The low similarity of the venom compounds we identified to those discovered by solely transcriptomic approaches in previous research, demonstrates the importance of complementary proteomic analyses. Also, the venom of additional pseudoscorpion species needs to be investigated to improve the understanding of venom variation in this group.

Chapter 2.2 contains our publication about activity tests with Checacin1, the first venom compound identified for the house pseudoscorpion. This compound is a linear cationic peptide corresponding to one of the most prominent ion signals in MALDI-TOF MS mass fingerprints of the venom. We tested synthetized Checacin1 and several of its fragments on different microbe strains, a mammalian cell culture and in an aphid feeding assay. The complete Checacin1 showed potent antimicrobial activity on both gram-negative and gram-positive bacteria, including a multi-resistant strain, and a significant reduction of aphid survival, comparable to that caused by a commercial insecticide. However, the observed cytotoxic activity for this peptide limits its potential as a lead compound. Checacin1 fragments exhibit much lower activities, which indicates that both the hydrophobic N-terminus and the cationic C-terminus are essential for the potency of Checacin1. As Checacin1 is only one out of seven checacins identified in the venom of *C. cancroides*, it is promising to test the remaining ones

in the same setup. Checacins show different expression levels and signal intensities in mass fingerprints of the venom, which might correlate with their respective efficiencies.

Chapter 2.3 shows our recent progress in fractionating pseudoscorpion venom for activity tests to identify the venom compounds responsible for the effects caused by the crude venom on selected ion channels described in chapter 2.1. The performed fractionation allowed for simultaneously collecting fractions for biotests and performing mass spectrometry (MS) analyses. Although the amount of applied venom was very low, many of the previously identified venom compounds could be recovered and efficiently separated during fractionation. For activity tests on the previously tested ion channels, those fractions containing the highest amount of previously identified venom compounds were selected. However, no activity could be measured for these fractions. In future research we could utilize an automated ligand binding assay for identifying the toxic fractions if these can be adapted to our ion channels of interest. In case the used venom amounts were insufficient, recombinant production systems might be the best solution to obtain pseudoscorpion toxins for activity tests.

In chapter 2.4 of this dissertation a publication is included that focuses on investigating sex-specific differences in the venom of the scorpion *Euscorpius italicus*. We were especially interested in the potential mating-related use of venom compounds. For this reason, we performed a comprehensive venom analysis for a scorpion species with a pronounced sexual dimorphism of the telson morphology, which is also known to perform an extensive sexual sting during the promenade a deux. For this scorpion species we could identify male-specifically expressed venom compounds; the up-regulation was also confirmed at the transcriptome level. Personal mating observations showed that females of this species cease all reluctant behavior towards male mating attempts after receiving a sexual sting. The identified compounds might play a key role for this behavior. Additionally, several compounds are down-regulated in male venom, mostly classified as potential neurotoxins. It is likely that these are harmful for the females. The sexual sting is performed by representatives of various scorpion families, and it is likely that this behavior represents a plesiomorphic trait. This raises the question if the closely related pseudoscorpions exhibit something similar. With respect to the venom composition, I could not observe any sex-specific differences in case of the house pseudoscorpion and no evidence for venom transfer during mating exists. However, cheliferid pseudoscorpions evolved another unique feature in this regard. During the promenade a deux so called ram's horn organs are extruded, which was shown to decrease female resistance and hence might fulfill a similar function as the sexual sting in scorpions.

With my dissertation I could advance neglected aspects of arachnid venom research. For a pseudoscorpion, I identified the first genuine venom compounds and for one of these we demonstrated potent activities on organismic level. In addition, for a scorpion with pronounced sexual dimorphism we could reveal those compounds most likely utilized in a mating-related context.

Zusammenfassung

Diese Dissertation befasst sich mit bisher vernachlässigten Aspekten der Giftforschung an Panskorpiones, welche Skorpione und Pseudoskorpione umfassen. Beide Gruppen haben einzigartige Giftübertragungssysteme entwickelt. Skorpione verwenden ihr verlängertes Metasoma mit einem für die Giftinjektion modifizierten Telson, wohingegen Pseudoskorpione zu diesem Zweck auf ihre mit Giftdrüsen und -zähnen ausgestatteten Scherenhände zurückgreifen. Die Erforschung von Skorpionsgift ist relativ weit fortgeschritten, insbesondere für die medizinisch relevanten Buthiden. Alle anderen Skorpionsfamilien sind in dieser Hinsicht jedoch weit weniger erforscht und einige Familien wurden von der Giftforschung bisher nicht berücksichtigt. Ein weiterer wenig erforschter Aspekt sind intraspezifische Unterschiede der Giftzusammensetzung innerhalb der Panscorpiones. Eine Ursache dieser Variation könnte paarungsbedingt sein, da viele Skorpione einen ‚sexual sting' während der promenade a deux durchführen. Geschlechtsspezifische Unterschiede in der Giftzusammensetzung wurden für viele Skorpionsarten beschrieben, wobei die beteiligten Giftstoffe und deren Gene nur selten identifiziert wurden. Pseudoskorpione wurden von der Giftforschung fast vollständig ignoriert. In einigen wenigen Studien wurden Aktivitätstest mit dem Giftgemisch durchgeführt oder die Giftzusammensetzung dieser Tiere untersucht, jedoch auf Basis rein transkriptomischer Ansätze. In einer Arbeit, die dieser Dissertation vorausging, haben wir ein Protokoll zur Giftextraktion von diesen winzigen Spinnentieren entwickelt und mit Hilfe von transkriptomischen und proteomischen Analysen die ersten Giftstoffe identifiziert.

Das Kapitel 2.1 dieser Dissertation umfasst eine Publikation, in der wir die erste vollumfassende Pseudoskorpions-Giftanalyse durchgeführt haben, welche sowohl durch proteomische als auch transkriptomische Daten gestützt wird. Hierdurch konnten wir die ersten genuinen Pseudoskorpions-Neurotoxine und eine Vielzahl neuartiger Substanzen identifizieren, sowie neurotoxische Wirkungen des Giftes auf spezifische Ionenkanäle nachweisen. Diese Studie ist der Ausgangspunkt für die Auswahl von potenziellen Toxinen für Aktivitätsscreenings, um die Wirkungsweise des Pseudoskorpion-Giftes besser zu verstehen und Substanzen zu identifizieren, die als Leitstrukturen für Arzneimittel geeignet sein könnten. Die von uns beschriebenen Giftkomponenten zeigen nur geringe Übereinstimmungen mit den zuvor identifizierten Komponenten, welche für die anderen Arten durch rein transkriptomische Analysen ermittelt wurden. Dies demonstriert die Notwendigkeit auch das Gift-Proteom zur Identifizierung der Giftkomponenten zu verwenden. Um die Giftvariation innerhalb dieser Gruppe besser zu verstehen, bedarf es zudem der Untersuchung des Giftes weiterer Pseudoskorpionsarten.

Im Kapitel 2.2 beschreibe ich unsere Veröffentlichung über Aktivitätstests mit Checacin1, der ersten für den Bücherskorpion identifizierten Giftverbindung. Dieses Toxin ist ein lineares kationisches Peptid, das einem der auffälligsten Ionensignale in MALDI-TOF MS Spektren des Giftes entspricht. Wir haben das synthetisierte Checacin1 und mehrere seiner Fragmente an verschiedenen Mikrobenstämmen, einer Säugetierzellkultur und in einem Fütterungsversuch mit Blattläusen getestet. Für das vollständige Checacin1 zeigte sich eine starke antimikrobielle Aktivität sowohl auf gramnegative als auch grampositive Bakterien, einschließlich eines

multiresistenten Erregers und eine signifikante Verringerung der Überlebensrate von Blattläusen, vergleichbar mit der eines kommerziellen Insektizids. Die ebenfalls beobachtete zytotoxische Aktivität dieses Peptids schränkt jedoch dessen Potenzial für die pharmazeutische Anwendung ein. Die deutlich geringere Aktivität der Checacin1-Fragmente deutet darauf hin, dass sowohl der hydrophobe N-Terminus als auch der kationische C-Terminus für die Wirksamkeit dieses Peptids wesentlich sind. Da es sich hierbei nur um das erste der sieben im Gift von *C. cancroides* identifizierten Checacine handelt, ist es vielversprechend, auch die Übrigen in gleicher Weise zu testen. Die Checacine weisen unterschiedliche Expressionslevel und Signalintensitäten in Massenfingerprints des Giftes auf. Dies könnte mit ihrer Wirksamkeit in Zusammenhang stehen.

Das Kapitel 2.3 zeigt unsere bisherigen Fortschritte bei der Fraktionierung von Pseudoskorpionsgift für Aktivitätstests. Hierdurch sollten die Fraktionen identifiziert werden, welche die in Kapitel 2.1 beschriebenen Wirkungen des Giftgemischs auf ausgewählte Ionenkanäle verursachen. Die durchgeführte Fraktionierung, ermöglichte die gleichzeitige Gewinnung von Fraktionen für Aktivitätstests sowie den Nachweis der enthaltenen Giftstoffe mittels MS. Trotz der geringen eingesetzten Giftmenge konnten viele der zuvor identifizierten Giftstoffe während der Fraktionierung nachgewiesen und effizient aufgetrennt werden. Für Aktivitätstests an den zuvor getesteten Ionenkanälen, wurden diejenigen Fraktionen ausgewählt, welche die höchste Menge der bereits beschriebenen Giftstoffe enthielten. Jedoch konnte für diese Fraktionen keine Aktivität gemessen werden. In zukünftigen Untersuchungen könnte ein automatisiertes ‚ligand-binding' Assay hilfreich sein, um eine umfassendere Testung der Fraktionen zu realisieren, wobei diese bisher nur für wenige Ionenkanäle optimiert wurden. Falls die verwendeten Giftmengen zu gering waren, könnte auf rekombinante Produktionssysteme zurückgegriffen werden, um ausgewählte Giftkomponenten für Aktivitätstests herzustellen.

Das Kapitel 2.4 dieser Dissertation zeigt eine Publikation, die sich mit der Untersuchung von geschlechtsspezifischen Unterschieden im Gift des Skorpions *Euscorpius italicus* befasst. Hierbei lag der Fokus auf dem potenziellen Einsatz von Giftstoffen im Kontext der Paarung bei Skorpionen. Aus diesem Grund haben wir eine umfassende Giftanalyse für eine Skorpionsart mit einem ausgeprägten Geschlechtsdimorphismus der Telsonmorphologie durchgeführt. Für diese Art ist auch ein umfangreicher ‚sexual sting' beschrieben worden. Mittels der Analyse des Giftes konnten wir bei diesem Skorpion Giftstoffe identifizieren, welche nur im männlichen Gift deutlich hochreguliert sind. Meine Beobachtungen der Paarung haben gezeigt, dass die Weibchen dieser Art nach einem erfolgreichen ‚sexual sting' jegliches Abwehrverhalten gegenüber männlichen Paarungsversuchen einstellen. Daher kann angenommen werden, dass die identifizierten Giftkomponenten hierbei eine Schlüsselrolle spielen. Darüber hinaus wurden im männlichen Gift mehrere Verbindungen herunterreguliert, welche größtenteils als potenzielle Neurotoxine klassifiziert wurden. Für diese Substanzen nehmen wir eine schädliche Wirkung auf die Weibchen an. Der ‚sexual sting' wurde für mehrere Skorpionsfamilien beschrieben und es ist wahrscheinlich, dass es sich hierbei um ein plesiomorphes Merkmal handelt. Dies führt zu der Frage, ob die nah verwandten Pseudoskorpione ein ähnliches Verhalten aufweisen. Die Giftzusammensetzung betreffend, konnte ich beim Bücherskorpion keine geschlechtsspezifischen Unterschiede feststellen und es gibt keine Hinweise auf eine

Giftübertragung während der Paarung. Die Pseudoskorpione dieser Familie haben in dieser Hinsicht jedoch ein weiteres einzigartiges Merkmal entwickelt. Während der Promenade a deux werden sogenannte ‚ram's horn organs' ausgestülpt, die nachweislich den Widerstand der Weibchen vermindern und somit eine ähnliche Funktion wie der ‚sexual sting' bei Skorpionen erfüllen könnten.

Mit dieser Dissertation konnte ich wertvolle Erkenntnisse über vernachlässigte Aspekte der Giftforschung an Arachniden gewinnen. Für eine Pseudoskorpionsart habe ich die ersten tatsächlichen Giftstoffe identifiziert und für eine dieser Komponenten konnten wir wirkungsvolle Aktivitäten auf organismischer Ebene demonstrieren. Außerdem haben wir für eine Skorpionsart mit ausgeprägtem Sexualdimorphismus diejenigen Giftkomponenten identifiziert, welche mit hoher Wahrscheinlichkeit im Kotext der Paarung eingesetzt werden.

1 Introduction

1.1 Animal Venomics – Advances and Potential for Application

Animal venoms are complex cocktails of bioactive compounds, dominated by peptides and proteins. Per definition a venom is stored in specialized glands and delivered to the target via injection, utilizing a venom apparatus (Nelsen et al., 2014). Evolution has optimized venom compounds mainly for predation, feeding and defense, though in some taxa venom is utilized intraspecifically, e. g. in a mating-related context (e. g. Binford et al., 2016; Whittington and Belov, 2007). Those substances responsible for the detrimental effects caused by venom are called toxins. These can, in principle, act on all physiological pathways and tissues, accessible via the blood stream (Fry et al., 2009). Neurotoxins are binding to different kinds of ion-channels, modulating signal transmission in various ways, whereas other toxins target for example different factors involved in the coagulation cascade (Fry et al., 2009). Within the animal kingdom, venom usage is described for at least 100 different subgroups including at least eight phyla (Schendel et al., 2019). Venom evolution has created an immense variety of different compounds with individual venoms comprising hundreds and even up to thousand different substances (e. g. Escoubas et al., 2006). The venom composition is a highly variable trait exhibiting considerable intraspecific variation between populations (Carcamo-Noriega et al., 2018; Rodríguez-Vargas et al., 2022; Schaffrath et al., 2018), sexes (e. g. Nystrom, 2019; Sousa et al., 2010), and ontogenetic stages (Cipriani et al., 2017; Machado Braga et al., 2020). The genetic driver of this variety is suspected to be a combination of gene duplication and recruitment into the venom gland, by which venom compounds evolve from ancestral non-toxin peptides and proteins (Fry et al., 2009; Wong and Belov, 2012). The field of venomics aims to unravel the vast complexity of venoms by large-scale approaches utilizing a combination of proteomic and transcriptomic workflows and characterizing the activity of isolated or produced venom compounds in various assays (von Reumont et al., 2022).

Initially, venom research was mainly driven by the motivation of understanding the devastating effects some toxins can unfold on mankind and to develop antivenom for saving human lives. By now, venoms have been recognized as a promising source for lead compounds that can be utilized for various purposes (e. g. Ghosh et al., 2019; Khalil et al., 2021; Robinson et al., 2017). Many venom compounds were found to exhibit potentially beneficial properties, comprising e. g. antimicrobial, anticancer or insecticidal activities (Herzig et al., 2020). Hence, such compounds hold a great potential for pharmaceutic applications and insecticide development. So far, at least eleven commercial drugs have been developed based on lead compounds identified in animal venoms (Bordon et al., 2020). Given the humungus amount of described venom compounds, the real potential still seems to remain almost untouched. Reasons for this might be the expenses involved in drug development combined with bureaucratic hurdles (Harrison et al., 2014). As venoms comprise an extensive toolkit of ion channel modulators, they likely hold promising candidates for treating diseases that involve specific ion channels. This has been demonstrated in recent studies that have identified compounds with promising activities against diseases like epilepsy (Chow et al., 2020), chronic pain (Yang et al., 2013), autoimmune diseases (Tarcha et al., 2017), stroke (Chassagnon et al., 2017), and diabetes

(Furman, 2012). Moreover, cell-lytic toxins can be highly specific, e. g. depending on the charge state of cell membranes, and thus beneficial for selectively targeting certain microbes and parasites (de Moura et al., 2022; Rincón-Cortés et al., 2022).

As indicated, venom research has long been biased towards those species posing a threat to humans. In addition, methodological constraints limited the discovery of novel compounds to relatively large species. Now, the advances in 'omics' approaches allow to discover venom compounds even from tiny creatures not exceeding a few millimeters in body size (Marchi et al., 2022; von Reumont et al., 2022). Accompanied by the development of modern synthesizers and optimized recombinant production, this has enabled the production of toxins, without the necessity to extract large quantities of venom form the animals (von Reumont et al., 2022).

These advances allow to sample across a wider phylogenetic range, which might be a promising strategy for maximizing success in screening for lead compounds (Lüddecke et al., 2019). In this regard, a comprehensive study on the venom of all major groups of centipedes found a unique set of venom compounds for each of the subgroups (Jenner et al., 2019). By now, several studies on neglected taxa revealed a plethora of novel compounds (e. g. Drukewitz et al., 2018; von Reumont et al., 2014a, 2014c; Walker et al., 2018), whose specific activities in many cases remain unknown. A promising group for identifying novel venom compounds are pseudoscorpions, a group of tiny venomous arachnids nearly completely overlooked by venom research. The phylogenetic placement of pseudoscorpions within Arachnida remained ambiguous for a long time, but by now a positioning of pseudoscorpions within Arachnopulmonata as a sister taxon of the 'real' scorpions was proposed (Ontano et al., 2021). This new clade was defined as Panscorpiones.

The major aim of this dissertation was to characterize the venom of understudied groups within Panscorpiones by (1) performing comprehensive venom analyses for selected pseudoscorpion and scorpion species combining proteomic and transcriptomic approaches, (2) performing first activity tests with pseudoscorpion crude venom and individual toxins on organismic and cellular level, and (3) identifying potential sex-specific differences and compare scorpion and pseudoscorpion venom in this regard.

1.2 Current State of Panscorpion Venom Research

The origin of Panscorpiones can be traced back to the Devonian based on the oldest discovered pseudoscorpion fossils (Harms and Dunlop, 2017). Scorpion-like fossils probably representing the common ancestor of Panscorpiones date back to the Silurian (Howard et al., 2019) with some extinct groups described as aquatic (e. g. Waddington et al., 2015). With over 4,000 described extant species belonging to 25 families (WPC, 2022) pseudoscorpions exhibit a slightly higher diversity compared to scorpions, for which at least 2,500 species are known that are classified into 22 families (Santibáñez-López et al., 2022). Regarding size, scorpions clearly outrange pseudoscorpions, since most scorpion species reach a size of a few centimeters, and their largest representatives of the genera *Heterometrus* and *Pandinus* can be over 20 cm long. In comparison, pseudoscorpions are tiny, with most species not exceeding a body length of a few millimeters and the larger species like *Garypus titanius* barely exceeding 1 cm. Both scorpions and pseudoscorpions inhabit crevice habitats, though the much smaller size of

pseudoscorpions enabled them to occupy even more inconspicuous niches like in between the bark and trunk of a tree. The absence of book lungs described for pseudoscorpions, probably evolved alongside this reduction in size (Dunlop, 2019).

Both, scorpions and part of the pseudoscorpions, rely on venom to overcome prey. In case of scorpions all described species exhibit the well-known elongated metasoma with the telson, modified for venom injection. Within pseudoscorpions, which lack the scorpion stinger, another unique venom delivery system has evolved, as Iocheiratan pseudoscorpions possess chelal hands equipped with venom glands for grasping prey and simultaneously injecting their venom (Chamberlin, 1924; Croneberg, 1888).

As some scorpions can cause severe envenomation and even fatalities, venom research on this group is relatively advanced, though with a clear bias towards Buthidae, which comprise nearly all scorpion species dangerous to humans. These cause an estimated number of 1.2 million envenomations leading to more than 3,000 fatalities every year (Isbister and Bawaskar, 2014). The medically most relevant buthids comprise the genera *Androctonus*, *Leiurus* and *Buthus* distributed across the 'old world' and the genera *Centruroides* and *Tityus*, inhabiting the 'new world'. The venom composition of representatives of all these genera have been investigated (e. g. Caliskan et al., 2006; Díaz et al., 2019; Erdeş et al., 2014; Furtado et al., 2020; Xu et al., 2014). In a review on proteomics approaches applied to scorpion and spider venom, Marchi et al. (2022) showed that most research on scorpion venom focused on the genera *Tityus* followed by *Centruroides*. Apart from buthids, most other venom studies focused on the family of Scorpionidae, which comprises some of the largest extant scorpions. Due to their size, large quantities of venom and RNA are easily accessible, and several studies provided whole proteomic or transcriptomic analyses of the venom compositions (Bringans et al., 2008; Deng et al., 2018; Diego-García et al., 2012; Ma et al., 2010). The remaining scorpion families are much less studied. Von Reumont et al. (2022) showed that for six of these scorpion families, compounds were deposited into the Uniprot database. For many of the non-buthid scorpion families at least a transcriptomic investigation of the venom composition was performed (e. g. Santibáñez-López et al., 2015), though in many cases proteomic data, confirming the presence of the identified compounds in the venom, is lacking (Smith and Alewood, 2015). As part of this thesis, I have reviewed the current state of scorpion venom research by performing a literature search for the non-buthid scorpion families using the search terms 'venom' AND '[family name]' in the search platform google scholar. In case this did not yield any result, I repeated the search for all genera of the respective families. The outcome is summarized in supplementary table SM 1.1. Figure 1 shows the current scorpion phylogeny (Santibáñez-López et al., 2022), in which I indicated the progress of venom research for the individual families. For some of the families not a single study has been performed on the venom and for families like Bothriuridae only a single compound has been identified so far (Ramos and Escobar, 2006).

The best characterized scorpion venom compounds are neurotoxins acting on different sites of sodium, potassium, calcium, and chlorine channels (e. g. Quintero-Hernández et al., 2013). Miranda and Lissitzky (1961) were the first to isolate scorpion neurotoxins showing that these compounds are mainly responsible for the toxic effect caused in mice. Most scorpion neurotoxins are peptides ranging in length from 31 to 70 amino acids and exhibiting three to four disulfide bridges (e. g. Rodríguez de la Vega and Possani, 2005; Rodríguez de la Vega and

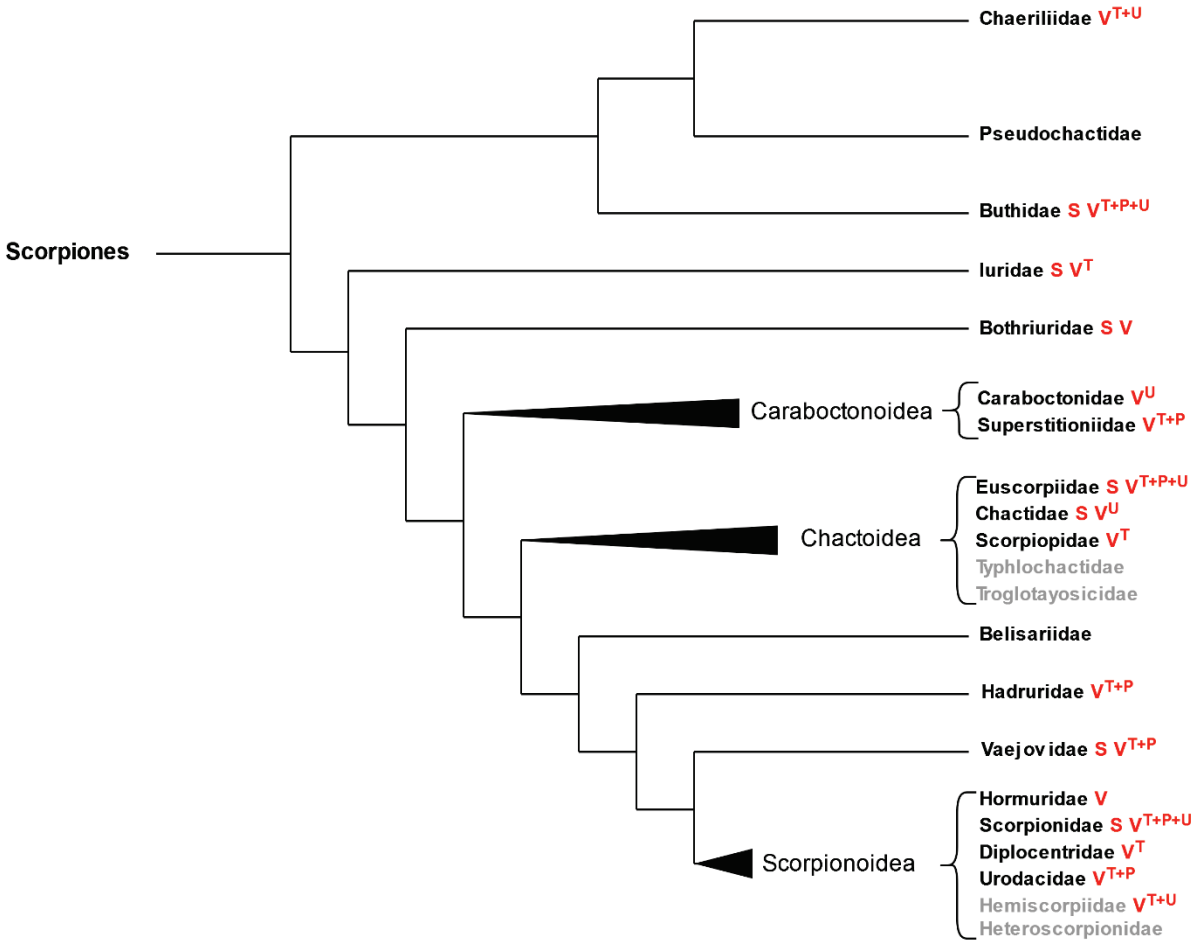


Figure 1. Scorpion phylogeny based on Santibáñez-López et al. (2022) indicating the venom research progress on family level. Scorpion families missing in the phylogeny of Santibáñez-López et al. were added based on Stockmann (2013) and these families are highlighted in grey. In case only single venom compounds were characterized, the respective family is labeled with a red V. For more comprehensive analyses an upper-case T is added for transcriptomic and upper-case P for proteomic analyses. If venom compounds were found in the Uniprotdatabase an additional upper-case U is added. The latter information is taken from von Reumont et al. (2022). Additionally, those families that perform a sexual sting are marked with an S.

Possani, 2004). Scorpion sodium channel toxins were classified into α-toxins acting on site 3 of the sodium channel, thereby inhibiting the ion flow (Bosmans and Tytgat, 2007; Gurevitz, 2012) and β-toxins acting on site 4 thereby causing a sustained ion flow through the channel (Escalona, 2013). In accordance, also the potassium channel toxins bind to several different sites of the channel and were originally classified into the sub-groups α, β, γ, and κ (Tytgat et al., 1999). Meanwhile, it has been discovered that at least the potassium channel toxins likely evolved from defensins, AMPs with conserved cystine framework (Zhu et al., 2014). Also, several non-disulfide-bridged peptides were found in scorpion venom which mostly act by disrupting membranes and exhibit antibacterial, antiviral and anticancer activities (Almaaytah and Albalas, 2014).

Due to their small size and harmlessness for humans, studies on the venom of pseudoscorpions are scarce. Dos Santos and Coutinho-Netto (2006) were the first ones to investigate biochemical aspects of pseudoscorpion venom by applying crude venom of *Paratemnoides nidificator* on preparations of synaptosomal membranes of rat cerebral cortex tissue, which revealed a

modulation of the receptor-binding of L-glutamate. However, venom was extracted by tissue homogenization and the actual bioactive compounds responsible for the observed effects were not identified. In 2014, pseudoscorpions were identified as one of the groups of neglected venomous animals for which not a single venom compound had been identified beforehand (von Reumont et al., 2014b). The first study investigating the venom composition of the pseudoscorpion *Synsphyronus apimelus* was then performed by Santibáñez-López et al. (2018), who applied a solely transcriptomic approach. They identified potential venom compounds by similarity to known arthropod toxins and the venom was found to be dominated by enzymes and to contain only a few potential peptide toxins. Up to that point, pseudoscorpion venom had not been investigated using proteomics which is however crucial for the identification of the genuine venom compounds. Especially novel compounds with low similarity to known substances can easily be overlooked by existing solely transcriptomic approaches which are additionally prone to the discovery of false positives (Smith and Undheim, 2018).

Conducting a proteomic analysis of pseudoscorpion venom used to be impeded by the small size of these arthropods, which is linked to minute venom yields and a complicated venom extraction. For this reason, I developed a sophisticated methodology to extract venom from pseudoscorpions and other tiny arthropods by application of electricity, which is illustrated in figure 2 (Krämer et al., 2019). This work was preceding this dissertation and provided the precondition for our advances on analyzing the venom of these arachnids. To implement our extraction methodology, we developed a device for simultaneously immobilizing pseudoscorpions under a layer of foam plastic and applying electricity with movable arms at the integument of the chelal hand. Since electricity alone was not sufficient to trigger the release of venom, it was necessary to insert one of the chelal fingers into a liquid-filled capillary. This suggests that the pseudoscorpion's venom delivery system possesses a mechanism preventing the wasteful release of venom. This idea is supported by the presence of a hair-like structure located at the basement of the venom teeth, which was described as lamina defensor (Chamberlin, 1924). It has been proposed that this structure is involved in venom release (Muchmore, 1981), though this remains to be proven. Utilizing our newly developed extraction methodology, we performed the first analysis of pseudoscorpion venom utilizing transcriptomic and proteomic approaches (Krämer et al., 2019). This led to the discovery of checacins, linear peptides which were classified as antimicrobial peptides (AMP) based on a similarity to Megicin-18, an AMP discovered in the venom of the scorpion *Mesobuthus gibbosus* (Diego-García et al., 2014).

1.3 Thesis Contribution

This dissertation builds up on the previously described state of venom research by closing gaps in pseudoscorpion and scorpion venom research. For the presented research, two species were selected, the house pseudoscorpion *C. cancroides* and the scorpion *E. italicus*.

The house pseudoscorpion was selected mainly for two reasons. First, it is relatively large compared to other pseudoscorpions and second it can be collected in quantities that allow to extract sufficient venom amounts. The house pseudoscorpion is mainly known for its potential use as pest control against the *Varoa* mite (van Toor et al., 2015). It was introduced worldwide due to its formerly natural occurrence in beehives (Harvey, 2014). In temperate regions,

however it is found only in association with human dwellings. For obtaining this species in high numbers, an effective method was proposed to collect it from locations like haylofts (Schiffer, 2017). For this purpose, grooved boards are laid out under which the animals accumulate after a short period of time.

To acquire a more profound insight into the extant of sex-specific differences present in scorpion venom, we selected an *Euscorpius* species for a comprehensive analysis of the venom composition of both sexes. *Euscorpius* is a suitable genus in this regard, as a distinctive sexual dimorphism, especially of the telson morphology is developed by matured specimens which is accompanied by differences in gland architecture (Sentenská et al., 2017). In addition, these scorpions are known to perform an extensive sexual sting during the promenade a deux, a ritualized mating dance (Braunwalder, 2005). It is noteworthy that *C. cancroides* belongs to the only pseudoscorpion superfamily which retained the promenade a deux (Ontano et al., 2021).

The remainder of the thesis comprises a main part with four subchapters This is followed by a discussion referring to each subchapter of the main part and an overall conclusion.

The first main chapter includes a publication comprising a comprehensive analysis of pseudoscorpion venom done with transcriptomic and proteomic approaches. This enabled the identification of the first pseudoscorpion-specific neurotoxins with confirmed presence in the venom proteome of the house pseudoscorpion *Chelifer cancroides*. Another finding was the high number of novel compounds without significant similarity to previously identified venom compounds. In addition, this study provides the first activity tests of pseudoscorpion crude venom on cellular level. This revealed an inhibition of an insect potassium channel and a modulation of the inactivation process of a sodium channel from the *Varoa* mite. In the second main chapter I present a study in which we focused on investigating the activity of the previously mentioned checacins. As these peptides exhibit a similarity to AMPs from scorpion venom, we tested their activity on different microbes, insects and mammalian cells. As a result, for peptides synthetized based on the sequence of Checacin1, we observed potent antimicrobial, insecticidal and cytotoxic activities. With the third chapter, I summarize our progress on fractionating pseudoscorpion crude venom for activity tests. My motivation was to narrow down which of the venom compounds correspond to neurotoxic activities of the venom described in chapter 1. The fourth chapter comprises a publication that provides a comprehensive analysis of the venom of *E. italicus*. For this scorpion we revealed the presence of sex-specifically expressed venom compounds.

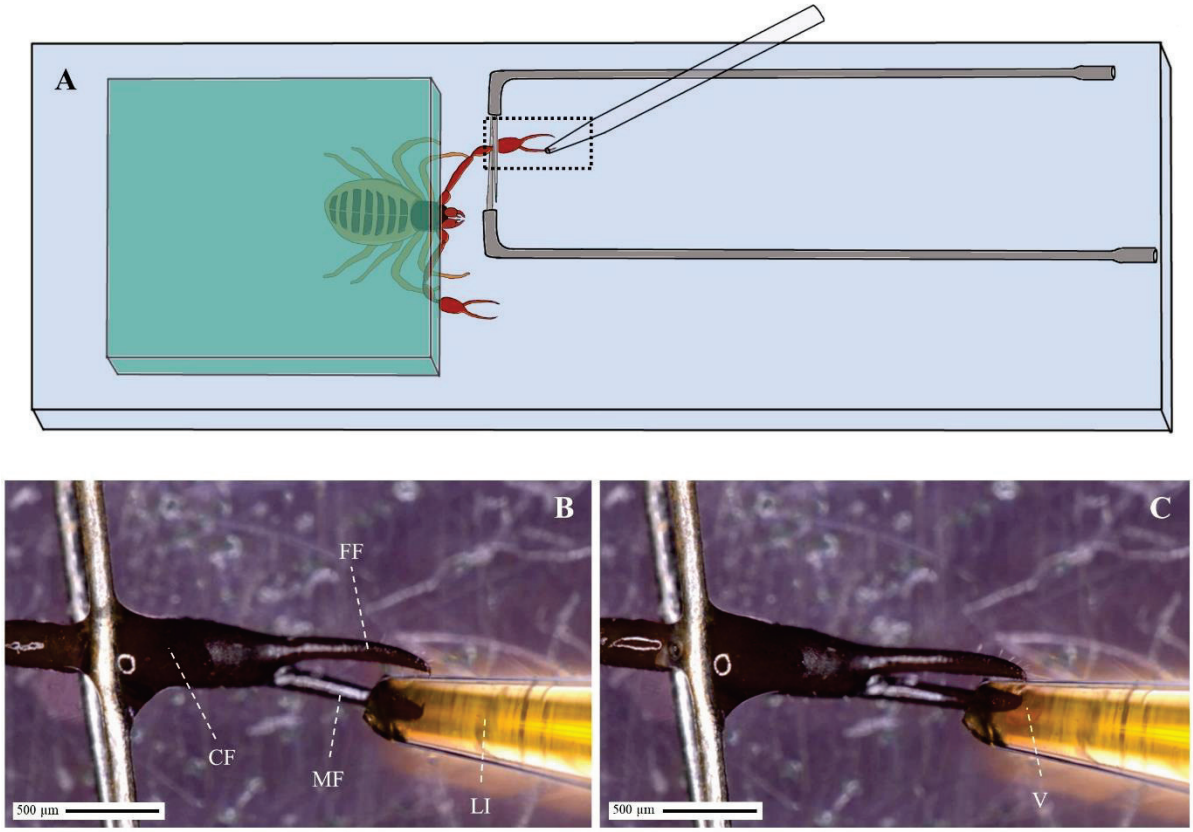


Figure 2. Venom extraction methodology for pseudoscorpions according to (Krämer et al., 2019). (**A**) Schematic drawing illustrating fixation of pseudoscorpions and application of electricity. Section highlighted in dotted square is shown in detailed photographs (**B**, **C**) documenting the insertion of one chelal finger into a liquid-filled capillary and the release of venom after application of electricity. FF: fixed finger, MF: movable finger, CF: conductive fluid, LI: liquid indicator, V: venom.

2 Main Part

2.1 A Pseudoscorpion's Promising Pinch: The venom of *Chelifer cancroides* contains a rich source of novel compounds

Jonas Krämer, Steve Peigneur, Jan Tytgat, Ronald A. Jenner, Ronald van Toor, Reinhard Predel

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Author contributions:

Jonas Krämer: Conceptualization, Investigation, Formal analysis, Writing – original draft; Steve Peigneur: Conceptualization, Investigation, Formal analysis, Writing – original draft; Jan Tytgat: Conceptualization, Writing – review & editing; Ronald Jenner: Resources, Writing – review & editing; Ronald van Toor: Resources, Writing – review & editing; Reinhard Predel: Conceptualization, Writing – original draft, Supervision.

Relevance for the dissertation:

This publication is a major contribution to pseudoscorpion venom research as it provides the first comprehensive analysis of a pseudoscorpion's venom composition that is also supported by proteomic data. To achieve this, I applied our previously developed method for extracting pseudoscorpion venom with which I obtained sufficient venom quantities for top-down and bottom-up proteomics. Together with transcriptome data, this allowed for identifying a large fraction of the venom compounds, comprising the first genuine pseudoscorpion neurotoxins and a plethora of novel substances. In addition, we could demonstrate the effect of pseudoscorpion crude venom on specific ion channels for the first time. This publication was the foundation for testing pseudoscorpion venom compounds in activity assays, of which the first one is described in chapter 2.2 of this dissertation.

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A Pseudoscorpion's Promising Pinch: The venom of *Chelifer cancroides* contains a rich source of novel compounds

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ABSTRACT

With pedipalps modified for venom injection, some pseudoscorpions possess a unique venom delivery system, which evolved independently from those of other arachnids like scorpions and spiders. Up to now, only a few studies have been focused on pseudoscorpion venom, which either identified a small fraction of venom compounds, or were based on solely transcriptomic approaches. Only one study addressed the bioactivity of pseudoscorpion venom. Here, we expand existing knowledge about pseudoscorpion venom by providing a comprehensive proteomic and transcriptomic analysis of the venom of *Chelifer cancroides*. We identified the first putative genuine toxins in the venom of *C. cancroides* and we showed that a large fraction of the venom comprises novel compounds. In addition, we tested the activity of the venom at specific ion channels for the first time. These tests demonstrate that the venom of *C. cancroides* causes inhibition of a voltage-gated insect potassium channel (Shaker IR) and modulates the inactivation process of voltage-gated sodium channels from *Varroa destructor*. For one of the smallest venomous animals ever studied, today's toolkits enabled a comprehensive venom analysis. This is demonstrated by allocating our identified venom compounds to more than half of the prominent ion signals in MALDI-TOF mass spectra of venom samples. The present study is a starting point for understanding the complex composition and activity of pseudoscorpion venom and provides a potential rich source of bioactive compounds useable for basic research and industrial application.

1. Introduction

Animal venoms are a rich source of bioactive compounds optimized by evolutionary processes for various purposes like subduing prey, defense against predators/microbes, and competition with conspecifics. As venoms contain substances that act on a wide variety of targets in different organisms, these represent an enormous reservoir for identifying lead compounds for developing novel pharmaceutics or pesticides (Herzig et al., 2020). In at least eleven cases, the determined structure of venom compounds has already been successfully used for developing commercial drugs (Bordon et al., 2020). The majority of these were developed on the basis of venom compounds from snakes, mainly because of the high venom amount and the research focus on snake venoms in the past (King, 2013). Within metazoans, venoms have evolved at least 100 times (Schendel et al., 2019) and solely for spiders more than 20 million venom compounds are estimated (King and Hardy, 2013). In the light of these numbers, the number of drugs developed to date based on venom compounds does not seem particularly high. However, only a small fraction of substances makes it to the final step of drug development, mainly due to lack of efficacy and side effects discovered in clinical trials during the process (Harvey, 2014). On top of that, venom research was biased towards harmful and relatively large-bodied (due to methodological constraints) taxa. Today, -omics approaches and increasing instrument sensitivity allow identifying venom compounds starting from very little amounts of substance, and modern synthesizers or recombinant manufacturing procedures allow sufficient quantities to be obtained for pharmaceutical or biotechnological approaches (Boldrini-França et al., 2017). Venom sampling across a wider phylogenetic range is very likely to increase the probability of identifying novel compounds with activities not observed before

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(Lüddecke et al., 2019). Even in well-studied venomous groups like spiders, uncommon venom compositions with novel venom compounds can still be found as demonstrated by recent findings on the venom composition of the wasp spider (Lüddecke et al., 2020). In addition, several studies on previously unstudied venomous animals (Drukewitz et al., 2018; von Reumont et al., 2020; 2014a; 2014b; Walker et al., 2018) also identified entirely novel bioactive compounds. This emphasizes the necessity to include a wide range of venomous animals into venom research in order to gain a more profound understanding of venom evolution and identify novel venom compounds with previously unknown targets or modes of action.

A very promising group for venom research are pseudoscorpions. Comprising more than 3600 species, pseudoscorpions are more diverse than the 'true' scorpions and offer the promise of a large library of bioactive compounds. Within these small terrestrial arachnids, a unique venom delivery system has evolved independently from those of scorpions or spiders, as pseudoscorpions of the suborder Iocheirata inject venom with the pincers of their pedipalps (Chamberlin, 1924). With this venom delivery system, pseudoscorpions (adults and nymphs) are capable of subduing prey even exceeding their own body size (Fig. 1). Depending on the subgroup of Iocheirata, venom glands can be present in both fingers or be reduced in either the fixed or the movable finger of the chelal hand (Harvey, 1992). The external parts of the venom delivery system are already well described, and comprise the venom tooth with lateral pore and the *lamina defensor*, a seta closely associated with the venom tooth (Chamberlin, 1924; Krämer et al., 2019). The internal parts consist of a narrow venom canal extending proximally to form one or more tubes (depending on the species), which are presumably surrounded by glandular tissue. Studies on chemical aspects of pseudoscorpion venom are still rare. A single study examined the activity of pseudoscorpion venom by testing the effect of crude venom from *Paratemnoides nidificator* on the binding of the neurotransmitter L-Glutamate to its receptor in rat brains (dos Santos and Coutinho-Netto, 2006). Two studies have investigated the potential venom compositions of the pseudoscorpion species *Synsphyronus apimelus* (Garypidae) and *Wyochernes asiaticus* (Chernetidae) by means of transcriptomic approaches (Lebenzon et al., 2021; Santibáñez-López et al., 2018). However, the study on *W. asiaticus* was based solely on a whole-body transcriptome. A comprehensive analysis of pseudoscorpion venom also comprising proteomics was hampered in the past by the small size of these animals, which mostly do not exceed a body length of 5 mm. In a previous study, we addressed this issue by developing a venom extraction procedure for pseudoscorpions followed by a combined transcriptomic and proteomic analysis of the venom of *Chelifer cancroides* (Cheliferidae) (Krämer et al., 2019). This enabled the identification of checacins, the first genuine venom compounds of pseudoscorpions that are potential antimicrobial peptides. However, for that study proteomic data was based solely on a top-down approach, with the identification of venom compounds limited to substances below 3 kDa. Therefore, the first aim of our current study was to identify the full set of major venom compounds from *C. cancroides* using a combined proteo-transcriptomic approach. Another objective was to compare the venom composition of *C. cancroides* and *S. apimelus*, which represent different pseudoscorpion families. Finally, a further goal was to provide the first activity tests at the cellular level with crude venom.

The oldest known fossils of pseudoscorpions date from 360 million years ago (Harms and Dunlop, 2017). Due to their early divergence from the sister group Scorpiones (Ontano et al., 2021) and their unique venom delivery system, we expect a fairly high number of novel venom compounds in *C. cancroides*.

2. Material and methods

2.1. Collection and rearing of pseudoscorpions

Specimens of *C. cancroides* used for proteomics were collected in North Rhine Westphalia, Germany. The methodology for collection and rearing is described in Krämer et al. (2019). For transcriptome analyses, 31 adult specimens were collected from honeybee hives at Lincoln, Canterbury, New Zealand. These animals were collected from refuges in the hives, in which they lived for two months with access to *Varroa* mites (*Varroa destructor*), psocids, wax moth larvae and other small arthropods. After collection, the specimens were transferred into a micro-tube each and kept at 4 °C.

2.2. Venom collection

Venom was collected as described in Krämer et al. (2019). To increase the yield, venom was extracted from both fingers of one chelal hand during one extraction procedure. Each venom sample was extracted into 1 µl of either Milli-Q water or ND96 buffer containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES (pH = 7.4).

2.3. Quadrupole Orbitrap mass spectrometry with nanoflow HPLC

Four Quadrupole Orbitrap mass spectrometry (MS) experiments were performed, three of these were bottom-up analyses with digested samples, the fourth experiment was performed without digestion step, but with reduction/alkylation of the sample. For the three bottom-up experiments, we used venom extracted from 24, 44 and 64 specimens, respectively. For the top-down experiment, venom was extracted from 12 specimens and mixed with an equal volume of urea buffer (8 M urea/50 mM triethylammonium bicarbonate buffer (TEAB)) for denaturation prior to reduction/alkylation. For desalting and removal of urea, poly (styrene divinylbenzene) reverse phase (SDB-RP)-StageTip purification

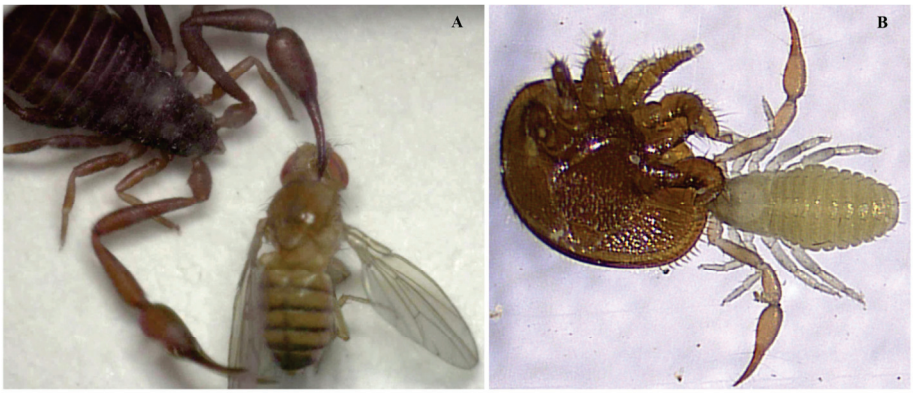

Fig. 1. Images of Chelifer cancroides subduing its prey. **A**) Adult specimen paralyzing a fruit fly (*Drosophila melanogaster*). **B**) First instar eating a captured Varroa destructor (photo: Sam Read).

was performed before Orbitrap MS analyses according to the StageTip purification protocol from the CECAD Proteomics Facility, University of Cologne (http://proteomics.cecad-labs.uni-koeln.de/Protocols.955.0.html). As the protein quantity of the venom samples for bottom-up analyses was too low to be measured, the single-pot, solid-phase-enhanced sample preparation (SP3) (Hughes et al., 2019) was utilized for digestion of venom samples, which is especially useful for low concentrated samples. For the SP3 procedure, sodium dodecyl sulfate was added to the venom samples with a final concentration of 5% and reduction/alkylation were performed according to the SP3 protocol (http://proteomics.cecad-labs.uni-koeln.de/Protocols.955.0.html). The SP3 procedure with integrated trypsin/LysC digestion was performed with 0.5 μg trypsin and 0.5 μg LysC and a beads/protein ratio of 10:1. Afterwards, the venom compounds/tryptic peptides were separated on an EASY nanoLC 1000 UPLC system (Thermo Fisher Scientific, Bremen, Germany). For this purpose, inhouse packed RPC18-columns with a length of 50 cm were used (fused silica tube with ID 50 μm ± 3 μm, OD 150 μm; Reprosil 1.9 μm, pore diameter 60 A°; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The UPLC separation was performed with a binary buffer system (A: 0.1% formic acid (FA), B: 80% acetonitrile, 0.1% FA): linear gradient from 2 to 62% in 110 min, 62–75% in 30 min, and final washing from 75 to 95% in 6 min (flow rate 250 nl/min). Re-equilibration was performed with 4% B for 4 min. The UPLC was coupled to a Q-Exactive Plus (Thermo Fisher Scientific) mass spectrometer. HCD fragmentations were performed for the 10 most abundant ion signals from each survey scan in a mass range of m/z 300–3000. The resolution for full MS1 acquisition was set to 70,000 with automatic gain control target (AGC target) at 3e6 and a maximum injection time of 80 ms. In order to obtain the HCD spectra, the run was performed at a resolution of 35,000, AGC target at 3e6, a maximum injection time of 240 ms, and 28 eV normalized collision energy; dynamic exclusion was set to 25 s.

2.4. MALDI-TOF MS

For MALDI-TOF MS analysis of reduced/alkylated venom samples, 10 μl diluted venom extracted from 10 specimens was used. One μl of this venom sample was mixed with an equal amount of ethanol/water/trifluoroacetic acid (TFA; 35/64.95/0.05) for MALDI-TOF MS analysis without prior reduction/alkylation. The remaining 9 μl were mixed with 9 μl urea buffer to ensure denaturation of the venom sample. Afterwards, the venom sample was reduced, alkylated and the urea was removed utilizing SDB-RP StageTips. For MALDI-TOF MS analysis, 0.3 μl of venom samples were directly spotted onto the sample plate for MALDI-TOF MS and mixed with the same volume of 10 mg/ml 2.5-dihydroxybenzoic acid (Sigma Aldrich, Steinheim, Germany) matrix, dissolved in 50% acetonitrile/0.05 % TFA. For an optimal crystallization of the matrix, samples were blow-dried with a hairdryer. An ultrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) was used in reflectron positive mode with overlapping mass ranges of m/z 800–4500 and m/z 3000−10,000. For an optimal signal-to-noise ratio, laser intensity and the number of laser shots were adjusted for each sample. Laser frequency was set to 666 Hz. For external calibration, a mixture containing proctolin ($[M+H]^+$, 649.3), Drm-sNPF-2^{12-19}, ($[M+H]^+$, 974.5), Pea-FMRFa-12 ($[M+H]^+$, 1009.5), Lom-PVK ($[M+H]^+$, 1104.6), Mas-allatotropin ($[M+H]^+$, 1486.7), Drm-IPNa ($[M+H]^+$, 1653.9), Pea-SKN ($[M+H]^+$, 2010.9), and glucagon ($[M+H]^+$, 3481.6) was used for the mass range of m/z 800–4500 and a mixture of bovine insulin ($[M+H]^+$, 5731.5), glucagon and ubiquitin ($[M+H]^+$, 8560.6) was used for the mass range of m/z 3000−10,000. Ion signals were identified by using the peak detection algorithm SNAP from the flexAnalysis 3.4 software package. In addition, each spectrum was manually checked to ensure that the monoisotopic peaks were correctly identified. MSMS experiments were conducted using Bruker LIFT™ technology without CID. Peptide sequences were identified by manual analysis of fragment ions and subsequent comparison of predicted (http://prospector.ucsf.edu) and experimentally obtained fragment patterns.

2.5. RNA extraction, transcriptome sequencing and de novo assembly of nucleotide sequences

Two transcriptomes were generated for the pedipalps, both based on the same 31 individuals of *C. cancroides*. One transcriptome is based on the chelal hands containing the venom glands, while the other (negative control without venom glands) is based on the two proximal segments (patella and femur) of the remaining pedipalps. The specimens were anesthetized by freezing prior to dissection either by keeping them at −18 °C for 24 h, at −80 °C for 5 min, or by snap freezing in liquid nitrogen. The pedipalps were severed at the trochanter and placed into a micro-tube containing 1 ml fresh RNA*later*™ (Qiagen, Hilden, Germany). Total RNA was extracted using the standard TRIzol protocol (ThermoFisher). Further sample processing and sequencing were performed by the sequencing facility of the Core Research Laboratories at the Natural History Museum in London. RNA was quantified using a Qubit RNA HS Assay Kit (ThermoFisher), and quality was checked with an Agilent TapeStation with RNA ScreenTape. The sequencing library was prepared with the Illumina MiSeq V3 kit following the manufacturer's protocol. Paired end sequencing (2 × 250bp) was performed on an Illumina MiSeq machine. Raw sequences were demultiplexed and adapters were removed using the MiSeq Reporter Software v.2.6 (Illumina). De novo assembly of RNA sequence data was performed with Trinity v2.8.5 (Grabherr et al., 2011) based on default settings. This assembly was used to search for peptide sequences obtained by Quadrupole Orbitrap and MALDI-TOF MSMS experiments. To assess the completeness of transcriptomic data, BUSCO 3 (Waterhouse et al., 2018) was used. The transcriptome data of the chelal hands has been submitted to NCBI (Bioproject: PRJNA752025).

2.6. Identification of venom compounds

Precursors of potential venom compounds were identified by matching the fragment spectra of Quadrupole Orbitrap MS analyses against the chelal hand transcriptome of *C. cancroides*, utilizing the software PEAKS 10 (PEAKS Studio 10; BSI, Toronto, Canada). PEAKS was run with a parent error mass tolerance of 10 ppm and 0.05 Da for fragment ions. As posttranslational modifications (PTMs), carboxymethyl was set as fixed modification and acetylation (N-terminus, Lys), amidation, carbamidomethylation, carboxylation (Glu), half of a disulfide bridge, oxidation (at Met, His, Trp) and pyroglutamate from Glu were accepted as variable modifications in the analysis. For enzymatically digested samples (bottom-up analyses), enzyme mode was set to 'Trypsin'. For the sample without digestion (top-down analyses) 'None' was selected as enzyme mode. To evaluate the significance of the hits, identified precursor sequences were each examined for the presence of a signal peptide with SignalP 5.0 (Almagro Armenteros et al., 2019) and for the presence of a stop codon. In a second step, we searched for the presence of the respective precursors in the negative control (transcriptome of the proximal pedipalp segments without venom glands). For all matches, the expression level in the transcriptomes was assessed with Kallisto (Bray et al., 2016). Another criterion for defining venom compounds was the presence of corresponding ion signals in MALDI-TOF mass spectra of venom samples. For this purpose, theoretical masses calculated for each of the potential bioactive venom peptides were searched against a list of MALDI-TOF ion signals, considering potential PTMs and cleavage sites (e.g., dibasic including quadruplet-cleavage sites (Kozlov et al., 2005), and cleavage at the 'LEAP'-motif described for *C. cancroides* (Krämer et al., 2019)). A classification based on similarity to compounds from the online database UniProt (The UniProt Consortium, 2021) was made. For this purpose, a local BLAST search of the amino acid precursor sequences was performed against the following UniProt databases. The Metazoa database

was searched with the term 'taxonomy:"Metazoa [33,208]' and the Tox-Prot database with the term 'taxonomy:"Metazoa [33,208]" (keyword:toxin OR annotation:(type:"tissue specificity" venom)). In the case of the Metazoa-database, an E-value of $1e^{-5}$ was used. For the search against the Tox-Prot database, an E-value of 10 was used. The matches were then classified based on the description of the BLAST hits in both databases, in case of the Tox-Prot-database only if the E-value was lower than 0.055. Then, the matches were filtered with respect to the quality of MS data, the coverage between transcriptomic and proteomic data (false discovery rate (−10lgP) > 30, coverage >7%) and the presence of a signal peptide. Finally, all identified venom precursors were analyzed with InterProScan (Blum et al., 2021) to perform a functional annotation.

2.7. Comparison of the venom compositions of C. cancroides and S. apimelus

To find orthologous precursors of venom compounds for *C. cancroides* and *S. apimelus,* several BLAST searches were performed. First, the precursors of venom compounds identified for *C. cancroides* were searched against the chelal hand transcriptome of *S. apimelus* with an E-value of 10^{-5}. The second step was to search the proposed precursors of venom compounds described for *S. apimelus* against the chelal hand transcriptome of *C. cancroides*. Finally, a BLAST search was performed to compare the precursor sequences of identified (*C. cancroides*) and proposed (*S. apimelus*) venom compounds with each other. To evaluate the significance of the BLAST hits, the bitscore was used, which is less dependent on database size. BLAST hits with a bitscore >40 were considered significant (Pearson, 2013).

2.8. Electrophysiological characterization of the crude venom

We followed the protocols described in detail previously (Camargos et al., 2011; Peigneur et al., 2021) For the expression of voltage-gated sodium (Nav) channels (VdNav1), the auxiliary subunits TipE and the Shaker IR in *Xenopus laevis* oocytes, the linearized plasmids were transcribed using the T7 mMESSAGE-mMACHINE transcription kit (Ambion). In total, 50 nL of cRNA (1 ng/nL) was injected into oocytes, which were incubated in ND96 solution, supplemented with 50 mg/L gentamycin sulfate. Recordings were performed using a Geneclamp 500 amplifier (Molecular Devices) controlled by a pClamp data acquisition system (Axon Instruments); bath solution was ND96. Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.7 and 1.5 MΩ. Elicited currents were sampled at 1 kHz and filtered at 0.5 kHz (for potassium currents) or sampled at 20 kHz and filtered at 2 kHz (for sodium currents) using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4 protocol. Currents were evoked by a 100 ms (Nav) or 500 ms (Kv) depolarization to the voltage corresponding to the maximal activation of the channels in control conditions from a holding potential of −90 mV. In general, current-voltage relationships were determined by 50-ms step depolarizations between −90 and 70 mV, using 5 mV increments. Toxin-induced effects on the steady-state inactivation were investigated by using a standard two-step protocol. In this protocol, 100 ms conditioning 5 mV step prepulses ranging from −90 to 70 mV were followed by a 50 ms test pulse to 0 mV. For current–voltage relationship studies of Kv channels, currents were evoked by 10 mV depolarization steps from −90 mV to 70 mV for 250 s from a holding potential of −90 mV. All data were obtained in at least six independent experiments (n ≥ 6). To test the effect of crude pseudoscorpion venom on different ion channels, 2 μl venom with concentrations of 2 μg/μl or 4 μg/μl were applied to measuring chambers containing the oocytes in 80 μl ND96 buffer. For determining the concentration of the *C. cancroides* venom samples a ND1000 nanodrop was used. The concentration of the venom samples was adjusted by dilution with ND96-buffer.

3. Results

3.1. Combined transcriptomic and proteomic analysis

Next-generation sequencing of samples from the New Zealand population of *C. cancroides*, after adapter removal, yielded 16,826,640 paired-end reads for the chelal hand (pedipalp) sample, and 8,983,558 paired-end reads for the proximal pedipalp segments sample (negative control). Sequence assemblies resulted in 80,317 contigs for the chelal hands transcriptome and 69,810 contigs for the proximal pedipalp segments transcriptome. Regarding completeness of transcriptome data, the chelal hand transcriptome contains 45.3% complete and 30.2% fragmented BUSCOs, whereas the proximal pedipalp segments transcriptome comprises 68.1% complete and 20.4% fragmented BUSCOs.

Matching of the proteomic data from four Orbitrap MS experiments (three samples with trypsin digestion, one sample without digestion) against the chelal hands transcriptome initially yielded 1270 hits. After quality filtering (coverage and P-value), removal of precursors without a signal peptide and redundant matches, 124 precursors contributing to the venom composition of *C. cancroides* were identified (Supplementary material 1). These precursors were first separated into precursors with cysteine-containing peptides and the remaining precursors. Both groups were further classified, based on sequence similarities to annotated sequences from online databases, into precursors of potential peptide toxins, antimicrobial peptides, enzymes, 'other' or, in case no matches were found within the databases, as 'novel' (Fig. 2). Where the InterProScan analysis resulted in functional annotations, this information is added in Supplementary material 1. Precursors of identified venom compounds with presumed orthology to annotated toxin sequences of other taxa were named according to rational nomenclature guidelines (King et al., 2008). For the precursors that were also detected in the negative control, a comparison of the expression levels of the corresponding trancripts of chelal hands and remaining pedipalp segments (negative control) is shown in Fig. 3.

Information on 11 precursors, whose products have been detected in the venom samples of *C. cancroides* and show sequence similarity to known arthropod toxins, is summarized in Table 1. All of these are cysteine-rich precursors with 3–5 disulfide bonds and the biochemically confirmed or predicted mature peptide toxins are in the mass range below 10 kDa. Precursor genes of CHTX-Cc1a and 1b, CHTX-Cc2a and 2b as well as those of CHTX-Cc8a and 8b likely represent paralogs, respectively. CHTX-Cc1a and 1b exhibit the best BLAST-matches to potential arthropod toxins, the latter classified as potential potassium channel toxins. An alignment of these sequences is shown in

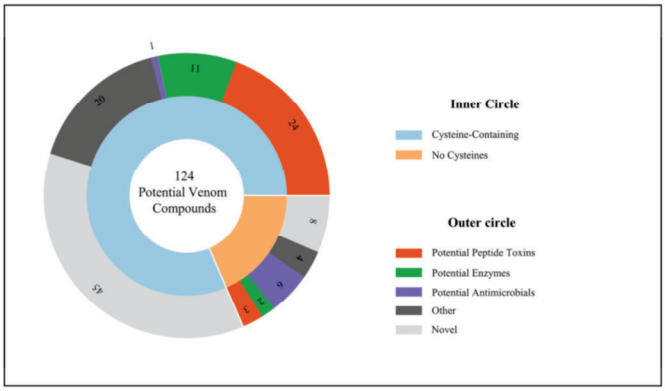

Fig. 2. Pie Chart showing a classification of the venom compounds identified for *C. cancroides* by a combined transcriptomic and proteomic approach. The inner circle shows a differentiation of the venom compounds based on cysteine content. In the outer circle, venom compounds are classified based on a presumed orthology to sequences in UniProt (The UniProt Consortium, 2021) entries.

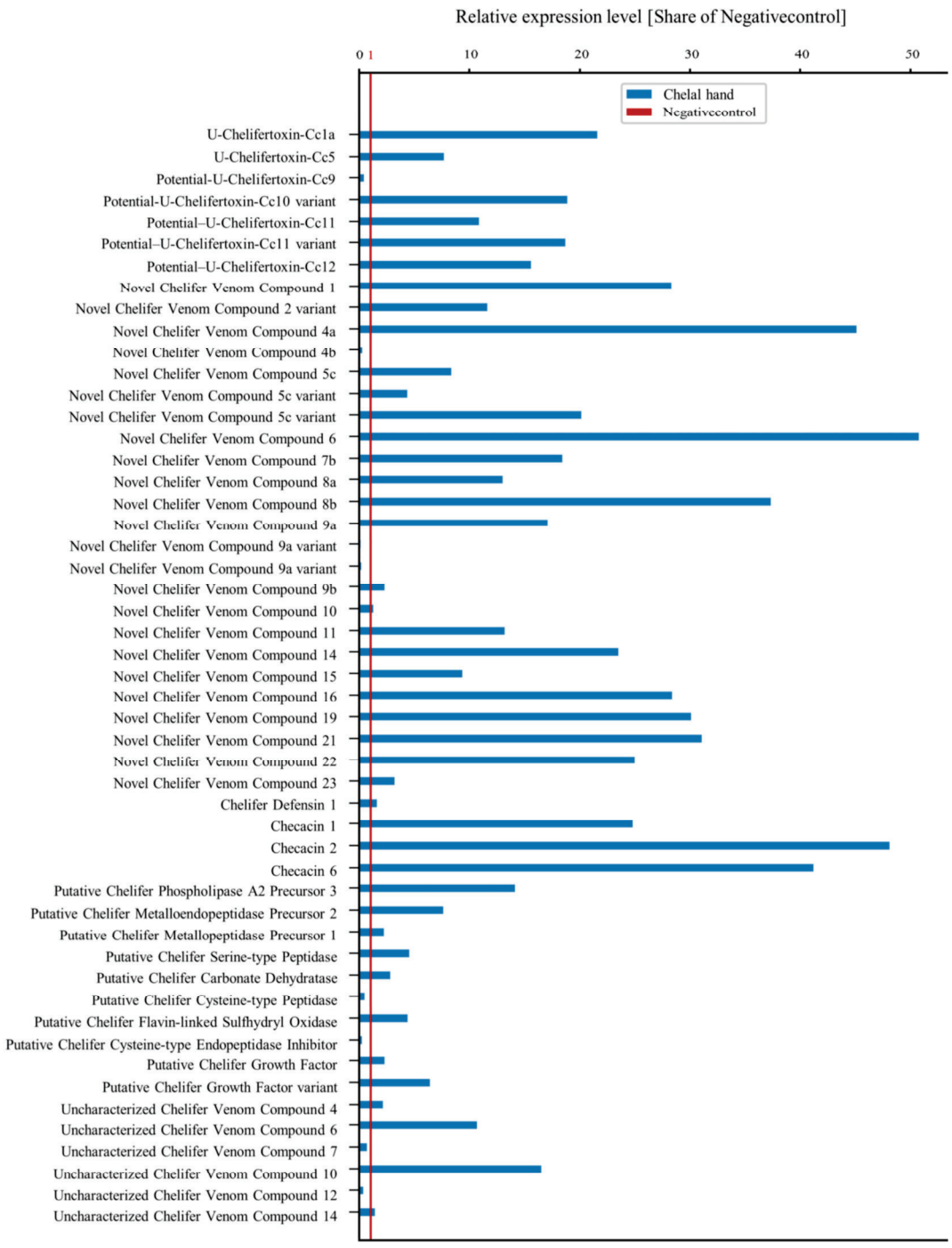

Fig. 3. Bar chart showing relative expression levels of venom precursors identified in a chelal hand transcriptome of *Chelifer cancroides*. Only the values of those precursors also present in the negative control are shown. Values are presented as share of the expression levels of respective precursors from the negative control. (Transcriptome of remaining pedipalp segments). Precursors were identified by a combined proteo-transcriptomic approach.

Supplementary material 2. All of the listed precursors either show significantly higher expression levels in chelal hands (i.e., in venom glands) compared to the remaining pedipalp segments or are absent in the negative control. Additional potential toxin precursors can be found in Supplementary material 1.

Fifty-two precursors with biochemical confirmation of corresponding peptides in venom samples, but without significant sequence similarity to annotated sequences in the UniProt databases are classified as Novel *Chelifer* Venom Compounds (NCVCs). Of these, a selection of 19 is shown in Table 2. Among these precursors are products of several paralogous genes (NCVS-4a/b/c, 7a/b, 8a/b, 9a/b), and except for NCVC-4b, all precursors showed increased expression levels in the *Chelifer* hand transcriptome. Three NCVCs (NCVC-4a, 5a, 8b) showed exceptionally high expression levels. Confirmed or predicted mature peptides are mostly cysteine-rich, only products of the precursors of NCVC-11 and 12 represent linear peptides, whereas a single disulfide bridge is present in NCVC-2 (Table 2). Both the linear peptides and the mature peptides of NCVC-1, 2 are C-terminally amidated.

Table 3 lists precursors of 8 potentially antimicrobial peptides. In addition to the three checacin precursors already described (Krämer et al., 2019), four new checacin precursors could be identified. The corresponding *checacin* genes always show much higher expression

Table 1
List of potential *C. cancroides* toxin precursors with sequence similarity to annotated toxins of arthropods. Precursors were identified by a combined proteomic and transcriptomic approach. Grey, signal peptide; blue, potential bioactive peptide; green, amidation signal; yellow, cysteine (half of the disulfide bond); red, potential cleavage site. Black underlined, confirmed by MSMS; red underlined, confirmed only by MSMS digested samples; dashed line, mass match in MALDI-TOF MS. The column Orbitrap MS indicates which of the four proteomic analyses provided confirmation of the precursor sequence: D, Bottom-up analysis with the digestion of samples with low (l, venom from 24 specimens), medium (m, venom from 44 specimens) or high (h, venom from 64 specimens) venom amount; ND, Top-down analysis without digestion. MALDI-TOF MS: Confirmation of precursor products in mass spectra of venom samples either by mass match (+) or MSMS (+*). The number of cystines (C–C) was confirmed by mass shifts of the respective ion signals in MALDI-TOF mass spectra after reduction/alkylation. Assumed PTMs include amidation (A), disulfide bridges (C–C) and modification of N-terminal glutamine to pyroglutamic acid (pQ).

Name	BLAST hit	Expression level [tpm]	Expression level negative control [tpm]	PTM	Predicted Mass [M+ H+]	Orbitrap MS	Confirmed disulfide-bridges (MALDI)	MALDI-TOF MS
U-Chelifertoxin-Cc1a*	Potassium channel toxin alpha-KTx Tx308 (*Buthus occitanus israelis*), 30%, Acc: B8XH30 MKCYLFVILLVVCAIGMDSVQGQKWACENGGAECDKMCRSIGKMGACSPGGPGVLLCRCI	2659	123	pQ,C-C	3905.75	D (h, m), ND	3	+*
U-Chelifertoxin-Cc1b*	Potassium channel toxin alpha-KTx 1.16 (*Mesobuthus eupeus*), 28%, Acc: C0HJQ8 MKCYLFIILLVVCAIGMDSVQGQKWRCDNGGEECYKMCRRIGKVGECSPGGPGVPLCRCI	716	-	pQ,C-C	4161.91	D (m), ND	3	+*
U-Chelifertoxin-Cc2a*	Toxin CSTX-17 (*Cupiennius salei*), 45%, Acc: B3EWT2 MSRLILFLCFSVLVMVSLAMAEDTPGEESEHISKRACVPDYGKCKQNGIKKNNCCNKVSCYCNLTFTNCYCKPPLFGK	2133	-	A,C-C	4616.02	D (l, m), ND	4	+
U-Chelifertoxin-Cc2b*	Toxin CSTX-17 (*Cupiennius salei*), 41%, Acc: B3EWT2 MSRLILFLCFSALVMVSLAMAEDTPGEEPEQISKRACVPDYGKCKWTSKGKKNNCCNDVSCYCNLSLTDCYCNPPIFG	54	-	A,C-C	4689.02	D (l, m)	4	+
U-Chelifertoxin-Cc3	Putative neurotoxin LTDF S-06 (*Dolomedes fimbriatus*), 34%, Acc: A0A0K1D8H2 MSKLIFALLFSGLVLASLVMAEEEEEETLEISKRSCIKEYGTCQWKGLGAKSQCCDNRNCVCNIALNNCKCKPSPSQLLAKVFG	679	-	A,C-C	5357.46	D (h, l, m), ND	4	+
U-Chelifertoxin-Cc4*	U28-Sparatoxin-Hju1n (*Heteropoda jugulans*), 41%, Acc: A0A4Q8KD95 MKVAFFVFLVVLSAAALAKAIEDGQEENMEISKRDTCLAVGDNCQGNTGKCCDGAKCVCRKDFILGFSGSHIITRCNCKK	13	-	C-C	4874.14	D (h, l, m), ND	4	-
U-Chelifertoxin-Cc5	Putative neurotoxin-II (*Lychasmucronatus*), 30%, Acc: D9U2B4 MAAVEMGRASWILAVLVLTAVFWTCEADALCDKGAETCNLSCYRKSYQLVGYCDRNRDGKTHCRCMKKSDASLIGR	84	10	A,C-C	5311.4	D (h, l, m)	3	-
U-Chelifertoxin-Cc6	U20-Liphistoxin-Lsp1a (*Liphistius* sp.), 43%, Acc: A0A4Q8K5N5 MWRCWWTVLLLWLVAEARYATWADFEAAHGRRPPQARALAACARAGPARDLCERCAKVTRSEVVFPFCCDDTRDVRAWCERFLDFGLQNL	23	-	C-C	8294.96	D (h, l, m), ND	3	-
U-Chelifertoxin-Cc7	U68-Liphistoxin-Lsp1a (*Liphistius* sp.), 30%, Acc: A0A4Q8K539 LTIVLALVILAVVAEAERKCFIHRRDCSKDECCAGVGIVGVCKKLAQAGEKCRIIDSFDCPCAKGLECLPFGIIRGICFKKKDETPAQDLAE	7	-	C-C	8351.00	D (h, m)	5	-
U-Chelifertoxin-Cc8a	Kappa-Theraphotoxin-Ct1a_1 (*Coremiocnemis tropix*), 37%, Acc: A0A482Z9G0 MYKFSVIFLLAAAVILVAAEYDDEDGRRYLATEKRSCSISKCNIQECCPGYVCRKGARHSSGSVCVNSG	149	-	C-C, A	3455.54	D (l, h), ND	3	-
U-Chelifertoxin-Cc8b*	Kappa-Theraphotoxin-Ct1a_1 (*Coremiocnemis tropix*), 37%, Acc: A0A482Z9G0 MYKFSVIFLLAAAVILVAAEYDDEDGRRYLATEKRSCSISKCNIQECCPGYVCRKGALRSSGSVCVDSGLTIL	219	-	C-C	3930.85	D(l)	3	-

levels in the chelal hands transcriptome than in the proximal pedipalp segments, although the expression level itself is quite different for the various *checacin* genes. All checacins are c-terminally amidated linear peptides. A cysteine-rich putative antimicrobial peptide without sequence similarity to checacins is named *Chelifer* defensin (Table 3). The corresponding precursor shows similarity to Tddefensin, which has been identified in the transcriptome of the scorpion *Tityus discrepans* (D'Suze et al., 2009). Different from the *checacin* precursor genes, the transcription level of the precursor gene for *Chelifer defensin* is not much higher in the chelal hand transcriptome compared to the transcriptome of the proximal pedipalp segments. Thirteen precursors biochemically identified in the venom of *C. cancroides* show similarity to enzymes from the UniProt database (Supplementary material 1). Eleven of these could be functionally annotated based on InterProScan results and were named accordingly. Most of these precursors are either absent from the proximal pedipalp segments or show higher expression levels in the

Table 2
List of selected precursors of novel venom compounds identified in the venom of C. cancroides. Listed precursors either show a corresponding MALDI-signal or were considered most evident after manual inspection of PEAKs-results (matches between proteomic and transcriptomic data). Additional precursors of novel venom compounds can be found in Supplementary 1. For further explanations see Table 1.

Name	Expression level [tpm]	Expression level negativecontrol [tpm]	PTM	Predicted Mass [M+ H+]	OrbitrapMS	Confirmed disulfide-bridges (MALDI)	MALDI-TOF MS
Novel*Chelifer* Venom Compound 1	680	24	A,C-C	5124.19	D (h, l, m), ND	3	+
	MKTFCLALLLVGVLAGVMETEAVVAGCPDESKCHAWCLSQFPKYQAVTTGFCVNSNRCACHVDTNEDPTGK						
Novel*Chelifer* Venom Compound 2*	2150	-	A,C-C	2672.40	D (h, m), ND	1	+*
	MKTFVVLFFGAVLLAFAAADIENEAALESEMLDLESDLAELLEAPSPIGILQCLGRKDTTWKECLNKNNKGK						
Novel*Chelifer* Venom Compound 3	376	-	C-C	2659.15	ND	2	+
	MSRLLVVLVVAAVVLTAVVSVEAETESEVMDESTVEESPECVCNPPESTCCFAKGQVYNKNKT						
Novel*Chelifer* Venom Compound 4a	26718	592	C-C	7924.93	D (h, l, m), ND	3	+
	MKYVALSLALVLCLAVLARAEDQGVQDGDVCIIDRVLGEIKCIGKGINKIYKSIFKSYQKCKEFCKQYEAQGYKCKQKGISDYKCTNK K						
Novel*Chelifer* Venom Compound 4b	8	28	C-C	7678.77	D (h, l, m), ND	3	+
	MKTWFYLAAVAAMLTLATRAEEDPPEGGKCIIDAVLDEIKCIGKAINKVYKNKFTSYQKCVKFCKDYEAKGFKCKSKGPLSDYKCTDK						
Novel*Chelifer* Venom Compound 4c	3775	-	C-C	7940.01	D (h, l, m), ND	2	+
	MNSCALFLLVVLSLCALSWAEEEKKKTVLDKVGEELKKVGQGMKDIYNNIYKSYNKCKDFCKQYESKGYTCQKKLLSVSDYKCAPKKP						
Novel*Chelifer* Venom Compound 5a*	11672	-	C-C	9409.56	D (h, l, m), ND	3	+
	MKVAVSLLCLLLAAVLAAVSCTADQHVQDEQELESPDGIVDWLKKELGDRADSIYKGTMRNPITKVYGKYQKCQEECKNKPDKRCKCQ MMRFLKKMEDKQEHKCMCISLLDHSMD						
Novel *Chelifer* Venom Compound 5b*	145	-	C-C	9392.6	D (h, l, m)	3	-
	MKVAVSLLCLLLAAVSCTADQLVQDEQELESPDVVLDWFKKEVGSRAESIYKGTMRNPVTKVYGKYQKCQEECKNKPDKRCKCQLSKF FRSMENKQEHKCMCISLLDRSMD						
Novel *Chelifer* Venom Compound 5c*	1038	124	C-C	9276.71	D (h, l, m)	3	-
	MKVAVSLLCLLLAAVSCTADQLVQDEQELESPDGVLDWLKKEIGDRAEAIYKGVLRNPVTKVYNKYLKCQDECKGKPDKRCKCQLSRF LKPYSEKQEHKCMCVDLLDKSFD						
Novel *Chelifer* Venom Compound 6	3069	60	C-C	6244.33	D (h, l, m)	3	+
	MKYLQIVCLLLALTVFASAFQQEEEELETELDELDTPGWGKLFGVIKKGARFVLKRGQKLMRNRKKCRAQCKDPAFHCKCDPISTKCKC VAN						
Novel *Chelifer*Venom Compound 7a	288	-	A, C-C	7938.98	D (h, l, m), ND	2	-
	MNMKILKILIIGLTITLNLLCSSNAADLQEDEGNTENEALPSFESYPVYDLSKGKPEKCPEGMGFYNGKCHKLHCAIPGYVLKDKKCVR KPKTLLKG						
Novel *Chelifer* Venom Compound 7b	611	33	A, C-C	8388.23	D (h, l, m)	2	-
	MNMKILKILIISLIITLNLVCSSNAAELQEEEGNTEYEALPSFAILLDMNPEHGKSVGKYEKCPEGTGRFNGECRILNCGIPGYVLKGD KCVPKRRRILKG						
Novel *Chelifer*Venom Compound 8a	297	22	C-C	9508.53	D (h, l, m)	3	-
	MRAAIVLGLLLAVALETTAASYLEAEDGGMLAWMRKELGDKSARLYGMFADPIKKVYGKYVKCQEECRGQADKRCRCQLLKALYSKENQ QAHKCMCVSMLSE						
Novel *Chelifer*Venom Compound 8b	33315	892	C-C	10581.28	D (h, l, m), ND	3	-
	MKSQLLVLCLCLAVAAAELQDLEKPDELEAGESEATYMQWITGEVGAKWAKAVYMAIKGQVVKVYQKYKTCQTTCTAPDKRCKCQLLRF LKPLSKKQEHKCMCKGMLEE						
Novel *Chelifer* Venom Compound 9a*	4420	258	C-C	10181.11	D (h, l, m)	3	-
	MNGLQWTVTALCLALFSASVQAAHVQDDQELEVGVTEAALLAWVTYEVGPKFAKAVYNALTGQIKKVYDKYLACQATCTAPDMRCNCQL LRFLYPMEKKQEKKCMCLNIKKN						
Novel *Chelifer* Venom Compound 9b	70	30	C-C	10140.9	D (h, l, m)	4	-
	MNGLQWTVTALCLALFSASVQAAHVQDDQELEVGVTEGALIAWITAEVCPKVAKAVYEALSGQITKVYDKYKACQATCTGANMKCHCQL LRFLKPMDKKQEHKCMCKNSNETSC						
Novel *Chelifer* Venom Compound 10	349	258	C-C	13085.92	D (h, l, m)	4	-
	MLLLVCALLVVAATGTSAQSCEVEKEWDIQQVFCNMKDGEKQFATCEEMMPDEAKEMIKNCNTAAQGENPSQTAFQYSCADCASLQKLK QCLSDNSMDQKIKNMNKSDQEKMAKSVVCVMSLYKKETGKDMEL						
Novel *Chelifer* Venom Compound 11	1000	75	A	3870.16	D (h, l, n); ND	-	+
	MKTLALVVCGLAVLLVASAEQEDSELGSYASDSLQSPLDEMMNQYANEDEESLSLESDLAYQWEQMELESPFWKKMKSFFKDKVIPKVQ QAYSLYNKLQHKLG						
Novel *Chelifer* Venom Compound 12	559	79	A	959.52	-	-	+
	MKNLLLCLFIFGLVLSNGVAFEDGNSVDELLESWAEESWVQEEKMPLESPGKPFQPMRG						

Table 3
List of precursors of potential antimicrobial peptides identified in the venom of *C. cancroides*. For further explanations see Table 1.

Name	BLAST hit	Expression level [tpm]	Expression level negative control [tpm]	PTM	Predicted Mass [M+ H+]	Orbitrap MS	MALDI-TOF MS
Checacin 1	Megicin-18 (*Mesobuthus gibbosus*); 47.2%; Acc: A0A059U8Y9 MKYLQIVCLVLSLAVLTSA**FPMEEQLSESELKE**LEAPFFGAIAKLAMKFLPAIYKQIQKKRKGRSLEAQ	3316	133	A	2937.76	D (h, l, n); ND	+*
Checacin 2	- MKYIQVVCLVLSMAVFTSAF**EVEDLTESELQE**LEAPFVGLLAKLAAYVIPQIVKRFQKKKGKRSLEWEDDDA	4563	94	A	2757.72	D (h, l, n); ND	+
Checacin 3	- MKYIQVVCLVLSMAVFTSA**FQVEELTESELQE**LEAPFIGIMATLASLVIPKLIEKIKQARGRRSLEEDELFF	1563	55	A	2652.63	-	+
Checacin 4	- MKYIQVVCLVLSMAVFTSA**FQVEDLTESELQE**LEAPFVGLLARLAAFVIPQIVKRFQKKNGKRSLEWEEE	341	-	A	2755.77	D (h, l, n)	+
Checacin 5	- MKYLQFVCLLLSLAVFTSA**FQVEEELSESELKE**LEAPFFGVIAKMAMKFLPAIFKQIQKKRKGRSLEDQ	2459	-	A	2967.75	D (h, l, n); ND	+
Checacin 6	- MKYLQIVCLVISLAVLASSFPLEEQLTESDLNELESLWRGTTHFVHQYKIMPFRKLVFKRRKNGRRG	146	3	A	5459.89	D (l, n); ND	-
Checacin 7	- MKYIQVVCLVLSMAVFTSA**FQVEELTESELQE**LEAPFFGAFAAIASLVIPKLIEKIKQARGRRSLEDEEFVF	717	-	A	2630.59	ND	+
Chelifer Defensin 1	Tddefensin (*Tityusdiscrepans*); 57%; Acc: P0CF77 MKLLGVVCLSALLLCLGFHMAEAISGANGCPMNEGRCEDHCMRRGRPGGHCGGSMRRSCICDSNLP	34	21	A, C-C	4570.86	D (h, l, n); ND	-

venom gland transcriptome. An exception is the putative *Chelifer* cysteine-type peptidase. Two of the precursors (*Chelifer* phospholipase A2 precursor 3 and *Chelifer* metalloendopeptidase precursor 1) show particularly high expression levels in the chelal hand transcriptome compared to the remaining enzyme precursors. For *Chelifer* metalloendopeptidase precursor 1, an alignment with sequences from the closest BLAST hits is shown in Supplementary material 2. Supplementary material 1 also includes 21 precursors classified as 'Other'. Three of these could be functionally annotated as cysteine-type endopeptidase inhibitors and growth factors based on interProScan results, and the precursors were named accordingly. The remaining precursors were classified as Uncharacterized *Chelifer* Venom Compounds. None of these precursors exhibit high expression levels in the chelal hand transcriptome.

3.2. Allocation of venom compounds to signals in MALDI-TOF MS

MALDI-TOF mass spectra allow rapid screening of venom compounds released from individual venom glands and provide sufficient information on (1) the relative abundance of venom compounds, (2) mature (main) products of the various precursors that contribute to the venom composition, (3) changes in the venom composition over time, and (4) the completeness of precursors already described, i.e., what is the percentage of ion signals in mass spectra that can be assigned to the known precursors. As shown in Fig. 4, most of the prominent ion signals in the mass spectra (m/z 900–10,000) are products of precursors with particularly high expression levels (see Table 1–3). Of the 11 precursors with assumed orthology to known arthropod toxins (Table 1), a number of ion signals were identified in MALDI-TOF mass spectra of venom samples that were mass-identical to products of precursors for U-chelifertoxin-Cc1a, Cc1b, Cc2a, Cc2b, and Cc3 (Fig. 4), and subsequent analyses of reduced/alkylated venom samples confirmed the expected number of disulfide bonds for these substances (Fig. 5). The mature U-chelifertoxins Cc-1a and Cc1b each comprise the complete precursor sequence without a signal peptide and are N-terminally blocked by pyroglutamate. The sequence of both peptides could be confirmed by MALDI-TOF MSMS fragmentations (Supplementary Material S3), in addition to the Orbitrap MSMS analyses. The precursors of U-chelifertoxin-Cc2a, 2b, and 3 each contain an internal Arg-Lys cleavage signal which is efficiently used to cleave an N-terminal precursor peptide from the mature C-terminally amidated U-Chelifertoxins.

For three of the 19 precursors representing potential novel venom precursors (including 7 paralogs; Table 2), the predicted masses of the mature peptides are above the analyzed mass range. Mass matches were found for products of eight of the remaining 13 precursors (Fig. 4). Due to the loss of long-chain peptides during Stage-Tip purification of

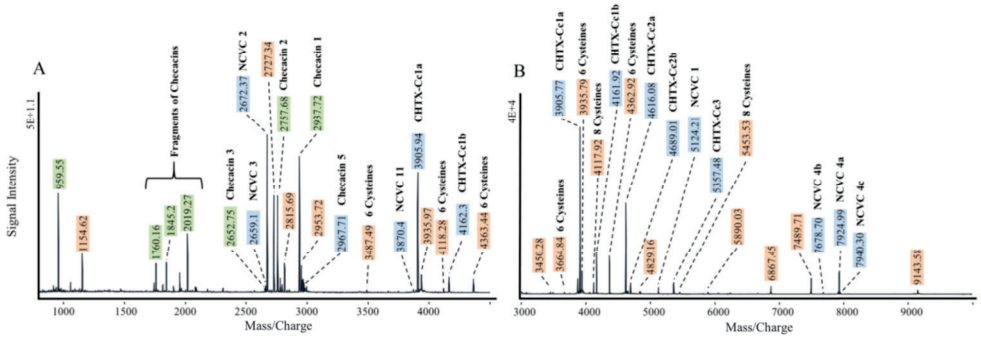

Fig. 4. MALDI-TOF mass fingerprints of venom samples of *C. cancroides* highlighting venom compounds that can be correlated with transcriptomic and Orbitrap MS data. Ion signals highlighted in green represent venom compounds identified in Krämer et al. (2019). Ion signals highlighted in blue are venom compounds identified with their precursors in the current study. Remaining signals are marked in beige. **A**) Lower mass range (m/z 800–4500). **B**) Higher mass range (m/z 3000–10,000). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

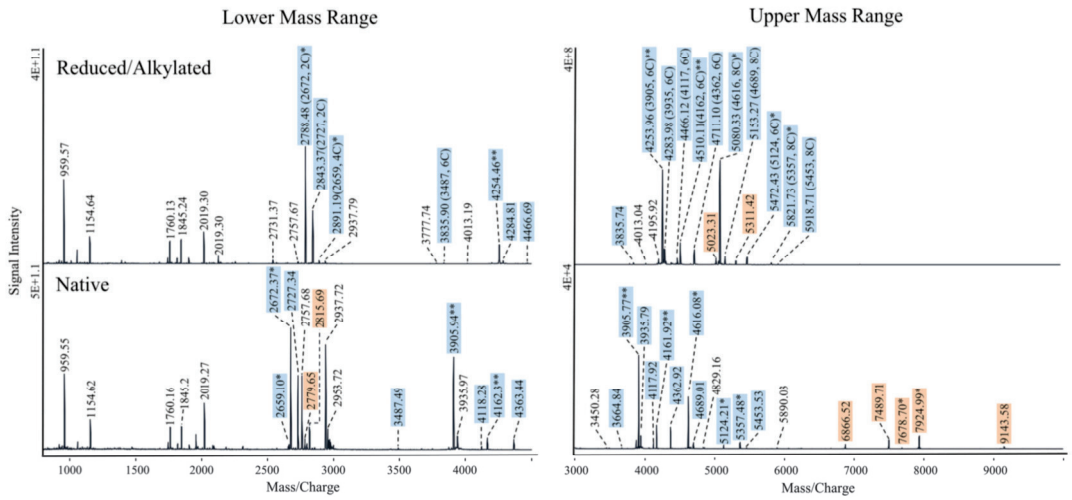

Fig. 5. Comparison of MALDI-TOF mass spectra of untreated and reduced/alkylated venom samples from *C. cancroides*. Ion signals suggesting a mass shift due to reduction/alkylation are highlighted in blue. For these ion signals, the original mass and the proposed number of cysteines is added in brackets. Ion signals without corresponding signal in untreated samples are highlighted in beige. All ion signals mass-identical to venom compounds identified by our combined transcriptomic and proteomic data are marked with '*'. In case the sequences of these peptides could be confirmed in the same samples by MALDI-TOF MSMS, they are labeled with '**'. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reduced/alkylated samples, the number of disulfide bonds could not be confirmed for most of these relatively large peptides. Confirmed disulfide bonds were, however, obtained for NCVC-1 and 2 (Fig. 5). The latter peptides are both amidated. While NCVC-1 comprises the complete precursor sequence (without signal peptide) downstream to the C-terminal Gly-Lys motif, mature NCVC-2 represents only the C-terminal sequence of the corresponding precursor (Table 2). The NCVC-2 precursor contains the LEAP cleavage motif described for *C. cancroides* checacin precursors (Krämer et al., 2019), but mature NCVC-2 is cleaved two amino acids C-terminally from that motif, i.e., C-terminally from Ser-Pro (Table 2).

The expression level of the paralogous *checacin* genes is highly different (Table 3), and this is also reflected in the ion signal intensity of the mature checacins (Fig. 4). Overall, ion signals mass-identical with checacins of six checacin precursors, always amidated at the C-terminus, were detected in the MALDI-TOF mass spectra (Fig. 4). These checacins are all N-terminally cleaved at the LEAP motif within the precursor sequence, and start with Phe as the N-terminal amino acid (Table 3). Truncated checacins cleaved predominantly at internal Lys or Arg-Lys were occasionally detected in the mass spectra, but with much lower signal intensity than those of full-length checacins. The lowest expression level was found for the *checacin* 6 gene, and the ion signals predicted for checacin 6 were not detectable at all. The precursor of checacin 6 does not contain an internal LEAP motif and the predicted mature checacin 6 therefore potentially contains a much longer N-terminus (Table 3).

All experimental data considered, the MALDI-TOF mass spectra suggest that a majority of the more enriched venom compounds of *C. cancroides* are identified for the mass range examined (Fig. 4).

3.3. Comparing the venom compositions of C. cancroides and S. apimelus

A recent study described potential venom precursors of the pseudoscorpion *S. apimelus* based on a chelal hand transcriptome and a BLAST search within this dataset using known toxins and other venom compounds from arthropods (Santibáñez-López et al., 2018). Are *C. cancroides* orthologs of these proposed venom precursor genes from *S. apimelus* responsible for the composition of the venom in *C. cancroides*? Supplementary material 4 lists BLAST results of searching the potential venom precursors described for *S. apimelus* in the chelal hand transcriptome of *C. cancroides* on the one hand, and in the venom compounds biochemically identified for *C. cancroides* on the other hand. Most of the potential *S. apimelus* venom precursors have a corresponding BLAST hit in the chelal hand transcriptome of *C. cancroides*. However, a majority of the predicted *S. apimelus* venom precursors did not show significant BLAST hits with the precursors that contribute substantially to the venom compounds biochemically identified here for *C. cancroides*. For example, many of the putative U8-agatoxin-like peptides described for *S. apimelus* yielded significant BLAST hits in the chelal hand transcriptome of *C. cancroides* (in some cases >90% sequence identity), though products of the corresponding *C. cancroides* genes were not found in our MS datasets. This suggests that these genes are not specifically expressed in the venom glands.

The results of a BLAST search in the chelal hand transcriptome of *S. apimelus* with those *C. cancroides* precursors whose products are specifically enriched in venom samples of *C. cancroides* are included in Supplementary material 1. It is noteworthy that some of the prominent *Chelifer* venom compounds with presumed orthology to arthropod toxins (e.g., CHTX-Cc1 and 2) did not yield significant matches in the *S. apimelus* transcriptome or resulted in BLAST hits with only moderate sequence similarity (high E-values). Only four of these compounds (see Table 1) exhibit significant similarity to venom precursors described for *S. apimelus* (CHTX-Cc 2a, 2b, 3, 7). In addition, most of the NCVCs identified in this study (see Table 2) did not yield significant BLAST-hits in the *S. apimelus* transcriptome, exceptions being e.g., NCVC 7a, 7b and 11. For the checacins (see Table 3), which belong to the most abundant venom compounds of *C. cancroides*, and are provisionally grouped among antimicrobial peptides due to a moderate similarity to scorpion megicin (Diego-García et al., 2014), no BLAST hits were identified in the *S. apimelus* transcriptome. The BLAST-hits with the highest similarity in both species were found for metalloprotease and phospholipase precursors (Supplementary material 4).

3.4. Electrophysiological characterization of the crude venom

In control conditions, the conductance over the membrane of the oocytes remains minimal over a large voltage range (Fig. 6A). At a concentration of 0.1 µg/µl, a venom-dependent increase in chord conductance was observed in non-injected oocytes with a reversal potential around 0 mV, which can be interpreted as evidence for a non-ion selective pore-forming activity or cytolysis as induced by the

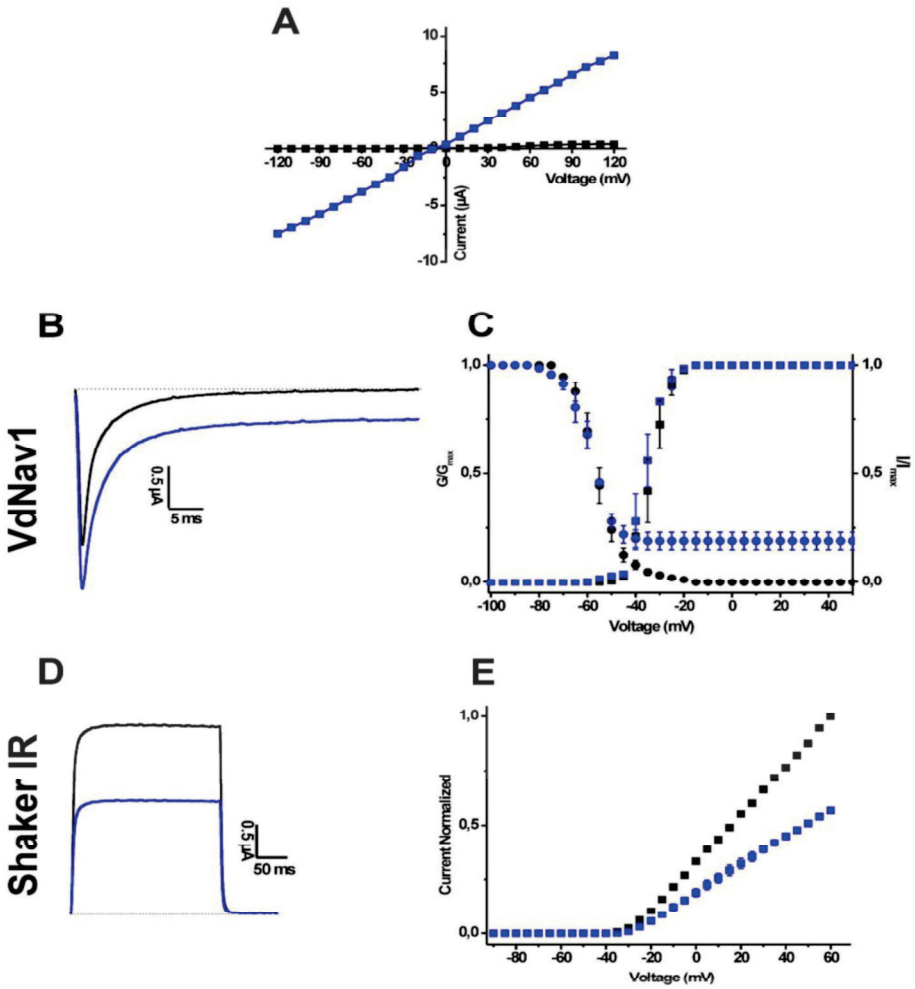

Fig. 6. Electrophysiological profiling of *Chelifer cancroides* total venom. **A**) In the absence of the venom, the conductance over the membrane of the oocytes remains minimal over a large voltage range, which is indicative for a healthy cell (control, black symbols). In the presence of higher concentrations of venom (>0.05 µg/µl) a venom-dependent increase in chord conductance was observed in non-injected oocytes with a reversal potential around 0 mV (blue symbols). **B**) Whole-cell current traces were recorded from *Xenopus laevis* oocytes expressing cloned VdNav1 in control or 0.05 µg/µl venom. The dotted line indicates the zero current level. Blue traces were recorded after the application of venom. **C**) steady-state activation (square symbols) and inactivation (circle symbols) curves in control conditions (black) and in the presence of venom (blue). **D**) representative whole-cell current through *Shaker* IR channels in control (black) and in the presence of 0.05 µg/µl venom (blue). **E**) Current-voltage dependencies of *Shaker* IR. Black symbols, control; blue symbols, after application of venom. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

C. cancroides venom (i.e. induction of 'leaky cells'). As the cytolytic damage of the oocytes at high concentrations makes it impossible to investigate potential ion channel modulation, we tested the venom at a concentration of 0.05 µg/µl on arthropod voltage-gated sodium (Nav) channels and voltage-gated potassium (Kv) channels. At this concentration, crude venom modulates the Nav channels from the mite *V. destructor* (VdNav). In the presence of 0.05 µg/µl venom an increase of the sodium peak current and a slowing down of the inactivation could be observed, resulting in sustained currents (Fig. 6B). A small but significant shift of the midpoint of activation was noted with $V_{1/2}$ values of -33.9 ± 0.1 mV and -36.1 ± 0.2 mV in control and venom conditions, respectively. The $V_{1/2}$ of inactivation shifted from -55.9 ± 0.17 mV in control to -58.6 ± 0.2 mV in the presence of the venom (Fig. 6C). In the presence of 0.05 µg/µl *C. cancroides* venom an inhibition of Kv channels from *Drosophila melanogaster* (*Shaker* IR) occurred with $42.3 \pm 2.5\%$ (Fig. 6D). At this concentration, no modulation of the activation was observed since the $V_{1/2}$ values yielded 17.9 ± 2.0 mV in control and 16.1 ± 1.6 mV after application of venom (Fig. 6E).

4. Discussion

The present study provides the first comprehensive analysis on the composition of a pseudoscorpion's venom based on a combined transcriptomic and proteomic approach. To obtain information on real venom compounds, we performed proteomics analyses of venom samples. Peptides were considered to be venom-specific if they could be identified by MS in venom samples and the corresponding precursors showed higher expression levels in the chelal hand transcriptome compared to the transcriptome of the proximal pedipalp segments. In the case of the rather tiny pseudoscorpions, venom analysis is complicated by two factors in particular. First, milking the crude venom was hampered in the past mainly because handling these small animals is challenging. This problem has been solved in previous experiments by developing a sophisticated extraction methodology for pseudoscorpions (Krämer et al., 2019). Second, the very low volume of venom released per milking (estimated to be 5 nl; see Krämer et al. (2019)) requires multiple venom extractions to obtain a sufficient amount for biochemical analyses. We found that venom from approximately 50 specimens of *C. cancroides* was sufficient (two venom samples per specimen) to perform bottom-up analyses including reduction/alkylation/digestion, and subsequent Orbitrap MS analyses. The MALDI-TOF MS required only single venom samples. As verified in parallel experiments, analyzing the venom of, for example, 64 instead of 44 individuals did not significantly increase the number of identified peptides anymore. Therefore, we may have identified a large proportion of the more prominent venom compounds. This is supported by the high number of identified venom compounds represented by their ion signals in the MALDI-TOF mass spectra of venom samples.

In total, peptides from more than 124 precursors were identified in the venom of *C. cancroides*. One hundred and seventeen of the corresponding genes were found with higher expression levels in the transcriptome of chelal hands compared to the transcriptome of the proximal pedipalp segments. Mature peptides derived from these precursors show few PTMs, among them disulfide-bonds in most of the venom peptides, except for the checacins and C-terminal amidations. C-terminal amidation delays proteolytic degradation by exopeptidases; N-

terminal pyroglutamate formation, which has been demonstrated for CHTX-Cc1, has a similar effect. The low number of PTMs is consistent with findings on spider and scorpion venom for which only disulfide bonds and C-terminal amidation are more frequent (Delgado-Prudencio et al., 2019; King and Hardy, 2013). The precursors derived from these genes were provisionally grouped into putative orthologs of known arthropod toxins, precursors of antimicrobial peptides, enzyme precursors, and novel precursors without significant similarity to known venom precursors of arthropods. Many of the precursors listed as orthologs of known arthropods toxins exhibit only moderate sequence similarity to their respective arthropod precursors, and there is a smooth transition to precursors listed as novel, i.e., those precursors without significant orthology to known precursors of arthropods. Nevertheless, all orthologs of arthropod toxins derived from these *C. cancroides* precursors are stabilized by disulfide bridges, and some of these cysteine-rich peptides (e.g., CHTX-Cc 2a, 2b, 3 and 4) display toxin-specific cysteine patterns (Extra Structural Motif (ESM) and Principal Structural Motif (PSM)) typical for ion channel toxins from spider venom (Kozlov et al., 2005). Such cysteine-rich toxins often exhibit ICK motifs as known from peptide toxins in, e.g., spiders (Langenegger et al., 2019) and scorpions. The corresponding peptides are named knottins and some knottins are known to block e.g., potassium channels with high specificity (e.g., Kuzmenkov et al., 2018). It is probable that at least some of the here-identified venom compounds also exhibit the ICK-motif.

Another group of peptides recently described from *C. cancroides* is the checacins (Krämer et al., 2019). These linear peptides have been classified as antimicrobial peptides based on their similarity to megicin, an antimicrobial peptide from the venom of *Mesobuthus gibbosus* (Diego-García et al., 2014). Checacins are characterized by relatively high net charges and nonpolar amino acids at the N-terminus. This led to the assumption of membrane disruption through pore formation as a potential mode of action of these peptides (Langenegger et al., 2019). Depending on their charge, such peptides can act not only on bacterial membranes/cell walls, but also on those of potential prey. As typical for non-selective toxins, checacins were highly abundant in the venom samples of *C. cancroides*. Another putative antimicrobial peptide identified in our study shows similarity to the defensin family. Defensins are disulfide-rich cationic peptides that are already known to be present in venoms (e.g., scorpion venoms (Zhu and Tytgat, 2004)), and possibly protect the venom compounds against a wide range of bacteria (Shafee et al., 2017). However, compared to checacins, *Chelifer* defensin is not particularly enriched in venom. The transcriptome data also suggest a low expression level of *Chelifer* defensin in the chelal hands, which is only slightly higher compared to the proximal pedipalp segments. In the present study, we identified four additional checacin precursors, bringing the total number to seven. The expression level of the *checacin* genes is very variable in *C. cancroides*, but mass matches were found in MALDI-TOF mass spectra for six of the seven predicted mature checacins. All checacins are C-terminally amidated, and the six checacins detectable in MALDI-TOF mass spectra are cleaved from the N-terminal propeptide downstream of a highly conserved LEAP motif. An identical cleavage motif was also observed in the precursor of NCVC-2. However, the mature NCVC-2 peptide is cleaved further downstream, C-terminally from LEAPSP. The Ser-Pro motif appears to function as a cleavage signal even in the absence of the preceding LEAP motif, at least our data on NCVC-3 suggest this. Precursors of NCVC-5 and 6 contain modified LEAP motifs (LESP, LDTP) that appear to efficiently separate the N-terminal propeptides from the cysteine-rich peptides. Other observed cleavage signals that can be attributed to regular intracellular proprotein convertases (Benjannet et al., 1991) include quadruplet (Kozlov et al., 2005) and dibasic motifs (mostly Arg-Lys), which are commonly known from neuropeptide precursors. These cleavage signals either separate the N-terminal propeptide from the potential toxin (CHTX-Cc 2, 3, 4, 8) or, in the case of some checacins (checacin 2, 4), result in cleavage after the C-terminal Gly, which provides the amide group for the preceding amino acid. In addition, dibasic Arg-Arg (Checacin 3, 6, 7) or monobasic Arg (Checacin 1, 5) also enable effective C-terminal cleavage of the checacins. The remaining venom peptides of *C. cancroides* either consist of the complete precursor (without signal peptide), or the mature peptides are not yet verified by MS analysis. A number of identified precursors for venom peptides show orthology to enzyme precursors. It is likely that at least the predicted *C. cancroides* phospholipases and metalloproteases are actively involved in envenomation. Both phospholipases and metalloproteases have been identified previously in many venoms (Carmo et al., 2014; Casewell et al., 2013; Ramos and Selistre-de-Araujo, 2006), and are often described as spreading factors that facilitate the dispersion of toxins by destroying either cell membranes or proteins. However, at least for phospholipases, the range of effects seems to be more complex, as phospholipase homologues cause a variety of pharmacological effects in the case of snake venoms, and also act as neurotoxins themselves (Manjunatha Kini, 2003).

A major achievement of our study is the documentation of the first specific effects of pseudoscorpion crude venom on insect and arachnid ion channels. Interestingly, our activity test with crude venom confirmed inhibition of insect voltage-gated potassium channels. This activity might be caused by CHTX-Cc1a and 1b. However, similarities only provide a first indication about the potential activity of a bioactive peptide/protein and are not sufficient to draw valid conclusions (Stevens et al., 2011). In the case of neurotoxins, small sequence differences can alter the target-sensitivity/-binding (e.g., Peigneur et al., 2012). In addition, even though CHTX-Cc1a and 1b exhibit similarity to scorpion alpha-KTX, both lack the described 'scorpion K_V channel toxin signature' which is important but not obligatory for toxin interaction with K_V channels (Zhu et al., 2014). Consequently, further studies are needed to test the specific activities of CHTX-Cc1a and 1b on molecular and cellular level.

The modulation of the inactivation process of VdNav1 channel from *V. destructor*, discovered in our activity tests, provides evidence for the presence of sodium channel toxins in the venom. Similar activities were previously described for spider, sea anemone and scorpion toxins binding at the neurotoxin binding site 3 of Nav channels (Stevens et al., 2011). It is conspicuous that no compounds with sequence similarity to these were found in the venom of *C. cancroides*. Of the compounds identified, CHTX-Cc8a and 8b can be speculated to cause the observed effect, as both showed similarity to Kappa-theraphotoxins. These usually bind to potassium channels, but most peptides of this family also act on sodium channels by modulating the inactivation in a similar fashion as observed for the crude venom of *C. cancroides* (e.g., Xiao et al., 2004). Otherwise, it might be that the pseudoscorpion toxins causing this effect belong to a novel structural family of Nav/Kv modulators or even act in a different way, e.g., on another/novel binding site. The first evidence for pore-forming cytolytic effects of the venom can be drawn based on the application of higher venom amounts to *Xenopus*-oocytes, resulting in venom-dependent increase in chord conductance.

The electrophysiological data suggest that *C. cancroides* represents an interesting new source of pesticidal compounds. Especially the modulation of VdNav1 channels from *V. destructor*, an important pest of honeybee hives, may provide new insights on how *C. cancroides* efficiently control *Varroa* mites. *C. cancroides* has previously been considered to protect honey bees from mite infestations (van Toor et al., 2015).

The potency of the venom also becomes evident from predation rates of first-instar *C. cancroides* given a choice of the similar sized psocids (*Liposcelis entomophila*) and the much larger *Varroa* mites (Fig. 1B) In an arena containing 15 each of healthy psocids and *Varroa*, 5 first-instar larvae that had been removed from culture and starved for two days killed on average 52% of psocids and 25% of *Varroa* within 4 h over 23 repeats (van Toor, unpublished).

A previous study on *S. apimelus* used transcriptome information obtained from an extract of the chelal hand to discuss the hypothetical venom composition of pseudoscorpions (Santibáñez-López et al., 2018). This allows us to make a detailed comparison of the potential venom

precursors proposed for *S. apimelus* with the peptides biochemically identified in *C. cancroides*. Notably, few predicted venom precursors of *S. apimelus* match precursors whose products were biochemically confirmed in the venom of *C. cancroides*. Although orthologs of many of these *S. apimelus* genes that are dominated by enzyme-coding genes were also found in the chelal hand transcriptome of *C. cancroides*, we identified only very few mature compounds from these precursors in the venom of *C. cancroides*. Vice versa, only a few significant BLAST-hits corresponding to precursors of confirmed venom compounds of *C. cancroides* were found in the chelal hand transcriptome of *S. apimelus*. There are several possible explanations for these findings: 1) The venom of these species is indeed highly different; not only because the peptide sequences derived from orthologous genes are quite different, but also because completely different genes are involved in venom production. For example, it has been postulated for centipedes (Jenner et al., 2019) that the venom composition differs significantly between higher-level taxa, such as the different orders of centipedes. *C. cancroides* and *S. apimelus* belong to the same suborder (Iocheirata) within the Pseudoscorpiones but represent different families. The lineages to which they belong have been separated for more than 200 million years (Benavides et al., 2019). 2) The venom compounds identified here for *C. cancroides* are still incomplete and further studies will show better agreement between the proposed venom precursors of *S. apimelus* and those of *C. cancroides*. 3) Information on venom precursors of *S. apimelus*, currently based on a solely transcriptomic approach, is still incomplete. 4) The actual composition of the venom of *S. apimelus* differs to a greater degree from that described in Sharma et al. (2019). It was shown that solely transcriptomic venom profiles can overestimate venom complexity substantially (e. g., Smith and Undheim, 2018). The correct answer which of the explanations fits best is probably somewhere in the middle, but it would certainly be interesting to verify which compounds actually appear in the venom of *S. apimelus* by proteomics analysis of released venom. Only then will it be possible to assess whether the venom composition of these pseudoscorpions has evolved mainly after the separation of their lineages or is more similar than it currently appears. However, what is already clear from our study on *C. cancroides* is the presence of a strikingly large number of novel venom compounds whose specific cellular targets still await functional deorphanization.

Ethical Statement for Solid State Ionics

Hereby, I Jonas Krämer consciously assure that for the manuscript 'A Pseudoscorpion's Promising Pinch: The Venom of Chelifer cancroides Contains a Rich Source of Novel Compounds' the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.
2) The paper is not currently being considered for publication elsewhere.
3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
5) The results are appropriately placed in the context of prior and existing research.
6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.
7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

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Credit author statement

Jonas Krämer: Conceptualization, Investigation, Formal analysis, Writing – original draft; Steve Peigneur: Conceptualization, Investigation, Formal analysis, Writing – original draft; Jan Tytgat: Conceptualization, Writing – review & editing; Ronald Jenner: Resources, Writing – review & editing; Ronald van Toor: Resources, Writing – review & editing; Reinhard Predel: Conceptualization, Writing – original draft, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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2.2 Antimicrobial, Insecticidal and Cytotoxic Activity of Linear Venom Peptides from the Pseudoscorpion *Chelifer cancroides*

Jonas Krämer, Tim Lüddecke, Michael Marner, Elena Maiworm, Johanna Eichberg, Kornelia Hardes, Till F. Schäberle, Andreas Vilcinskas and Reinhard Predel

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Conceptualization, J.K., A.V., T.L. and R.P.; methodology, T.L., M.M.; formal analysis, J.K., M.M., K.H., E.M., J.E.; investigation, K.H., E.M., J.E; resources, K.H., T.F.S., A.V.; writing—original draft preparation, J.K. and R.P.; writing—review and editing, T.L., A.V., K.H., M.M., T.F.S.; visualization, J.K.; supervision, R.P..; project administration, T.L.; funding acquisition, A.V. All authors have read and agreed to the published version of the manuscript.

Relevance for the dissertation:

With this publication, we have achieved to perform the first activity tests with individual compounds from pseudoscorpion venom on organismic level. For Checacin1, a peptide I had preliminarily classified as AMP, we could demonstrate potent activities against several bacteria including a multi-resistant strain. The tested peptide also strongly reduces aphid survival in a feeding assay, with an effect size comparable to that of a commercial pesticide. With these findings, I can present the first functional characteristics of a pseudoscorpion venom compound, which was one of the major goals of this thesis. Checacin1 was one of the first compounds I identified in the venom of the house pseudoscorpion, and now we are one step closer to understanding its role in the venom composition. As Checacin1 also showed cytotoxic effects on mammalian cells, the potential for applications as insecticide or antibiotics may be limited, but these test results nevertheless demonstrate that pseudoscorpion venom is a promising source for bioprospecting.

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Antimicrobial, Insecticidal and Cytotoxic Activity of Linear Venom Peptides from the Pseudoscorpion *Chelifer cancroides*

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Abstract: Linear cationic venom peptides are antimicrobial peptides (AMPs) that exert their effects by damaging cell membranes. These peptides can be highly specific, and for some, a significant therapeutic value was proposed, in particular for treatment of bacterial infections. A prolific source of novel AMPs are arthropod venoms, especially those of hitherto neglected groups such as pseudoscorpions. In this study, we describe for the first time pharmacological effects of AMPs discovered in pseudoscorpion venom. We examined the antimicrobial, cytotoxic, and insecticidal activity of full-length Checacin1, a major component of the *Chelifer cancroides* venom, and three truncated forms of this peptide. The antimicrobial tests revealed a potent inhibitory activity of Checacin1 against several bacteria and fungi, including methicillin resistant *Staphylococcus aureus* (MRSA) and even Gram-negative pathogens. All peptides reduced survival rates of aphids, with Checacin1 and the C-terminally truncated Checacin1$^{1-21}$ exhibiting effects comparable to Spinosad, a commercially used pesticide. Cytotoxic effects on mammalian cells were observed mainly for the full-length Checacin1. All tested peptides might be potential candidates for developing lead structures for aphid pest treatment. However, as these peptides were not yet tested on other insects, aphid specificity has not been proven. The N- and C-terminal fragments of Checacin1 are less potent against aphids but exhibit no cytotoxicity on mammalian cells at the tested concentration of 100 µM.

Keywords: pseudoscorpion venom; antimicrobial peptides; antimicrobial activity; cytotoxic activity; insecticidal activity; checacin; megicin

Key Contribution: The recently discovered Checacin1; which represents an abundant antimicrobial peptide from the venom of the pseudoscorpion *C. cancroides*; shows potent antimicrobial and insecticidal activity

1. Introduction

One of the more prevalent medical challenges of our time is the emerging resistance of pathogenic bacteria to antibiotics. In the EU more than 33,000 deaths per year are attributed to infections with multi-resistant bacteria [1]. A possible strategy to address this issue is the discovery of novel compounds with antimicrobial activity. In this regard, antimicrobial peptides (AMPs) are a promising substance class. In contrast to established antibiotics, which normally act on specific cellular processes, AMPs usually exert their effect by disrupting the cell membrane of their target [2]. The specificity of these peptides depends, inter alia, on net charge and hydrophobicity [2] and many exhibit not only antimicrobial but also cytotoxic activities, which can hamper successful drug development [3].

A rich source for the discovery of novel AMPs are animal venoms. This has already been demonstrated by the discovery of many AMPs in the venom of, e.g., spiders, scorpions, snakes, insects, and cone snails [4–10]. Many of the AMPs discovered in venoms were found to be effective against a wide range of bacteria, including Methicillin-resistant Staphylococci e. g. [11,12]. For some AMPs, an apparent potential for the application against multi-resistant bacteria was already described. These AMPs include marcin and its orthologs from scorpion venom, which were effectively used to treat mice infected with multi-resistant bacteria [13]. Interestingly, the main reasons for rejecting commercial development of scorpion AMP-based antibiotics were rather cost-related or the lack of improvement in efficiency compared to conventional antibiotics, but not the frequent problem of cytotoxicity against human cells [7]. Another application of these compounds is their use as specific pesticides. For instance, oral administration of AMPs from scorpion venom was shown to effectively reduce the number of pea aphids within three days by killing the aphid's symbiotic bacteria [14]. Hence, AMPs might be promising candidates for alternative pesticides against aphids, which belong to the most important agricultural pests [15]. To increase the chances of identifying novel and highly efficient AMPs, sampling across a wider taxonomic range is advisable.

One group of venomous animals that has been poorly studied regarding its venom composition but has recently been proposed as a potential priority group for bioprospection is the order of pseudoscorpions. Pseudoscorpions, for which more than 3600 species [16] were described, belong to the arachnids and thus share a common ancestor with other venomous lineages, such as spiders and scorpions [17]. Although the more prominent venomous arachnids usually overshadow the pseudoscorpions in the general perception, they nevertheless represent an important group for bioprospection [18]. Within these tiny arthropods, which mostly do not exceed a body length of a few millimeters, a unique venom delivery system has evolved: members of the suborder Iocheirata possess pedipalps equipped with a venom delivery system for subduing their prey. Knowledge about the venom composition of these animals is still limited to a very few species. In the past, the small size and associated handling problems, as well as the miniscule venom yield, have made a proteomic analysis of the venom particularly difficult. Initial studies comprised solely transcriptomic analyses, which provided first insights on the potential venom composition of two pseudoscorpion species [19,20]. However, the possibility of false positives cannot be ruled out when de novo transcriptomic approaches are solely used for the inference of venom compositions [21]. Very recently, a method for extracting pseudoscorpion venom has been developed and thus the implementation of proteomic approaches for studies on pseudoscorpion venom became possible. This enabled for the first time a comprehensive proteo-transcriptomic analysis of the venom composition of the house pseudoscorpion *Chelifer cancroides* [22,23]. These works also led to the discovery of checacins, the first potential AMPs identified from the venom of pseudoscorpions [22]. Checacins were classified as potential AMPs based on their similarity to megicin, an antimicrobial peptide found in the venom of the scorpion *Mesobuthus gibbosus* [24]. As high therapeutic potential has been described for megicin and orthologous sequences [13], checacins might also have beneficial properties in this regard. In our study, we examined

the activity of Checacin1 and some Checacin1 fragments in different assays. First, we tested their antimicrobial activity on different strains of Gram-negative and Gram-positive bacteria as well as on different fungi. We also determined the cytotoxicity of the tested peptides to mammalian cells. Finally, their potential for use as pesticides was investigated using aphid feeding assays.

2. Results

An overview of the peptides tested in the bioassays is given in Figure 1. In the venom of *C. cancroides*, Checacin1 is highly abundant, whereas the N- and C-terminal fragments Checacin1$^{1–11}$ and Checacin1$^{12–25}$ occur naturally, but with lower abundance [18].

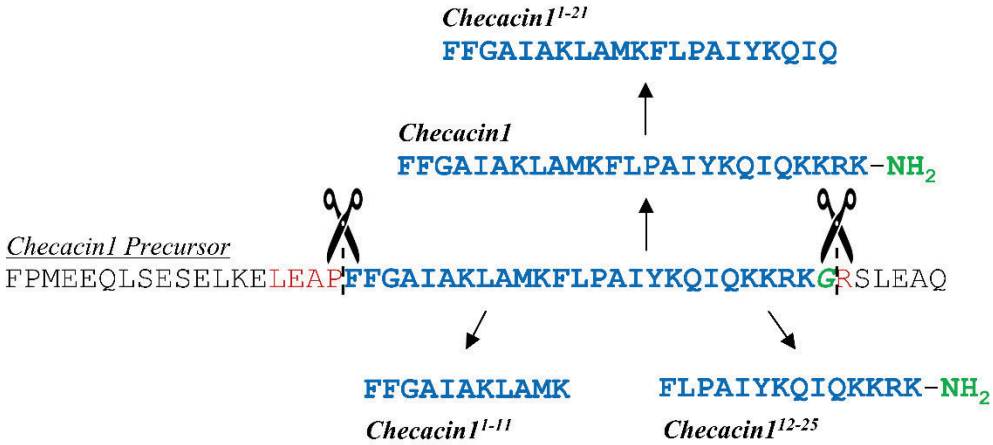

Figure 1. Precursor sequence of Checacin1 (without signal peptide) and the sequences used for the bioassays. Native Checacin1 is N-terminally cleaved at a highly effective 'LEAP' motif [22], which is typical of most checacin precursors [23]. The C-terminal cleavage of the Checacin1 progenitor is upstream of a monobasic (Arg) cleavage site and has a C-terminal amidation site (Gly). The preceding KKRK motif does not function as a cleavage signal [22]. Checacin1$^{1–11}$ and Checacin1$^{12–25}$ are naturally occurring fragments of Checacin1, while Checacin1$^{1–21}$ has not been detected in the venom of *C. cancroides* [22]. However, for the orthologous Megicin 18 from the scorpion *M. gibbosus*, the C-terminal cleavage was postulated to be upstream of such a tetrabasic motif [24].

2.1. Checacin1 Is Highly Active against Bacteria and Fungi

The minimum inhibitory concentration (MIC) of Checacin1 and the truncated Checacin1 fragments was determined for a diverse panel of Gram-negative and -positive bacteria as well as two fungal indicator strains (Table 1). In order to explore the translational potential of checacins regarding the development of antibiotics, clinically relevant microorganisms were included in the screening. Among these are methicillin-resistant *Staphylococcus aureus* (MRSA) and most importantly *Pseudomonas aeruginosa* as well as *Escherichia coli*. *Mycobacterium smegmatis* was screened as surrogate test organism for *Mycobacterium tuberculosis*. Yeasticidal efficacy was determined using *Candida albicans* as a surrogate strain for *Candida auris*. Only full-length Checacin1 and the C-terminally truncated Checacin1$^{1–21}$ (only on *S. aureus*) exhibited an inhibitory effect on the tested organisms. Checacin1 inhibited the growth of all tested microbes, with the lowest MIC values found for *E. coli* and MRSA. No difference of Checacin1 potency towards *E. coli* could be observed when tested in bicarbonate supplemented medium (CAMH-C). With a MIC of 6.25 µM, *C. albicans* is notably affected by application of Checacin1, while the growth of the filamentous ascomycete *Aspergillus flavus* was not inhibited.

Table 1. Minimum inhibitory concentrations (MIC) of Checacin1 and Checacin1 fragments determined for different microbes (*Ec*: *Escherichia coli*, *Pa*: *Pseudomonas aeruginosa*, *Ms*: *Mycobacterium smegmatis*, *Sa*: *Staphylococcus aureus*, *Af*: *Aspergillus flavus*, *Ca*: *Candida albicans*). CAMH-II: cation-adjusted Mueller Hinton II medium; CAMH-C: CAMH-II with 44 mM sodium bicarbonate; TEM-1: TEM-1 beta-lactamase expressing strain; MRSA: methicillin-resistant *Staphylococcus aureus*; BTG: BacTiter-Glo™-assay; MTT: microtiter turbidity assay.

Compounds	**MIC(μM)**						
	Ec		***Pa***	***Ms***	***Sa***	***Af***	***Ca***
	ATCC 35218		ATCC27853	ATCC607	ATCC33592	ATCC9170	FH2173
	TEM-1	TEM-1			MRSA		
	CAMHII	CAMH-C	CAMHII	CAMHII	CAMHII	CAMHII	CAMHII
Checacin1	1.6–0.8	1.6	12.5	25	1.6	50	6.25
Checacin1$^{1–11}$	>50	>50	>50	>50	>50	>50	>50
Checacin1$^{12–25}$	>50	>50	>50	>50	>50	>50	>50
Checacin1$^{1–21}$	>50	>50	>50	>50	12.5	>50	>50
Rifampicin	4.9	19.4	38.9	38.9–19.4	>77.8	NA	NA
Tetracycline	4.5	36–18	>144.1	0.14	>72	NA	NA
Gentamycin */Isoniazid''	2.1–1	1–0.5	1	29.2–14.6''	0.5–0.26	NA	NA
Tebuconazole	NA	NA	NA	NA	NA	0.19	0.19–0.09
Amphotericin B	NA	NA	NA	NA	NA	1.1	8.6–4.3
Nystatin	NA	NA	NA	NA	NA	1.1	8.6
readout	MTT	MTT	MTT	BTG	MTT	BTG	BTG

* Calculated for gentamycin C1 (25876-10-2) as gentamycin (1405-41-0) is a substance mixture.
'' *Mycobacterium smegmatis* was tested against isoniazid instead of gentamycin.

2.2. Orally Administered Checacin1 is Active against Pea Aphids (Acyrthosiphon pisum)

To evaluate aphid survival after oral application of Checacin1, aphids were monitored for three days. The aphid survival is compared to application of MeOH (negative control) and the pesticide Spinosad (positive control) in a survival curve (Figure 2, Table S1). Checacin1 and, to lesser degree also Checacin1$^{1–21}$, caused a rapid decrease of the aphid survival rate similar to that of Spinosad (Figure 2a,b). Both killed about half of the aphids (54% and 58% respectively). For the Checacin1 fragments (Checacin1$^{1–11}$, Checacin1$^{12–25}$; Figure 2d,e), the effect on aphid survival is much less distinct compared to the negative control, but still significant.

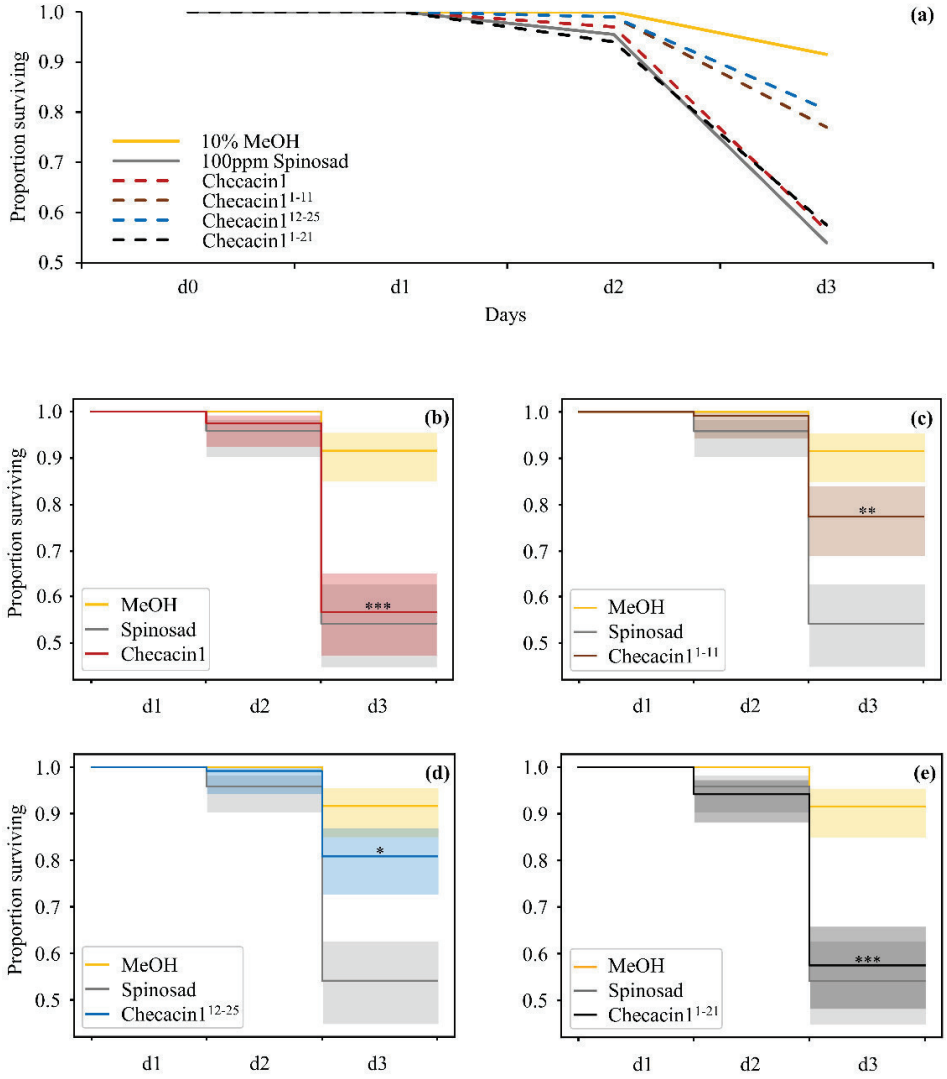

Figure 2. Survival curves of pea aphids (*A. pisum*) after oral administration of Checacin1 and its fragments. Aphids were monitored for three days. As negative control, aphids were fed with 10% methanol (MeOH) and a commercial pesticide was used as positive control (Spinosad). (**a**) Line chart comparing mean survival rates of all tested components with negative and positive control. Checacin1 and Checacin1$^{1–21}$, but not the shorter Checacin1 fragments, were recovered as insecticidal (**b**–**e**) Survival rates of individual components with included 95% confidence interval. Significant differences to the negative controls were assessed by a log-rank test and are indicated by '*' for $P < 0.1$, '**' for $P < 0.05$ and '***' for $P < 0.01$ (Table S1).

2.3. Cytotoxic Activity of Checacin1

For assessing the cytotoxicity of Checacin1, a CellTiter-Glo® cell viability assay was conducted (Figure 3, Table S2). Cytotoxic effects on the MDCK II cells were mainly observed for Checacin1. Whereas Checacin1$^{1–11}$ and Checacin1$^{12–25}$ caused no visible effects to the cell culture (Figure 4a,b) at the highest applied concentration (100 µM), Checacin1 and C-terminally truncated Checacin1$^{1–21}$ caused a nearly complete disintegration of the cell layer (Figure 4c,d). This was confirmed for both peptides by the significantly lower luminescence compared to the negative control. This is indicative of a strong decline of the cell's ATP content (Figure 3). For Checacin1, significant cytotoxic effects could also be demonstrated at the lower concentrations 50 µM and 25 µM, however with a relatively

high standard deviation in case of the measured luminescence for the 25 µM concentration. For C-terminally truncated Checacin1$^{1–21}$, a concentration of 50 µM does not cause significant cytotoxic effects anymore.

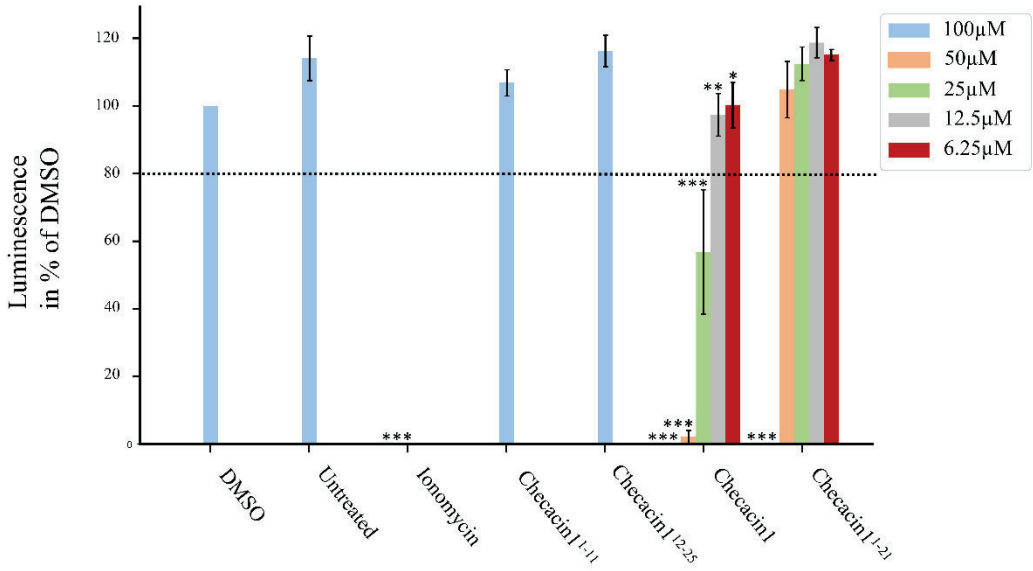

Figure 3. Cell viability assay of MDCK II cells treated with varying concentrations of Checacin1 and Checacin1 fragments. Cell viability was assessed based on CellTiter–Glo® (Promega GmbH, Walldorf, Germany) which measures luminescence as an indicator for ATP amount. The luminescence signal of the 100 µM treatment of Checacine1 and Checacin1$^{1–21}$ was at 0% and therefore not detectable. Luminescence was normalized to the DMSO control; data are presented as mean ± SD (n = 4–6). DMSO: dimethyl sulfoxide. Significant differences to the negative control (Untreated) were assessed by t-statistics and are indicated by '*' for P < 0.1, '**' for P < 0.05 and '***' for P < 0.01.

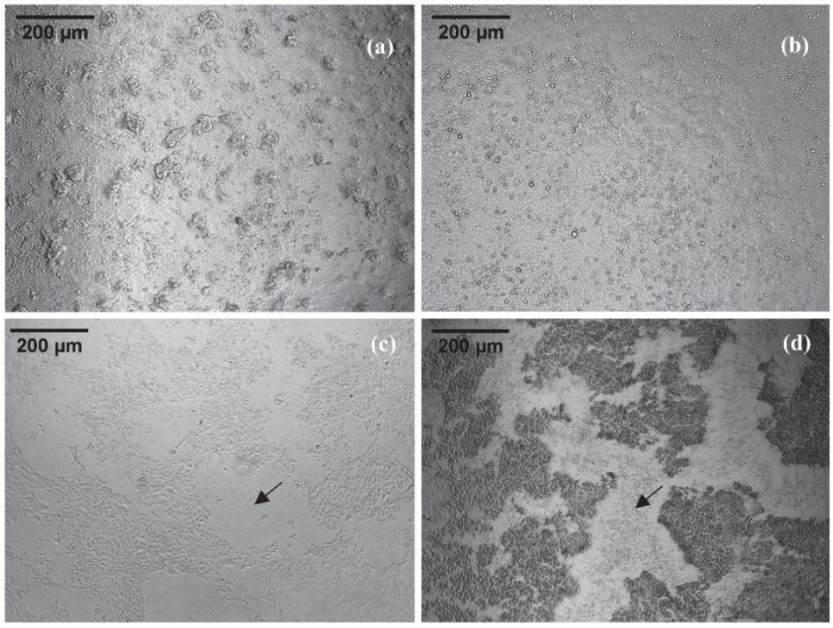

Figure 4. Madin–Darby canine kidney II (MDCK II) cell culture after application of 100 µM Checacin1 and its truncated forms. (**a**,**b**): intact cell layer after application of Checacin1$^{1–11}$ and Checacin1$^{12–25}$ (**c**,**d**): disintegrated cell layer after application of Checacin1$^{1–21}$ and Checacin1. Arrows indicate areas of disintegrated cell layers.

3. Discussion

This study provides first insights into the bioactivity of checacins, AMPs identified in the venom of the pseudoscorpion *C. cancroides*. By now, precursors of seven checacin genes were identified from this species [22,23]. These checacin genes show highly different expression levels which is also reflected by the relative intensities of the ion signals of the mature peptides in MALDI-TOF mass fingerprints. For our activity tests, we selected Checacin1, which is the most prominent checacin in terms of expression level as well as signal intensity in MALDI-TOF mass spectra of venom samples [22]. Additional peptides used in this study are truncated forms of Checacin1. Two of these peptides represent naturally occurring fragments of Checacin1, whereas synthetic Checacin1$^{1-21}$ is a peptide that was not yet found in the venom of *C. cancroides*. This peptide was tested because AMPs from orthologous scorpion genes (megicin, marcin etc.) were described without the existing basic C-terminus [20]. These peptides have already been synthesized and activity tests resulted in promising antimicrobial activities, including a successful therapy of mice infected with multi-drug-resistant Gram-positive bacteria [13]. Figure 5 shows an alignment of partial checacin precursors from *C. cancroides* with those of partial precursors of the scorpion AMPs megicin and marcin.

As many AMPs found in arachnid venoms, checacins are linear cationic peptides. Such peptides mostly exert their toxic effects by binding to cell membranes and causing the formation of pores, leading to cell death [25]. A crucial factor for the pore forming ability is the hydrophobic N-terminus, whereas the specificity for different membranes is mainly determined by the net charge of the peptide [25]. As bacterial cell membranes exhibit a more negative net charge than, e.g., animal cell membranes, cationic peptides bind to these with higher affinity [26]. In the venom, such peptides were first believed to function solely as conserving agents which protect the venom peptides against a wide range of microorganisms [27]. As linear cationic peptides often also exhibit cytotoxic activities on the potential prey, an active participation as, e.g., spreading factors [28] in envenomation cannot be excluded and the already postulated 'dual-use'-concept [29] might be the best approximation to explain the mode of action of the majority of these peptides.

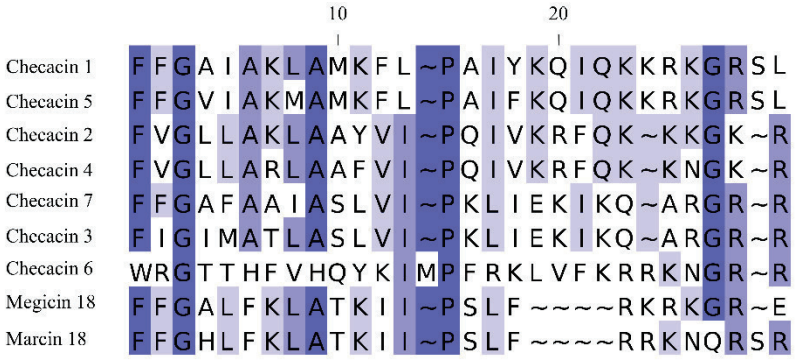

Figure 5. Alignment of the checacin motif, identified in pseudoscorpion venom with orthologs discovered in scorpion venom. Sequences include glycin as amidation signal (if present) and the C-terminal cleavage site. The coloration indicates percentage of identity.

In our tests, antimicrobial activities were only observed for full length Checacin1 and in part for Checacin1$^{1-21}$. The highest applied concentrations of the shorter checacin fragments did not cause any antimicrobial effects on the tested strains. This can be explained by the following assumptions: in case of Checacin$^{1-11}$, the lower net charge reduces antimicrobial activity, whereas Checacin1$^{12-25}$ is lacking the hydrophobic N-terminus. Checacin1$^{1-21}$ is also much less active against the tested strains and only efficient on *S. aureus*. As this peptide lacks the C-terminus, the reduced antimicrobial activity might also be explained by the reduced positive net charge. The C-terminal amidation improves peptide stability by protecting the peptide from proteolytic cleavage. In the case of the checacin

precursors, the use of the monobasic Arg cleavage signal following Gly may have been subject to positive selection during the evolution of the checacins. It was shown in a previous study, that synthetic scorpion AMPs are effective against aphids by acting on their symbiotic bacteria [14]. Interestingly, both Checacin1 and Checacin1$^{1–21}$ killed aphids with similar efficiency, though the antimicrobial efficacy of Checacin1$^{1–21}$ is much weaker. The N- and C-terminal fragments of Checacin1 also had a weak but significant effect on aphid survival without noticeably affecting any of the microbes tested. This indicates that at least some of the tested Checacin1 fragments exert their insecticidal activity on aphids by directly affecting the aphid cells and not the symbiotic bacteria. Nevertheless, the aphid's symbionts were not yet among the tested strains, and the peptide concentrations used in the aphid assay were mostly higher than the concentrations applied in the antimicrobial tests. Regarding the cytotoxicity for mammalian cells, mainly Checacin1 showed cytotoxic effects in the tested concentration range. Cytotoxic effects caused by AMPs were observed frequently in the past, e.g., [21]. Interestingly, the mechanism by which AMPs act on most mammalian cells is usually apoptosis and not cell lysis as observed for bacterial cells and erythrocytes [30]. A requirement for pharmaceutic application of AMPs is a MIC lower than the minimal concentration causing cytotoxic effects in mammals [31]. In case of Checacin1, the minimal cytotoxic concentration is between 25 μM and 12.5 μM, which is one order of magnitude above the observed MICs of *E. coli* and *S. aureus*. Due to this minimal therapeutic window, pharmaceutical application would require further optimization. However, in the current study cytotoxicity was tested with MDCK II cells from dogs and the effect on human cells has still to be examined. Bacalum and Radu (2015) recommended to test cytotoxicity on erythrocytes and lymphocytes to consider apoptotic effects caused by AMPs [30]. A yet unresolved question regarding differential expression of the checacins is to what extent the relative abundance of the checacins correlates with specificity or efficiency toward their target cells. In this regard, three hypotheses seem plausible: (1) The highly abundant checacins are the most efficient, which would explain the higher energy investment of increased production. (2) Less abundant checacins are more efficient and due to their higher efficacy, higher concentrations are not necessary to fulfil their functions. It has been postulated previously, that venoms contain toxins with low abundance, that are equally important as the products of highly expressed venom compounds e.g., [32]. (3) The ratio of checacins in the venom might lead to an optimal efficiency. This idea might be supported by findings that demonstrated synergistic effects of venom compounds, e.g., [33].

4. Conclusions

The venom of neglected arthropods provides an additional source of novel AMPs, which are promising candidates for developing alternative pesticides or antibiotics. For full-length Checacin1, the most prominent AMP present in the venom of the pseudoscorpion *C. cancroides*, we observed promising antimicrobial activities against several clinically relevant strains of bacteria and fungi. In feeding assays, Checacin1 and C-terminal truncated Checacin1$^{1–21}$ efficiently reduced aphid survival, suggesting that these peptides have potential as novel pesticides, although their selectivity for pest insects needs to be explored further. As known for linear cationic peptides, the cell membrane disrupting capability of Checacin1 relies on its hydrophobic N-terminus as well as on the high net charge, which is demonstrated by the much lower efficacy observed for Checacin1 fragments. So far, we have only tested the activity of one out of seven known *C. cancroides* checacins. As these peptides differ substantially in terms of gene expression level, it would be interesting to test, how this is correlated with the efficiency and specificity of the different checacins.

5. Material and Methods

5.1. Peptide Synthesis

Synthetic peptides were obtained from DGpeptides Co., Ltd. (Hangzhou, China). Purities and identifiers used by the company are shown in Table 2.

Table 2. List of antimicrobial peptides synthesized based on peptides identified in the venom of the pseudoscorpion *Chelifer cancroides*.

Component/Company ID	Sequence	Purity
Checacin1/D-4040	FFGAIAKLAMKFLPAIYKQIQKKRK *	96.75%
Checacin1$^{1–11}$/D-4041	FFGAIAKLAMK	97.84%
Checacin1$^{12–25}$/D-4042	FLPAIYKQIQKKRK *	98.07%
Checacin1$^{1–21}$/D-4043	FFGAIAKLAMKFLPAIYKQIQ	96.87%

* Sequence with C-terminal amidation.

5.2. Feeding Assay on Pea Aphids (A. pisum)

Screenings for potential insecticidal activities were performed as feeding assays on age synchronized pea aphids (*A. pisum,* clone LL01, 5 days old) as described by Heep and colleagues [34,35]. Pea aphid nymphs were fed on an artificial diet [36], containing the tested checacins (100 ppm), in specialized feeding chambers [37] for three days. We used diet mixtures containing 10% methanol as negative control and Spinosad (100 ppm) as positive control. Pea aphid survival was scored daily. The feeding assay was performed in three biological replicates per substance and control, each containing a total of 60 *A. pisum* specimen.

5.3. Antimicrobial Activity Assays

The antimicrobial properties of the synthesized checacins were evaluated against a diverse set of indicator strains. The used method for minimum inhibitory concentrations (MIC) determination is derived from the methodology proposed by the EUCAST committee. The antibacterial/antifungal MIC value is defined as the lowest concentration of an agent that inhibits the growth of a microorganism by 85% relative to the growth controls (High value). The medium background (low value) is subtracted from all measurements (*AU* = absorption units; luminescence for cell viability assays). Relative growth inhibition is calculated according to:

$$rel.growth\ inhibtion\ [\%] = 100 \times \left(1 - \frac{AU_{sample} - AU_{Low}}{AU_{High} - AU_{Low}}\right)$$

AU = absorption units, low = medium blank, high = growth control.

The peptides were dissolved in sterile ultra-pure water (0.055 µS/cm) and tested in a 12 step dilution series ranging from 50 to 0.02 µM. All concentrations were tested in triplicate.

Escherichia coli ATCC35218, *Staphylococcus aureus* ATCC33592 MRSA and *Pseudomonas aeruginosa* ATCC27853 were incubated overnight (37 °C, 180 rotations per minute (rpm)) and subsequently diluted to 5 × 10^5 cells/mL in cation adjusted Mueller Hinton II medium (Becton Dickinson, Sparks, NV, USA). For *E. coli* ATCC35218 an additional cell suspension was prepared in cation adjusted Mueller Hinton II medium supplemented with 44 mM sodium bicarbonate. As positive controls dilution series of rifampicin , tetracycline and gentamycin (all Sigma Aldrich, St. Louis, MS, USA), ranging from 64–0.03 µg/mL, were used. Bacterial suspensions without peptide or antibiotic control were used as negative controls. After assay incubation (37 °C, 180 rpm, 85% relative humidity (r.H.)), cell growth was assessed by measuring the turbidity with a microplate spectrophotometer at 600 nm (LUMIstar® Omega BMG Labtech, Ortenberg, Germany). Growth inhibition was calculated relative to the absorption of the controls. The pre culture of *Mycobacterium*

smegmatis ATCC607 was incubated in brain–heart infusion broth (Becton Dickinson, Sparks, NV, USA) for 48 h, at 37 °C and 180 rpm before the cell concentration was adjusted in Mueller Hinton II medium. Isoniazid (Sigma Aldrich, St. Louis, MS, USA) was used instead of gentamycin as third positive control. Cell viability was evaluated after 48 h (37 °C, 180 rpm, 85% r.H.) via ATP quantification (BacTiter-Glo™, Promega, Madison, WI, USA) according to the manufacturer's instructions. *Candida albicans* FH2173 was incubated for 48 h at 28 °C and 180 rpm before the pre culture was diluted to 1×10^6 cells/mL in Mueller Hinton II medium. For *Aspergillus flavus* ATCC9170, a previously prepared spore solution was used to adjust the assay inoculum to 1×10^5 spores/mL. Assays were incubated for 48 h at 37 °C and 180 rpm. For both, tebuconazole (Cayman Chemical Company, Ann Arbor, MI, USA), amphotericin B and nystatin (both Sigma Aldrich, St. Louis, MS, USA) were used as a positive control (64–0.03 μg/mL). Readout was performed by way of ATP quantification.

5.4. Cytotoxicity Assays

5.4.1. Cell Culture

Madin–Darby canine kidney II (MDCK II) cells [38] were maintained in Dulbecco's modified Eagle's medium (DMEM GlutaMAX, ThermoFisher, Waltham, MA, USA) supplemented with 1 % penicillin/streptomycin (ThermoFisher) and 10 % fetal calf serum (ThermoFisher) and grown in an incubator at 37 °C with 5 % CO_2. The MDCK II cell line was kindly provided by Prof. Dr. Friebertshäuser (Philipps University Marburg, Institute of Viorology, Marburg, Germany).

5.4.2. Cell Viability Assays

Checacins and Ionomycin (Cayman Chemicals, Ann Arbor, MI, USA) were dissolved in DMSO and Aprotinin (Carl Roth, GmbH & co KG, Karlsruhe, Germany) was dissolved in water (stock solutions: 10 mM).

For the cell viability assay MDCK II cells were seeded in a 96-well plate and treated at a confluence of 90% with the indicated inhibitors (100 μM per well) or DMSO control. The plate was incubated at 37 °C and 5% CO_2. The cell viability was assessed by measuring the ATP content using the CellTiter-Glo Luminescent Cell Viability assay (Promega GmbH, Walldorf, Germany) according to the manufacturer's instructions. Luminescence was measured using a black 96-well plate in a Synergy H4 microplate reader (Biotek, Waldbronn, Germany, now part of Agilent Technologies). Relative light units (RLU) were normalized to DMSO control set to 100%. Measurements were conducted with four to six replicates and standard deviations were calculated.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/toxins14010058/s1, Table S1: 'Aphid Raw Data', Table S2: 'Cytotox Raw Data'.

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2.3 Fractionation of Pseudoscorpion Venom for Activity Tests on Specific Ion Channels

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To realize the fractionation of the tiny venom amounts obtainable from pseudoscorpions, I collaborated with Dr. Jeroen Kool an expert in this field. In his lab, efficient pipelines for venom fractionation with simultaneous MS detection had been established. The fractionation of pseudoscorpion venom was performed by Julien Slagboom. I formally analyzed the generated raw data and measured the resulting fractions with MALDI-TOF MS, thereby identifying the most suitable fractions for biotests. These were performed by Steve Peigneur in the same set-up as described in chapter 2.1.

Relevance for the dissertation:

In chapter 2.3 I describe our progress on fractionating pseudoscorpion venom for biotests with the aim to test the resulting fractions on specific ion channels. The idea was to narrow down which of the house pseudoscorpion's venom compounds exhibit neurotoxic effects. By performing a successful high performance liquid chromatography (HPLC) run with pseudoscorpion venom, we are one step closer to this goal. Many of the previously identified venom compounds could be found in the resulting fractions. However, no effects could be measured on specific ion channels, which is probably due to material loss during fractionation and related processing. The results shown in this chapter are part of ongoing research and have not been published yet.

Fractionation of Pseudoscorpion Venom for Activity Tests on Specific Ion Channels

1. Introduction

Modern approaches in venomics allow to analyze venom compositions even from tiny animals like pseudoscorpions, ticks, or minute ant species (Garcia et al., 2020; Hurka et al., 2022; Krämer et al., 2019). However, characterizing their venom compounds with respect to their activity remains a challenging task. Traditionally, this required the purification of toxins utilizing repetitive separation workflows, e. g. with high performance liquid chromatography (HPLC) (von Reumont et al., 2022). By now, this can be circumvented by producing the toxins of interest based on their sequence using synthesizers or recombinant expression (von Reumont et al., 2022). However, generating single toxins is still elaborate and, especially for toxins with complex tertiary structures or various posttranslational modifications, it can be hard to identify the suitable expression system (Amorim et al., 2018; Structural Genomics Consortium et al., 2008). As venoms mostly contain hundreds of different compounds, it would be very costly to produce all identified toxins. In addition, venomous animals belonging to understudied groups often express a high amount of novel venom compounds (e. g. Drukewitz et al., 2018; Krämer et al., 2021; von Reumont et al., 2017) with no significant similarity to well annotated toxins in the databases. In these cases, the traditional approach of toxin purification is still very useful to narrow down which fractions of a venom extract contain the toxins responsible for a certain activity. In case of very small venomous animals, this, however, is complicated by the low amount of venom produced by a single specimen.

In this work, we performed a fractionation of pseudoscorpion venom utilizing a HPLC coupled to a highly sensitive mass spectrometer with the aim (1) to assess whether previously identified venom compounds can be found in the resulting fractions, (2) to determine how effectively the toxins of interest are separated and finally (3) to identify those fractions containing neurotoxins responsible for activities measured beforehand with crude venom on specific ion channels.

2. Material and Methods

2.1. Venom collection and storage

Specimens of *C. cancroides* were reared as described in Krämer et al. (2019), who also describe the applied methodology for extracting the venom. For the fractionation, venom was extracted into 1 µl of Milli-Q water per extraction. Two samples were obtained for HPLC analysis, one from 20 extractions for subsequent MALDI-TOF MS and the other from 40 extractions for the biotests. Venom was stored at -20°C until fractionation.

2.2. RP-HPLC and Mass spectrometry

For fractionating pseudoscorpion venom extracts, reverse HPLC was applied utilizing an Agilent 1260 Infinity II LC System by Agilent Technologies Netherlands B.V. (Amstelveen, The Netherlands). The software Agilent OpenLab CDS was used to set separation parameters. Sample injection was done with the 1260 Infinity II Multisampler and a flow rate of 0.5 mL/min. The HPLC column was a 4.6 mm x 100 mm Waters XBridge Peptide BEH C_{18}

analytical column with 300 Å pore size and 5 µm particle size. As mobile phase eluent A (98% H_2O, 2% ACN, 0.1% TFA) and eluent B (98% ACN, 2% H_2O, 0.1% TFA) were used with a gradient comprising a linear increase of mobile phase B from 1% to 20% in 5 minutes followed by an increase from 20% to 40% B in 55 minutes, and a subsequent increase of mobile phase B from 40% to 90% in 4 minutes. Then an isocratic elution was performed for 5 minutes at 90% B. Starting conditions (1% B) were reached linearly in 1 minute and the column equilibration was achieved within 10 minutes at 1% B.

UV detection was done with a 1260 Infinity II Variable Wavelength Detector, following column separation. For simultaneously collecting fractions for activity tests and analyzing the HPLC output with mass spectrometry, an analytical adjustable flow splitter (from Analytical Scientific Instruments US) partitioned the effluent volume with a ratio of 1/10. The higher amount was collected with a 12 second interval per fraction using a FractioMateTM FRM100 nanofraction collector (Spark Holland and VU Amsterdam, Emmen and Amsterdam, the Netherlands). The lower amount was transferred to an in-line MaXis II Quadrupole time-of-flight (QTOF) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) for mass spectrometric analysis. MS was performed in positive mode and electrospray ionization (ESI) was applied for ion generation with the following source parameters: (i) capillary voltage 3.5 kV, (ii) source temperature 200 C, (iii) nebulizer at 0.8 Bar and (iv) dry gas flow 6 L/min. One average mass spectrum was generated per second in a mass range from 500-5500 m/z.

2.3. Data analysis

Analyzing the mass spectrometric data generated by the MaXis was done with the following procedure. Mass spectra were averaged from the TIC (total ion current)-MS chromatogram. Charge states were assigned to the observed masses by deconvoluting the average mass spectra, which also provided the average masses for the signals. For those masses corresponding to previously identified venom compounds, extracted ion chromatograms were generated plotting the highest intensity charge state. The software Compass (Bruker Daltonics, Billerica, MA, USA) was used for analyzing the MS data.

2.4. Reconstitution of fractions and subsequent MALDI-TOF-MS

To measure the collected HPLC fractions with MALDI-TOF-MS, these were reconstituted by adding 20 µl 80 % acetonitrile to the fractions. Then, sample volume was reduced to 3 µl using a Speed Vac (Hetovac) at room temperature for 3min and finally the samples were acidified by adding 0.3µl of 1 % trifluoroacetic acid. For measuring the samples with MALDI-TOF-MS, the same workflow was applied as described in Krämer et al. (2021).

2.5. Activity tests on specific ion channels

For measuring the effect of selected pseudoscorpion venom fractions on specific ion channels, the fractions were applied to oocytes expressed with the potassium channel Shaker and a sodium channel from the *Varoa* mite. Changes in the ion current were measured with the two-microelectrode patch clamp method. The methodological workflow was done as described in Krämer et al. (2021).

The fractions selected for the activity tests were reconstituted in 14µl ND96 buffer and for an individual measurement 2µl were applied to the measuring chamber containing the oocyte.

3. Results and Discussion

Figure 1 depicts the chromatograms generated based on UV absorbance for the HPLC runs with pseudoscorpion venom from 20 extractions (Figure 1A) and 40 extractions (Figure 1B), respectively. Mass spectrometric data generated during the run, allowed to allocate previously identified venom compounds (Krämer et al., 2021) to the chromatogram peaks in figure 1. A large fraction of these compounds could be identified and separated efficiently.

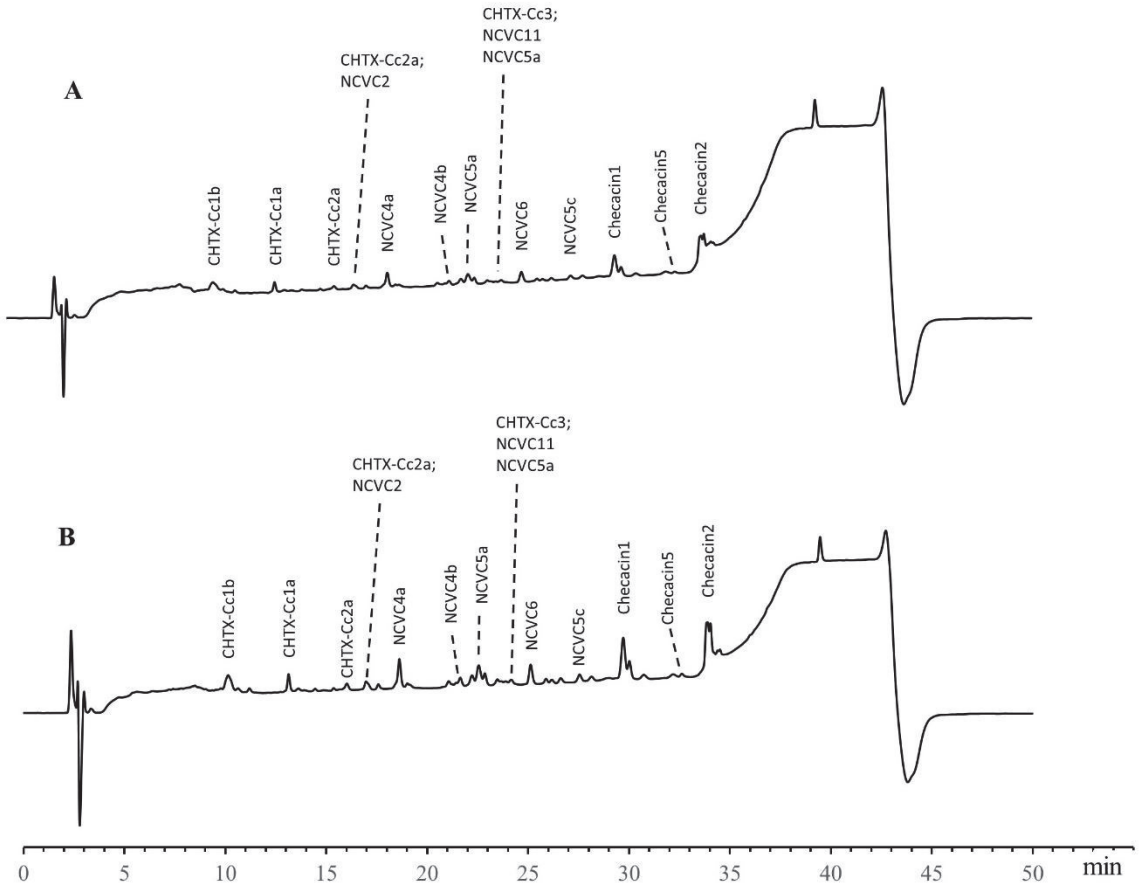

Figure 1: HPLC chromatograms generated based on UV absorbance at 220 nm for venom samples of the house pseudoscorpion *C. cancroides* based on extractions from 20 (**A**) and 40 (**B**) specimens.

To identify those fractions containing the previously identified venom compounds, the MS data generated by the coupled MaXis were utilized. The workflow is demonstrated in figure 2 for one chromatogram peak in which the previously identified Checacin1 was found alongside an unidentified compound with a mass of 9143Da for which a corresponding signal was present in MALDI-TOF mass spectra. Figure 2A shows a chromatogram which is based on the signal intensities detected by the MaXis throughout the HPLC-run, which correspond to the peaks detected based on UV absorption (Figure 1). The mass spectrum depicted in figure 2B shows signals measured during the time span marked with the red square in figure 2A. These signals mainly correspond to different charge states of Checacin1 and the compound with a mass of 9143Da.

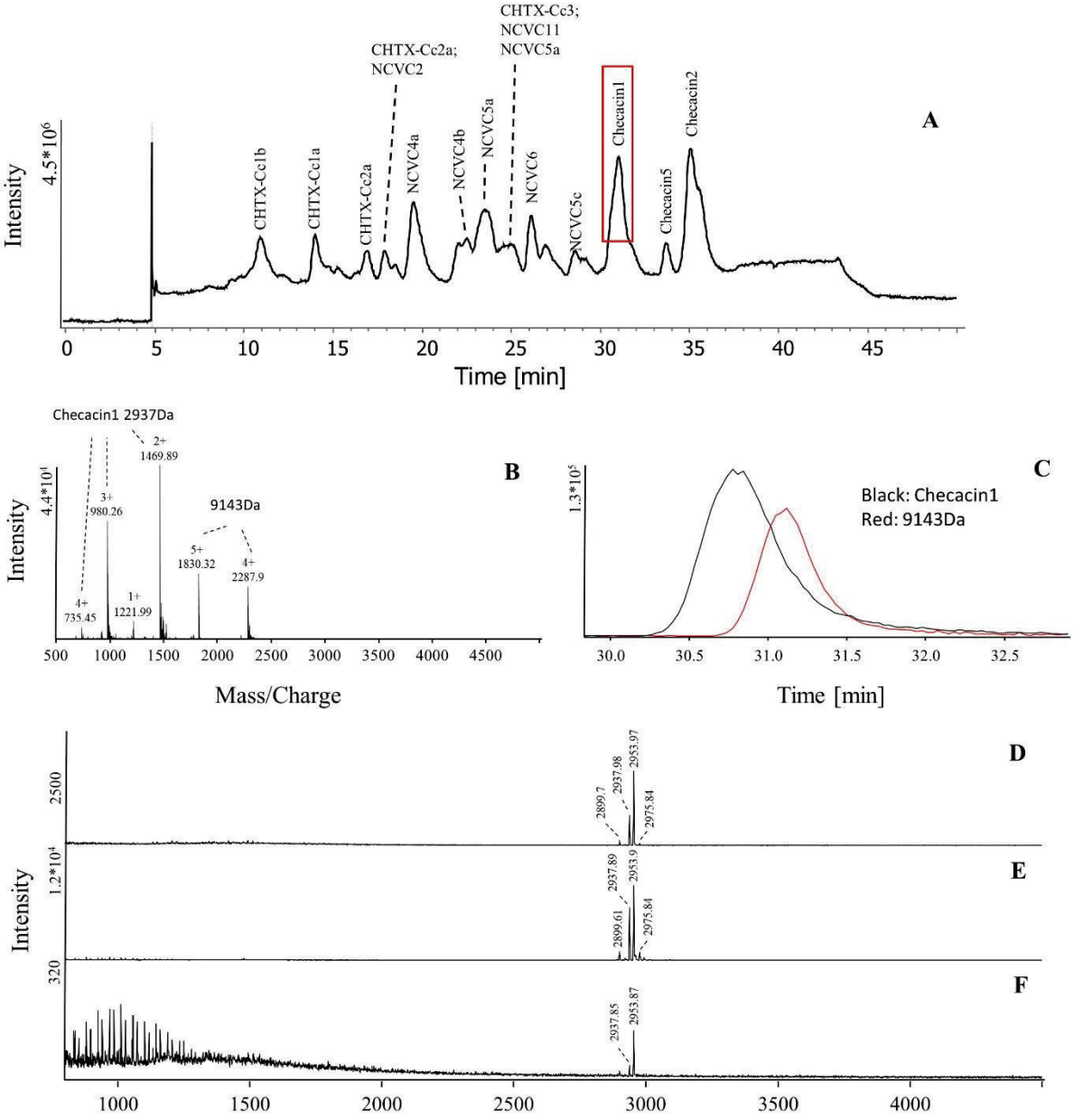

Figure 2: Overview of mass spectrometric data generated during and after the HPLC run with venom from the pseudoscorpion *C. cancroides*. (**A**) MS chromatogram based on TIC (total ion current). (**B**) Mass spectrum averaged for the marked part of the MS chromatogram. (**C**) Extracted ion chromatograms obtained for the highest charge states of the two most abundant venom compounds from the spectrum shown in (**B**). (**D**-**F**) MALDI-TOF mass spectra of HPLC-fractions, which contain signals of Checacin1.

For these compounds figure 2C shows when they were detected and at which time the intensities were maximal during the HPLC run. To verify the presence of the venom compounds of interest in the fractions after HPLC, the fractions obtained for the 20-extraction sample were measured with MALDI-TOF MS. Figure 2D-F show MALDI-TOF MS spectra for those fractions containing signals of Checacin 1. Based on the venom compounds retrieved in MALDI-TOF MS spectra, the delay time between fraction spotting and MS-measurement was estimated to be approximately 40s. Table 1 provides an overview of the compounds identified for the dominant chromatogram peaks and lists the corresponding spotted fractions which were estimated to contain these based on the delay time. Many of the venom compounds detected with MALDI-TOF MS before fractionation could not be retrieved in the MALDI-TOF MS spectra of the fractions after HPLC. For the activity tests, fractions from the HPLC-run with 40 pseudoscorpion venom extractions were used. From these, twelve fractions were selected for which the highest content of the previously identified venom compounds was assumed. These fractions are marked (*) in table 1.

The fractions were tested on oocytes expressed with an insect potassium channel or a sodium channel from the *Varoa* mite. Previously, for the crude venom of the house pseudoscorpion an inhibition of the potassium channel had been demonstrated, whereas for the sodium channel a modulation of the inactivation process had been observed (Krämer et al., 2021). The activity tests with the venom fractions did not cause any modulation of these channels. The tests were performed in the same lab, by the same researcher. Various reasons might explain these findings. First, the concentration of the tested fractions might have been too low. The process of HPLC separation and the afterwards performed evaporation of the resulting fractions involves a substantial loss of material (Flatt, 2019). This might also explain the few venom compounds found in MALDI-TOF-MS spectra of the reconstituted fractions (table 1). Another explanation could be that the neurotoxins responsible for the observed effect were not part of the tested fractions. To address these issues, on the one hand, a larger venom sample may be required to compensate for the loss of material during fractionation. On the other hand, a more comprehensive identification of bioactive compounds might be achieved using off- or on-line screening system with plate-reader based bioassays (Arrahman et al., 2022; Kool et al., 2011; Otvos et al., 2016). However, the established screening systems are configured for certain ion channels like the nicotinic acetylcholine receptor and cannot easily be modified for other ion channels. The limiting factor for activity tests with pseudoscorpion venom fractions remains the small material amount combined with the laborious extraction procedure. As an alternative, activity tests can be focused on single compounds, produced by synthesizers or recombinant systems. However, the selection of suitable candidate compounds is complicated due to the low similarity of the identified venom compounds to known toxins. Another idea might be to apply the organoid approach established for the production of snake venom (Post et al., 2020) to pseudoscorpions, which would be a novel but costly way to obtain higher quantities of crude venom.

Table 1. List of previously identified venom compounds detected during HPLC with the coupled MaXis MS. The detection time is the time interval during which the compound was detected. Based on the assumed delay time between detection with MaXis and spotting the fractions with the proposed highest amount of each compound were selected. In the MALDI column, those fraction IDs are listed for which the compounds could be retrieved in MALDI-TOF MS spectra after reconstitution. Those fractions used for activity tests are labeled with '*'.

Venom Compound (mass [Da])	Detection time MS [min] (Highest intensity)	Fraction (Spotting time)	MALDI
CHTX-Cc1b (4161)	10:30-11:30 (11:00)	O4 (10:09-10:21)*	Absent
CHTX-Cc1a (3905)	13:48-14:30 (14:06)	A5 (13:15-13:27)*	A5
CHTX-Cc2a (4616)	16:30-17:30 (17:00)	O5 (16:09-16:21)*	Absent
CHTX-Cc2b (4689)	17:45-18:30 (18:03)	M6 (17:11-17:23)*	Absent
NCVC2 (2672)	17:42-18:18 (17:57)	M6 (17:11-17:23)*	M6
NCVC4a (7924)	19:12-20:12 (19:36)	E6 (18:50-19:02)*	E6
NCVC4b (7678)	22:15-23:15 (22:42)	K7 (21:57-22:09)*	Absent
NCVC5a (9409)	23:00-24:30 (23:36)	O7 (22:46-22:58)*	Absent
CHTX-Cc3 (5357)	24:57-25:42 (25:15)	J8 (24:26-24:38)*	Absent
NCVC11 (3870)	24:00-25:00 (24:30)	M8 (23:48-24:00)	Absent
NCVC6 (6244)	25:48-26:48 (26:12)	E8 (25:28-25:40)*	Absent
NCVC5c (9276)	28:00-30:00 (28:36)	G9 (27:44-27:56)*	Absent
Checacin1 (2937)	30:18-32:00 (31:05)	N10 (30:12-30:25)*	O10, N10, M10
Checacin5 (2967)	33:12-34:00 (33:42)	A10 (32:55-33:07)	Absent
Checacin2 (2757)	34:36-36:18 (35:12)	G11(34:21-34:33) F11(34:09-34:21)*	D11, E11, F11, G11

4. Conclusion

With this work we demonstrate the successful fractionation of minute venom amounts for one of the smallest venomous animals ever studied. Normally much higher venom amounts are applied in such workflows (e. g. Arrahman et al., 2022). During the applied HPLC run the pseudoscorpion venom could be efficiently separated and previously identified compounds could be identified. These compounds were mostly found distributed among different fractions. However, effects that had been measured after application of crude venom to specific ion channels could not be reproduced with the tested venom fractions. This might be explained by

the material loss involved in HPLC-separation. Potential pathways for future research are i) obtaining larger amounts of starting material for the fractionation, ii) testing the resulting fractions more comprehensively or iii) relying on recombinant production to generate single toxins.

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2.4 Equipped for Sexual Stings? Male-Specific Venom Peptides in *Euscorpius italicus*

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Author contributions:

Conceptualization, J.K. and R.P. (Reinhard Predel); methodology, J.K.; validation, J.K., R.P. (Ricardo Pommerening) and R.P. (Reinhard Predel); formal analysis, J.K. and R.P. (Ricardo Pommerening); investigation, R.P. (Ricardo Pommerening); resources, R.P. (Reinhard Predel); data curation, J.K.; writing—original draft preparation, J.K. and R.P. (Reinhard Predel); writing— review and editing, R.P. (Ricardo Pommerening); visualization, J.K.; supervision, R.P. (Reinhard Predel); project administration, J.K. and R.P. (Reinhard Predel). All authors have read and agreed to the published version of the manuscript.

Relevance for the dissertation:

This publication paves the way for understanding sex-specific venom compositions within Panscorpiones. For a sexually dimorphic scorpion that is known for extensive sexual stings, we demonstrated the strong up-regulation of certain venom compounds in male venom, accompanied by the down-regulation of other compounds. During the work with these animals, I observed their mating behavior and noted that females ceased all defensive behavior towards male mating attempts after the sexual sting. The identified male specific venom compounds might be responsible for this effect and are interesting candidates for activity tests with the females. In addition, this paper provides the first analysis of the venom composition for the *Euscorpius* genus, for which not a single venom compound had been described beforehand.

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Equipped for Sexual Stings? Male-Specific Venom Peptides in *Euscorpius italicus*

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Abstract: In the animal kingdom, intraspecific variation occurs, for example, between populations, different life stages, and sexes. For venomous animals, this can involve differences in their venom composition. In cases where venom is utilized in the context of mating, the differences in composition might be driven by sexual selection. In this regard, the genus *Euscorpius* is a promising group for further research, as some of these scorpions exhibit a distinct sexual dimorphism and are known to perform a sexual sting during mating. However, the venom composition of this genus remains largely unexplored. Here, we demonstrate that *Euscorpius italicus* exhibits a male-specific venom composition, and we identify a large fraction of the substances involved. The sex specificity of venom peptides was first determined by analyzing the presence/absence patterns of ion signals in MALDI-TOF mass spectra of venom samples from both sexes and juveniles. Subsequently, a proteo-transcriptomic analysis provided sequence information on the relevant venom peptides and their corresponding precursors. As a result, we show that several potential toxin precursors are down-regulated in male venom glands, possibly to reduce toxic effects caused to females during the sexual sting. We have identified the precursor of one of the most prominent male-specific venom peptides, which may be an ideal candidate for activity tests in future studies. In addition to the description of male-specific features in the venom of *E. italicus*, this study also includes a general survey of venom precursors in this species.

Keywords: *Euscorpius*; intraspecific variation; sex specificity; venom composition; proteomic and transcriptomic analysis; toxin

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1. Introduction

Intraspecific variation in venom composition is a widespread phenomenon, observed throughout the animal kingdom [1]. Diet can be an important factor in this variation, which has been demonstrated, e.g., for snakes [2].

For clinically relevant species, studying this variation is crucial to ensure the development of effective antidotes [3,4]. Apart from this, understanding the variation within a species can help to identify substances with specific functions, which might be overlooked otherwise. In this regard, it was shown that sex-specific differences in venom composition can have a strong effect on their biochemical, insecticidal and neurotoxic properties [5]. Hence, it may be useful to also consider intraspecific venom variation when searching for compounds suitable for the development of specific pharmaceutics or pesticides.

Sex-specific differences in venom composition have been demonstrated for several arthropod taxa such as spiders, scorpions, or centipedes [6–8]. Different prey preferences due to sexual dimorphisms (e.g., size differences) might be a crucial evolutionary driver of these variations. In terms of understanding the functional purpose of sex-specific venom compositions, spiders are among the best studied groups. As has been demonstrated for the Sydney funnel web spider, the sex specificity of the venom composition can have drastic effects on its efficacy; compounds lethal to humans are only present in the venom of

males [9]. Evolutionarily, this might be explained by the development of behavioral differences between the sexes, as males cover long distances in search of females and are thus much more exposed to predators. A reverse situation was discovered for *Loxoceles* spiders, for which females are more lethal to humans [10]. It is also suspected for some arthropods that sex-specific compounds are involved in mating-related functions. An example are spiders of the *Tetragnatha* group, in which courtship behavior involves interlocking of their chelicera [11]. Possibly, the venom is used for communication in this case [12].

Much less is known about sex specificity of scorpion venoms than that of spiders. Even though sex-specific differences in venom composition have been postulated for several scorpion species [8,13,14], only a few compounds involved were identified [15,16]. As some male scorpions perform a sting during mating ('sexual sting'), the compounds transferred might have a direct relevance for mating. For instance, Olguín-Pérez et al. [14] confirmed that venom transference takes place during the sexual sting of *Megacormus gertschi* and revealed sex-specific differences in the venom composition. They hypothesized that the male-specific compounds function to sedate the female to reduce the risk of cannibalism.

To extend the knowledge about sex-specific venoms in scorpions, the genus *Euscorpius*, which is completely harmless to humans, appears very promising. Members of this genus, which occurs mainly in Europe, often show a pronounced sexual dimorphism; and for some species of this group, the execution of a sexual sting has been demonstrated [17]. For *Euscorpius alpha*, in addition to distinct sex-specific differences in the size and morphology of the telson, differences in the structure of the venom glands were described [18]. However, scorpions of this genus are generally still largely unexplored in terms of their venom composition.

In our study, we performed a comprehensive analysis of the venom composition of *Euscorpius italicus* (Scorpiones: Euscorpiidae), with a focus on identifying sex-specific peptides and the genes encoding them. Our expectation of finding sex-specific venom compounds is based on the previously described morphological differences of the venom delivery system between the sexes of this genus.

2. Results

The sexual dimorphism previously described for *E. alpha* was also observed in *E. italicus* (Figure 1). The telsi of males are inflated and considerably larger than those of females (Figure 1a). In addition, the tip of the stinger appears to be more curved. The morphology of the telson of last stage juveniles resembles that of females. Figure 1c,d document this transition of telson morphology from juvenile to male *E. italicus*. As venom was collected from marked specimens before and after the adult molt, we were also able to document the transition in venom composition from juveniles to males in these individuals (see below). The transition not only affected the venom composition, but also the amount and consistency of venom released during electrical stimulation (Figure 2). Basically, the quantity of venom released increased significantly in males (Figure 2a, Supplementary Material Figure S4) and the venom became translucent (Figure 2b).

2.1. MALDI-TOF Mass Spectra Reveal a Male-Specific Ion Signal Pattern

We analyzed MALDI-TOF mass spectra (hereafter referred to as mass fingerprints) from repeated venom extractions of six adults (two males, four females) and four last instar juveniles (two juvenile males, two juvenile females). Representative mass fingerprints (m/z 3000–10,000) are shown in Figure 3. No substantial differences were observed in venom mass fingerprints from females and juveniles. However, mass fingerprints of venom released from males appeared to systematically differ from those of juveniles and females (Supplementary Material Figure S1). Especially in the mass range of m/z 3000–10,000, several prominent ion signals typical of mass fingerprints from females/juveniles were not detected. On the other hand, mass fingerprints from the venom of males show additional signals that were not observed in mass fingerprints of venom from females/juveniles. In Figure 3, these signals are highlighted. We also analyzed the transition in venom

composition from juveniles to males using the same individuals before and after the adult molt (Supplementary Material Figure S2).

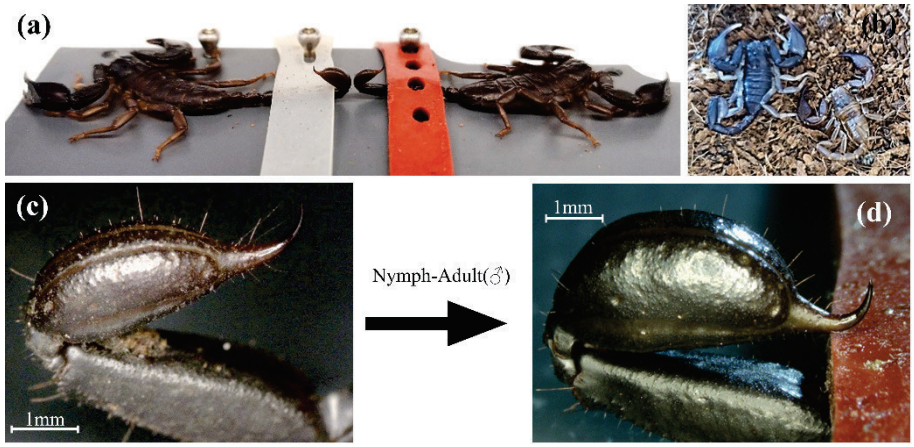

Figure 1. External telson morphology of *E. italicus*. (**a**) Comparison of male (left) and female (right) specimens with differently shaped telsi. (**b**) Male after adult molt with last instar exuvia. (**c**,**d**) Telson morphology of a single individual before (**c**) and after (**d**) molting into a male.

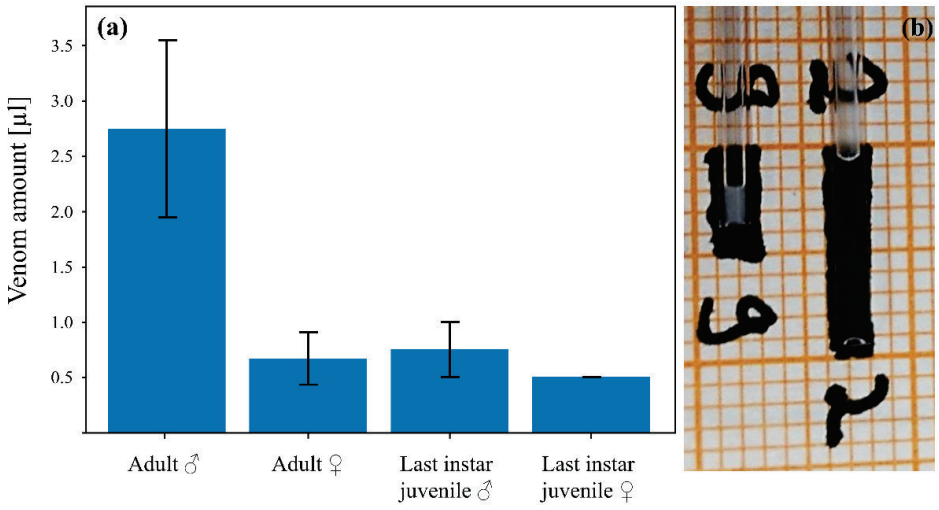

Figure 2. Comparison of venom amount and consistency of the sexes of *E. italicus*. (**a**) Mean venom amount (error bars represent standard deviation) released by adult males (6 extractions; 2 specimens), adult females (12 extractions; 4 specimens), and juveniles (12 extractions; 4 specimens) during venom extraction. The values are based on three repeated extractions per specimen with an interval of one week between the extractions. (**b**) Venom consistency in males (right capillary; translucent) and females (left capillary; milky).

2.2. Combined Proteo-Transcriptomic Analysis with a Focus on Identifying the Male-Specific Changes of the Venom

This study comprises a comprehensive venom analysis of male and female *E. italicus* with a focus on identifying products of genes with sex-specific expression. The basic information for assigning sex-specific compounds and their respective genes was obtained by repetitive MALDI-TOF mass spectrometry (MS) analyses of venom samples (see above). These experiments showed that in adult males the venom composition changed dramatically, while in females the venom composition was largely identical to that of juvenile scorpions. The changes in the venom of male *E. italicus* suggest both up- and down-regulation of genes.

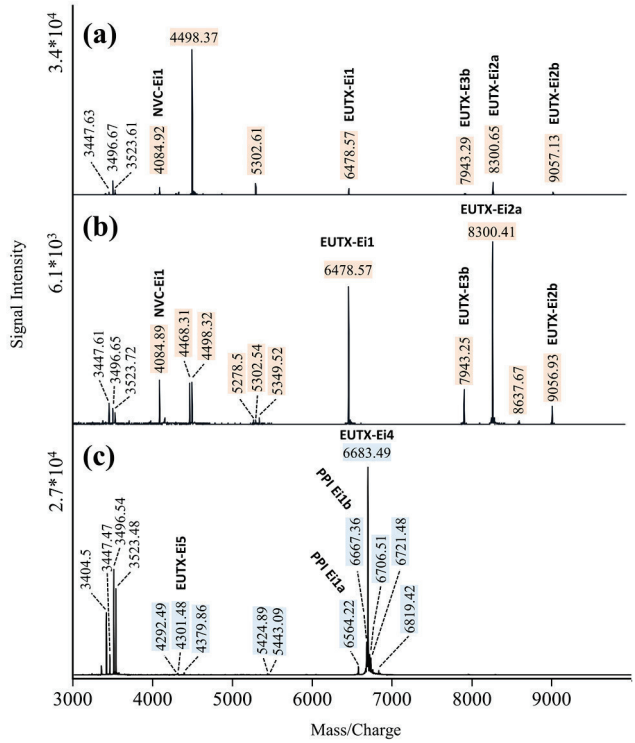

Figure 3. Comparison of MALDI-TOF mass fingerprints (m/z 3000–10,000) of venom extracted from juvenile (**a**), female (**b**), and male *E. italicus* (**c**). Male-specific ion signals are highlighted in blue; ion signals that are missing in mass spectra of venom from males are highlighted in beige.

This information was used to identify the corresponding gene products using a proteotranscriptomic approach in which proteomic venom analyses using Quadrupole Orbitrap MS were matched with transcriptome data generated from telsi of both sexes with their venom glands and an additional transcriptome generated from tail tissue without telson (negative control). Next-generation sequencing resulted in 18,806,043 paired-end reads for the female telson transcriptome, 20,946,933 paired end reads for the male telson transcriptome, and 22,576,333 paired end reads for the negative control, each after removal of adapter sequences. The corresponding assemblies contain 85,185 contigs for the female telson transcriptome, 66,778 contigs for the male telson transcriptome, and 80,811 contigs for the negative control transcriptome. The assessment of transcriptome completeness revealed 86.9% complete BUSCOs and 3.2% fragmented BUSCOs for the male telson transcriptome, 87.2% complete BUSCOs and 3.2% fragmented BUSCOs for the female telson transcriptome, and 94.1% complete BUSCOs and 1.0% fragmented BUSCOs for the negative control transcriptome.

For venom samples of both sexes, proteome data of two Quadrupole Orbitrap MS experiments (one experiment with trypsin digestion and one without) were matched to the transcriptome assemblies. After quality filtering, removal of precursors without signal peptide and redundant matches, 326 precursors were identified that contribute to the venom composition of both sexes of *E. italicus*. The complete list of venom precursors can be found in Supplementary Material Table S1. Based on similarity to sequences from the UniProt database, these were classified as precursors of potential neurotoxins, enzymes, antimicrobial peptides (AMPs), others and, for no matches with the database, as novel (Figure 4). Figure 4 also illustrates the percentage of precursors for which up- or down-regulation in males could be confirmed in the present study. Details of these precursors, including the most similar Blast hits in the UniProt database, are shown in Table 1. All in all, eleven precursors were identified, with the majority down-regulated in males. Although the corresponding mature peptides could not be detected in MALDI-TOF mass spectra of venom samples from males and their precursors show much higher expression levels

in the telson transcriptome of females, the expression level in male telson transcriptome was still higher compared to the negative control. Consistent with this, the proteomic data from Quadrupole Orbitrap MS also revealed low amounts of these peptides in male venom samples, indicating significant quantitative differences rather than presence/absence. Most of these precursors, which are down-regulated only in males, process cysteine-rich peptides, classified here as potential neurotoxins based on their similarity to corresponding scorpion sequences (Table 1). Of these, EUTX-Ei2a/EUTX-Ei2b and EUTX-Ei3a/EUTX-Ei3b are likely products of paralogous genes. For EUTX-Ei2b, we classify the peptide as a potential neurotoxin, although the most similar BLAST hit at UniProt lists an AMP of the scorpion *Hadrurus spadix* (Table 1). This classification is based on the presumed relationship of EUTX-Ei2b to EUTX-Ei2a, the latter showing in turn a high similarity to a sodium channel toxin of the scorpion *M. gertschi*. Regarding the precursor processing, the potential mature neurotoxins either correspond to the complete precursor sequences without signal peptide (EUTX-Ei1, EUTX-Ei3a), use the C-terminal Gly as amidation signal (EUTX-Ei3b), or the C-termini result from cleavage at a C-terminal tribasic cleavage site (EUTX-Ei2a, EUTX-Ei2b).

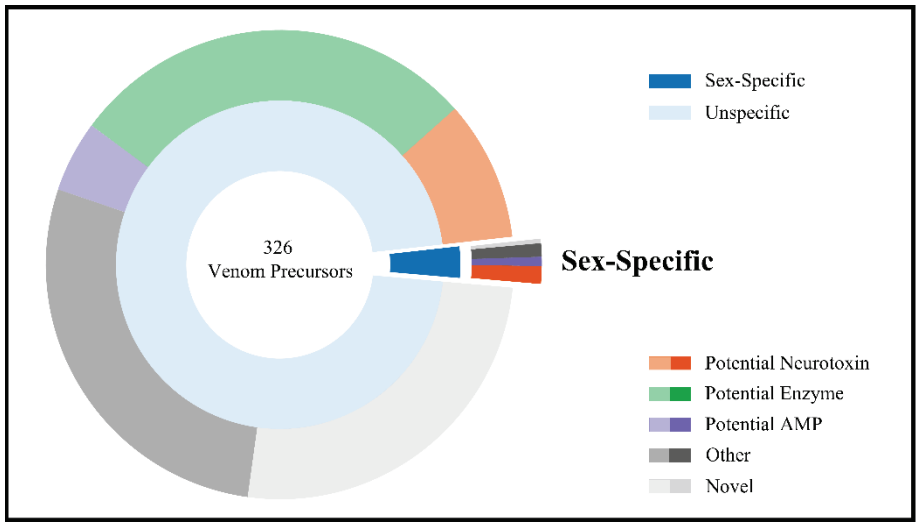

Figure 4. Pie chart highlighting the fraction of venom precursors that were confirmed to be down- or up-regulated ("sex-specific") in males of *Euscorpius italicus* (inner circle). In the outer circle, venom compounds are classified based on similarities to sequences from UniProt [19] entries.

Three precursors down-regulated in males are characterized by the absence of cysteines and are provisionally assigned here as AMP precursors (AMPs-Ei1, 2a, 2b). Their mature peptides are short and located in the precursor between the signal peptide and a monobasic (AMP-Ei1) or dibasic cleavage site (AMP-Ei2a, 2b), the former as part of a quadruplet motif [20]. The peptides of the likely paralogous AMP-Ei2a and AMP-Ei2b genes are amidated and thus better protected against degradation. Their C-terminal sequence resembles that of insect SIFamides, which are described as neuropeptides [21]. Processing of all putative AMP precursors potentially also releases one or more short peptides located C-terminally from the aforementioned cleavage sites, but these products were not detected by MS. One of the precursors down-regulated in males and yielding no significant BLAST hits in the UniProt database was classified as novel (Novel Venom Compound Ei1). The corresponding mature peptide with two cystines (C-C) results from cleavage at an N-terminal quadruplet motif, the C-terminal amino acid in the precursor sequence functions as amidation signal (Table 1).

Table 1. Information on venom precursors up- [↑] or down-regulated [↓] in males of *E. italicus*. Specificity was assessed based primarily on repeatedly generated MALDI-TOF mass spectra of venom samples from both sexes and juveniles but was also supported by the statistically less significant transcriptomic and Quadrupole Orbitrap MS experiments. Precursors were identified by a combined proteomic and transcriptomic approach. Grey, signal peptide; blue letters, potential bioactive peptide; green and italic, amidation signal; yellow, cysteine (half of the disulfide bond); red letters, cleavage site. Black underlined, confirmed by MSMS; red underlined, confirmed only by MSMS of digested samples; dashed line, mass match in MALDI-TOF MS. The column Orbitrap MS indicates which of the four analyses confirmed the precursor sequence. D, digested samples; ND, samples without digestion; m, venom from males; f, venom from females. Assumed PTMs include amidation (A) and disulfide bridges (C–C).

Name	BLAST Hit	Expression Level ♂ [tpm]	Expression Level ♀ [tpm]	Expression Level Negative Control [tpm]	PTM	Predicted Mass $[M+H]^+$	Orbitrap MS	Male Specificity (MALDI)
U-Euscorpiustoxin-Ei1	Putative potassium channel toxin (*M. gertschi*), 63%, Acc: A0A224XGQ4	349.45	4136.68	-	C-C	6478.97	D (m/f), ND (m/f)	↓
MVKQLVAAFLVIMLISSLVDAKKTFMEKAKSVFSKAGNKIKEIAGKSEYMCPVVSSFCEQHCARQEKSGFCDFNKCTCS								
U-Euscorpiustoxin-Ei2a	Putative sodium channel toxin (*M. gertschi*), 71%, Acc: A0A224XBU0	2771.21	13,995.7	-	C-C	8301.05	D (m/f), ND (m/f)	↓
MNAKLTVLLFLAMVAIASCGWINEKRVQSYIDEKIPNGVMKGAIKAVVHKIAKNEYGCVANIDTVSQCNKHCIAAGSEKGVCHGTKCKCDKELSYRRK								
U-Euscorpiustoxin-Ei2b	Antimicrobial peptide (*Hadrurus spadix*), 66%, Acc: A0A1W7RB12	766.38	6848.29	-	C-C	9056.51	D (m/f), ND (m/f)	↓
MQIRCSILLLLMISSFCSCGILREKYFHQAVDKVAPMIPLPVVSQVVGNVAKQIVHKFAKNEALCMFNKDVAGMCDKSCKEAGKSNGICHGTKCKCDKPLSYKKK								
U-Euscorpiustoxin-Ei3a	La1-like protein 15 (*Urodacus yaschenkoi*), 51%, Acc: L0GB04	8058.69	29,307.7	2.73	C-C	8637.12	D (m/f), ND (m/f)	↓
MKRLQVAALVCLLLCALFSLSAGAGEICEANGLSIPVGQDKQDPKSCDLYKCIMQNNRLVLDKFSCATLKRKRGCKIVPGDSKAAFPKCCPTSNCRGAQWDQ								
U-Euscorpiustoxin-Ei3b *	Putative La1-like peptide (*M. gertschi*), 68%, Acc: A0A224X3N5	2320.13	18,041.9	3.49	A, C-C	7942.79/ 7956.88	D (m/f), ND (m/f)	↓
MENALGGVMLGSLLLLSLFSASLAIGEKCETGQHVID/EVGKQVQDSKSCTLYKCINYNRKYALETLTCASQKLKSGCRSIPGAANTPFPNCCPTVICQG								
Antimicrobial Peptide Ei1	Putative non-disulfide bridge peptide (*M. gertschi*), 81%, Acc: A0A224XFL9	2015.04	9050.66	-	-	2586.38	D (m/f), ND (m/f)	↓
MHFNKTLLVIFLSYLLVTDEAEAFWGFLAKLATKVVPSLFGSSSEKSKREIENFFEPYQKDLDLELDRFDRFLSKLDLN								

Table 1. *Cont.*

Name	BLAST Hit	Expression Level ♂ [tpm]	Expression Level ♀ [tpm]	Expression Level Negative Control [tpm]	PTM	Predicted Mass [M+ H]+	Orbitrap MS	Male Specificity (MALDI)
Antimicrobial Peptide Ei2a	Antimicrobial peptide UyCT3 (*Urodacus yaschenkoi*), 59%, Acc: L0GCI6	1228.17	11,051.7	-	A	1474.82	D (m/f), ND (m/f) -	↓
	MKNQFVILIIAVVLLQLFSPSEAILSDIWNGIKGLFGKRGLFPQRPLINRDQFDDVFDDDLSAADLKFLQELLK							
Antimicrobial Peptide Ei2b	Antimicrobial peptide UyCT3 (*Urodacus yaschenkoi*), 65%, Acc: L0GCI6	637.46	2871.02	-	A	1504.83	D (m), ND (m/f)	↓
	MKNQFVILVIAVVLLQLFSPSEAILSDIWNGIKSIFGKRGLRNLDRFDDLFDDDVSDADLKVLQELFR							
Novel Venom Compound Ei1*	-	5.1	3418.27	-	A, C-C	4085.01/ 4086.00	D (f), ND (m/f)	↓
	MKCYLAVLVLLLVCAVLPDQTCGIENGRKSPNFCRNKCLKEYIPNNCVGYCERVLKEKEK/EKEG*							
U-Euscorpiustoxin-Ei4	Putative Na+ channel toxin (*Superstitionia donensis*), 50%, Acc: A0A1V1WBR1	8482.11	60.96	17.54	A, C-C	6683.95	D (m/f), ND (m/f)	↑
	MKWCTVFMFCLVILVHEFQDVYGEKEGYPLDATRNIYQCYDLGENDYCEKKCKEFGGHGYCYGFACYCKYIRDDVKIWKG							
Putative Protease Inhibitor Ei1a	Chymotrypsin-elastase inhibitor ixodidin-like (*Ixodes scapularis*), 37%, Acc: A0A2R4SV19	1712.29	218.501	88.11	A, C-C	6563.75	D(m)	↑
	MTNLRETVANMKTLAVTLLTLAAFQLVLPYPQPEESSPPENCGENELFYGRRTCAPICNDEVCKKPSREPTACFAICYQGCYCKEGYGRNRRNETCVKCE							
U-Euscorpiustoxin-Ei5	AKTx (*Hadrurus spadix*), 35%, Acc: A0A1W7RB23	1812.84	23.48	163.27	C-C	4301.78	D (m), ND (m)	↑
	MNLIIIFTLLLSSPFIEVEGSQVNARASCTNSGVCRSSTCPSRGCRSGKCINRKCTCYYC							

* Allelic difference (relevant amino acids highlighted in green).

Three precursors were identified as up-regulated in males, two of which were classified as potential neurotoxins based on the BLAST hits in the UniProt database (Table 1). With EUTX-Ei4, we identified the precursor associated with the most prominent male-specific MALDI-TOF ion signal in terms of intensity. The corresponding mature peptide with three cystines is the complete precursor without signal peptide, the C-terminal Gly is used as amidation signal. The much less abundant EUTX-Ei5 also results in a mature peptide comprising the complete precursor without signal peptide but does not have a C-terminal amidation. The third precursor, up-regulated in males, is classified as precursor of a cysteine-rich protease inhibitor. The mature peptide is C-terminally amidated, its sequence in the precursor is between the signal peptide and a short C-terminal precursor peptide that is N-terminally cleaved at a monobasic cleavage site (Arg).

3. Discussion

Using a combination of proteomics and transcriptomics, we performed the first comprehensive venom analysis for a scorpion of the species-rich genus *Euscorpius*. In addition to the general goal of assessing the complement of genes involved in venom production, our special focus was on analyzing the presumed sexual dimorphism of the venom of *Euscorpius* at a molecular level. A marked dimorphism on the morphological level was previously described for *E. alpha* [18] and comprises much larger male telsi and sex-specific differences in the cell architecture of the venom glands. For *E. italicus*, a similar sexual dimorphism, at least with respect to the telson morphology, has already been described [22] and was also observed by us for the specimens used in the current study. That this dimorphism also relates to an altered cellular composition of the venom gland seems plausible, as the male venom released after electrical stimulation has a much higher quantity and transparency. The transparent nature of the venom we observed in male *E. italicus* resembles the 'prevenom' described for *Parabuthus transvaalicus* (Buthidae), which has a lower protein concentration [23]. For *E. alpha*, it has been shown that the male venom glands contain much less of the granule-rich glandular cells that are assumed to produce a more concentrated venom [18]. In our search for sex-specific compounds in the venom of *E. italicus*, we found that only the composition of male venom changes after adult molt. This corresponds to the observed changes in telson morphology of males. In females, apart from size, there are no morphological changes compared to the juvenile stages. Thus, the sex specificity in the venom of *E. italicus* is in fact male specificity. No reproducible differences in the venom composition of females and juvenile scorpions could be found, regardless of whether the juveniles developed into females or males.

Using MALDI-TOF MS, we could conclusively demonstrate that the transparent male venom of *E. italicus* is indeed less complex in terms of peptides. In our subsequent structural elucidations, we focused exclusively on those precursors whose peptide ion signals were clearly different in MALDI-TOF mass spectra from those of females or juveniles. In our approach, we considered the peptides enriched in the venom with a molecular mass of up to 10,000 Da; other substance groups were not included. In the chosen mass range, we were able to identify most precursors of those venom peptides that are either up- or down-regulated in male venom. As verified by Q-Exactive MS and transcriptomics, up- and down-regulation of the respective genes is essentially a quantitative phenomenon. Small amounts of the peptides not detected by MALDI-TOF MS in venom samples from males or juveniles/females are nevertheless always present in both sexes. Similarly, Binford et al. [24] found relative differences in abundance of venom precursors for the spider *Tegenaria agrestis*. It is possible that sex-specific differences in venom composition observed in the past will also turn out to be merely quantitative differences when more sensitive analytical approaches are applied.

The evolutionary driver of a male-specific venom composition in *E. italicus* could be sexual selection [25]. For spiders, it was initially assumed that chemical differences in venom composition result from different feeding preferences of the sexes [26], which was corroborated by frequently observed sexual dimorphisms [27,28]. However, when

Binford et al. [11] wanted to confirm this hypothesis for spiders belonging to the genus *Tetragnatha*, this correlation could not be confirmed. Interestingly, these spiders perform a special courtship behavior in which the chelicera are interlocked. Venom transfer during this procedure is likely, and a function of sex-specific venom compounds in mating is therefore hypothesized [11]. A special courtship behavior in the form of a sexual sting during mating has also been described for *Euscorpius* [17]. This behavior is performed as part of the ritualized mating dance, known as promenade a deux [29], and has meanwhile been observed in several scorpion families [30]. Compared to a predatory/defensive sting, the duration of the sexual sting is extraordinarily long, ranging from few minutes [31] up to 50 min [14]. That the sexual sting involves venom injection into the female was confirmed at least for a Mexican scorpion, which also belongs to the family Euscorpiidae [14]. Given the more transparent male venom of that species, it has been hypothesized that venom injected during sexual sting might contain less toxins to prevent harm to females [23]. Our findings support this assumption, as several of the compounds down-regulated in male venom were classified as potential neurotoxins.

However, as shown here, male venom of *E. italicus* is not simply a diluted solution with less toxins, but a few genes are highly up-regulated, leading to a male-specific enrichment of their mature products in the venom. This enrichment strongly suggests a utilization in a mating-related context. Regarding possible functions of these compounds, several ideas are plausible. Their use could solely have a beneficial effect on the male fitness, e.g., by sedating females to facilitate sperm transfer and reduce the risk of cannibalism [32]. On the other hand, females could benefit, e.g., by assessing male suitability based on the injected compounds [14]. Finally, injection of male-specific compounds might promote fertilization or help relax female musculature to facilitate the mating procedure [23]. In *E. italicus*, the most prominent male-specific venom peptide (EUTX-Ei4) shows similarity to a sodium channel toxin. To unravel the function of such compounds, which may be linked to the sexual sting, future studies should address the effects caused by their injection in more detail. In a first step, male or female crude venom could be injected into females of *E. italicus*; in further experiments synthetic venom peptides could then be used to decipher their specific function(s). It appears reasonable that venom peptides down-regulated in males could be harmful to females, while venom peptides up-regulated in males cause changes in female behavior. Based on the observed down-regulation of several putative neurotoxin genes in male venom glands, also a reduction in venom toxicity for prey might be assumed. Male *E. italicus* do eat and use their stinger to subdue prey (personal observation). A reduced venom toxicity might be compensated by the relatively large male chelae though. In future studies, it might be interesting to test for behavioral difference during prey capture (e.g., frequency of stinging) and to compare male and female venom toxicity to prey.

4. Materials and Methods

4.1. Acquisition and Rearing of the Animals

Ten specimens of *E. italicus* were obtained from an online shop (https://www.vogelspinnen.shop/, accessed on 24 November 2020). These were offspring from specimens originating from Slovenia. Originally, two of these animals were males, while the rest were either females or juveniles. During this study, several juveniles had their adult molt. Two became males, one became female and the remaining juvenile was sacrificed for RNA extraction before the final molt. The scorpions were kept individually in plastic boxes equipped with coconut substrate and a piece of wood as a hideout. Once a week, they were fed with *Acheta* nymphs and water was provided to ensure sufficient moisture. In case the animals were used for venom extraction, feeding was performed immediately afterwards.

4.2. Sex Determination of the Animals

For males, determination of sex and maturity was unambiguous due to the presence of several male-specific morphological traits (telson, pectine organ, and chelal hand [22,33]).

For the remaining specimens (including the one used for RNA extraction), females were identified based on missing male-specific traits. This also enabled differentiating the sex of subadults, as some male-specific traits can already be observed in subadults (male-specific characteristics of pectine organ, presence of genital papillae). Differentiation from instars was mainly based on size (specimens exceeding a body length of 20 mm without metasoma were regarded as adults). As the size alone is a weak criterion for estimating maturity, at least some specimens classified as mature might have been subadult. At least for two females, observations of mating behavior supported their maturity (Supplementary Material Figure S3); instars and unreceptive females successfully fend off male mating attempts [34].

4.3. Venom Collection

To extract the venom, the scorpions were immobilized with rubber bands using the device shown in Figure 5. The release of venom was triggered by applying electricity (12 V, 250 µs pulse width, and 150 Hz pulse rate) to the telson integument using a promed tens device (Promed GmbH, Farchant, Germany) equipped with modified tweezers [35]. Conduct gel was used to improve electrical current flow. Venom was collected into glass capillaries (inner diameter 1 mm; Hilgenberg GmbH, Malsfeld, Germany) and transferred in 20 µL of Millipore water. Storage of venom samples was at −20 °C.

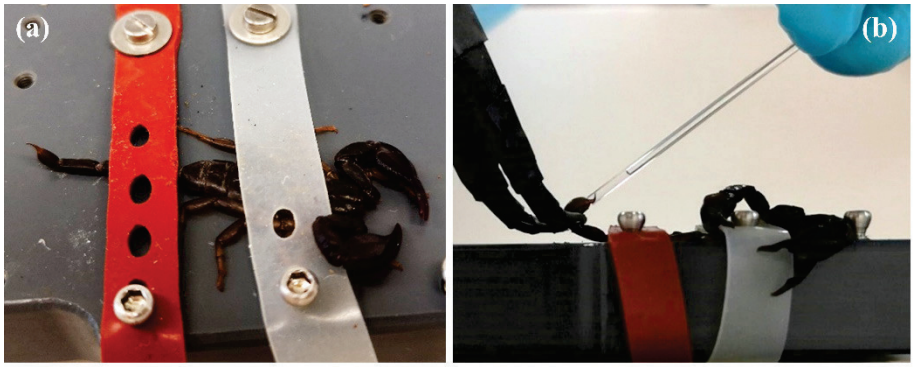

Figure 5. Venom extraction from *E. italicus*. (**a**) Fixation of specimens with rubber tubes. (**b**) Application of electricity with modified pincers and collection of released venom into glass capillaries.

4.4. Quadrupole Orbitrap MS with Nanoflow HPLC

For each sex, two Quadrupole Orbitrap MS experiments were performed, one bottom-up analyses with digested samples and the other without digestion step, but with reduction/alkylation of the sample. For the top-down experiments, venom samples were mixed with an equal volume of urea buffer (8 M urea/ 50 mM triethylammonium bicarbonate buffer) for denaturation prior to reduction/alkylation. For desalting and removal of urea, poly (styrene divinylbenzene) reverse phase (RP)-StageTip purification was performed before Orbitrap MS analyses according to the StageTip purification protocol from the CECAD Proteomics Facility, University of Cologne (http://proteomics.cecad-labs.uni-koeln.de/Protocols.955.0.html, accessed on 15 February 2021). The protein concentration of venom samples (diluted in 20 µL Millipore water) for bottom-up analyses was measured with Direct Detect^{TM} Spectrometer (Merck, Germany), which resulted in a final concentration of 4.935 µg/µL for the diluted male venom and 2.372 µg/µL for the diluted female venom used for the Quadrupole Orbitrap MS. Digestion of venom samples for the bottom-up analyses was performed according to the in solution digestion protocol (http://proteomics.cecad-labs.uni-koeln.de/Protocols.955.0.html, accessed on 15 February 2021). For both sexes, 25 µg of venom was used. Trypsin/LysC digestion was performed with 0.5 µg trypsin and 0.5 µg LysC. Afterwards, the venom compounds/tryptic peptides were separated on an EASY nanoLC 1000 UPLC system (Thermo Fisher Scientific, Bremen, Germany). For this purpose, inhouse packed RPC18 columns with a length of 50 cm

were used (fused silica tube with ID 50 µm ± 3 µm, OD 150 µm; Reprosil 1.9 µm, pore diameter 60 A°; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). UPLC separation was performed with a binary buffer system (A: 0.1% formic acid (FA), B: 80% acetonitrile, 0.1% FA): linear gradient from 2 to 62% in 110 min, 62–75% in 30 min, and final washing from 75 to 95% in 6 min (flow rate 250 nL/min). Re-equilibration was performed with 4% B for 4 min. The UPLC was coupled to a Q-Exactive Plus (Thermo Fisher Scientific) mass spectrometer. HCD fragmentations were performed for the 10 most abundant ion signals from each survey scan in a mass range of m/z 300–3000. The resolution for full MS1 acquisition was set to 70,000 with automatic gain control target (AGC target) at 3×10^6 and a maximum injection time of 80 ms. In order to obtain the HCD spectra, the run was performed at a resolution of 35,000, AGC target at 3×10^6, a maximum injection time of 240 ms, and 28 eV normalized collision energy; dynamic exclusion was set to 25 s.

4.5. MALDI-TOF MS

MALDI-TOF mass spectra were generated for two venom samples extracted from each of the ten specimens with a one-week interval. Venom samples used for MALDI-TOF MS were diluted in a mixture of ethanol and TFA (final concentration: 35%/0.1%) to achieve optimal analyte/matrix ratios. As reference the volume of extracted crude venom was used. Optimal dilutions were tested beforehand in dilution series performed with venom from both sexes and juveniles. Subsequently, 0.3 µl was directly spotted onto the sample plate and mixed with the same volume of 10 mg/ml 2.5-dihydroxybenzoic acid (Sigma Aldrich, Steinheim, Germany) matrix, dissolved in 50% acetonitrile/0.05% TFA. For an optimal crystallization of the matrix, samples were blow-dried with a hairdryer. An ultrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) was used in reflectron positive mode with overlapping mass ranges of m/z 800–4500 and m/z 3000–10,000. For an optimal signal-to-noise ratio, laser intensity and the number of laser shots were adjusted for each sample. Laser frequency was set to 666 Hz. For external calibration, a mixture containing proctolin ($[M+H]^+$, 649.3), Drm-sNPF-$21^{2–19}$, ($[M+H]^+$, 974.5), Pea-FMRFa-12 ($[M+H]^+$, 1009.5), Lom-PVK ($[M+H]^+$, 1104.6), Mas-allatotropin ($[M+H]^+$, 1486.7), Drm- IPNa ($[M+H]^+$, 1653.9), Pea-SKN ($[M+H]^+$, 2010.9), and glucagon ([M+H]+, 3481.6) was used for the mass range of m/z 800–4500 and a mixture of bovine insulin ($[M+H]^+$, 5731.5), glucagon and ubiquitin ($[M+H]^+$, 8560.6) was used for the mass range of m/z 3000–10,000. Ion signals were identified by using the peak detection algorithm SNAP from the flexAnalysis 3.4 software package. In addition, each spectrum was manually checked to ensure that the monoisotopic peaks were correctly identified.

4.6. RNA Extraction, Transcriptome Sequencing and De Novo Assembly of Nucleotide Sequences

For both sexes, one transcriptome was generated for the telson comprising the venom glands. As a negative control, one transcriptome was generated for parts of the scorpion tail without the venom glands. RNA extraction was performed with TRIzol (Thermo Fisher Scientific, Darmstadt, Germany) according to the standard manufacturer's user guide. Libraries were prepared starting from 1 µg of total RNA with the Illumina® TruSeq® stranded RNA sample preparation Kit. Vallidation and quantification of libraries were performed with the Agilent 2100 Bioanalyzer. Paired-end sequencing was performed using an Illumina TruSeq PE Cluster Kit v3 and an Illumina TruSeq SBS Kit v3—HS on an Illumina HiSeq 4000 sequencer. Adapter removal and quality trimming of resulting raw files was performed using Trimmomatic 0.3.2 [36]. Afterwards, de novo assembly of RNA sequence data was performed with Trinity v2.8.5 [37] based on default settings. This assembly was used to search for peptide sequences obtained by Quadrupole Orbitrap and MALDI-TOF MSMS experiments. To assess the completeness of transcriptomic data, BUSCO 4.1.4 [38] was used. Transcriptomic data were submitted to the National Center for Biotechnology Information (NCBI, Bioproject: PRJNA877843).

4.7. Identification of Venom Compounds

Identification of venom compounds was performed similarly as described in [39]. Precursors of potential venom compounds were identified by matching the fragment spectra of Quadrupole Orbitrap MS analyses against the telson-transcriptomes of male and female *E. italicus*, utilizing the software PEAKS 10 (PEAKS Studio 10; BSI, Toronto, ON, Canada). Then, precursors were characterized by predicting a signal peptide with SignalP 6.0 [40], checking for the presence of a stop codon, searching for respective precursors in the negative control (BLAST search against the remaining telson transcriptome with an E-value of 1×10^{-5}) and estimating the expression level in transcriptomic data with Kallisto [41]. Additionally, the precursors were classified based on similarities to compounds from the online database UniProt [19], by performing local BLAST searches in the Metazoa database (search term: 'taxonomy:"Metazoa [33, 208]') and the Tox-Prot database (search term 'taxonomy:"Metazoa [33, 208]" (keyword:toxin OR annotation:(type:"tissue specificity" venom))). As E-values, 1×10^{-5} was used for the search in the Metazoa database and 0.1 for the Tox-Prot database. Classification of the matches was performed based on the description of the BLAST hits, for the Tox-Prot database only in case the E-value was lower than 1×10^{-5}. Finally, the matches were filtered with respect to the quality of MS data, the coverage between transcriptomic and proteomic data (false discovery rate ($-10\lg P$) > 30, coverage > 7%) and the presence of a signal peptide. To perform a functional annotation all identified venom precursors were analyzed with InterProScan [42].

To verify the presence of the identified venom precursors in the venom, theoretical masses calculated for each of the potential bioactive peptides (predicted from the identified venom precursors) were searched against a list of ion signals from MALDI-TOF mass spectra of the venom.

The presence of corresponding ion signals in mass fingerprints of the venom was also utilized to assess the sex specificity of venom compounds. For this estimation, only highly reproducible MALDI-TOF ion signals were considered (occurrence in at least 50% of the spectra). This assessment was based on the generation of repeated MALDI-TOF mass spectra for two adult males, four adult females and four juveniles.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms231911020/s1. Reference [43] is cited in the supplementary materials.

Author Contributions: Conceptualization, J.K. and R.P. (Reinhard Predel); methodology, J.K.; validation, J.K., R.P. (Ricardo Pommerening) and R.P. (Reinhard Predel); formal analysis, J.K. and R.P. (Ricardo Pommerening); investigation, R.P. (Ricardo Pommerening); resources, R.P. (Reinhard Predel); data curation, J.K.; writing—original draft preparation, J.K. and R.P. (Reinhard Predel); writing—review and editing, R.P. (Ricardo Pommerening); visualization, J.K.; supervision, R.P. (Reinhard Predel); project administration, J.K. and R.P. (Reinhard Predel). All authors have read and agreed to the published version of the manuscript.

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3 Discussion

The major aim of this dissertation was to investigate understudied aspects of venom research of the Panscorpiones group. On the one hand, I focused on characterizing the venom of pseudoscorpions, a group which had been nearly completely neglected by venom research. On the other hand, I wanted to enhance the knowledge about sex-specific venom compounds within Panscorpiones. In the following, the achieved advances on identifying pseudoscorpion venom compounds and their activity will be discussed in the context of modern venomics. The sex-specific expression discovered for the scorpion species will be discussed in a phylogenetic context regarding other scorpion families and pseudoscorpions.

3.1 A Pseudoscorpion's Promising Pinch: The venom of *Chelifer cancroides* contains a rich source of novel compounds

In the first study, we characterized the venom of the house pseudoscorpion by utilizing a combination of proteomic and transcriptomic workflows. This enabled us not only to perform the first comprehensive investigation of the venom composition for this species, but also to identify the first pseudoscorpion neurotoxins for which the presence in the venom is confirmed by proteomic data. This work complements a preliminary study in which we established the methodology for extracting pseudoscorpion venom for proteomics and identified the first genuine venom compounds which we named checacins (Krämer et al., 2019). Beforehand, only one other study had investigated the venom composition of a pseudoscorpion (*S. apimelus*), utilizing a solely transcriptomic approach (Santibáñez-López et al., 2018). For this species, venom compounds were identified based on similarity to toxins from online databases, resulting in a venom composition dominated by enzymes with only a few peptide toxins. Interestingly, none of the peptide toxins described in our study show similarities to the peptide toxins identified for *S. apimelus*. However, blasting these toxins against the whole transcriptome of the house pseudoscorpion produced matches with high similarity. This might be an indication, that the peptide toxins identified for *S. apimelus* are not part of the actual venom composition but could be false positives. This is a known issue of applying solely transcriptomic approaches for the identification of venom compounds (Smith and Undheim, 2018). On the other hand, the transcriptomic data provided for *S. apimelus* did not show any clear matches with the toxins identified for *C. cancroides*. This indicates either a strong deviation of the venom composition between these two species, or an incompleteness of the generated transcriptomic data. Venom compositions even within a group can be subject to pronounced deviations from the 'regular' venom composition, as shown in case of the wasp spider *Argiope bruennichi* (Lüddecke et al., 2020). *S. apimelus* and *C. cacroides* are not closely related (Benavides et al., 2019) and hence a relatively strong deviation in their venom can be expected. Future research could analyze the venom of *S. apimelus* by utilizing a proteomic approach to confirm which compounds are genuinely part of the venom composition. In a different study, also the venom composition of a pseudoscorpion from the Chernetidae family was investigated with a solely transcriptomic approach (Lebenzon et al., 2021), but a detailed comparison with the compounds identified for *S. apimelus* and *C. cancroides* still needs to be done.

One achievement of the first included study was to provide first insights about the neurotoxic effects caused by crude venom of the house pseudoscorpion by measuring its activity on selected ion channels. Applying the pseudoscorpion venom to a potassium channel (Shaker) from *Drosophila* expressed in oocytes caused a clearly decreased ion current through these channels, which was detected with the two-microelectrode patch clamp method. This indicates that the venom contains potent potassium channel blockers, which might be responsible for the fast paralysis observed for *Drosophila* after venom injection by the house pseudoscorpion (personal observation). In addition, the venom modulated the inactivation process of a sodium channel form the *Varoa* mite, a pest of the honeybee. The house pseudoscorpion is known for its potential as a biological pest control against this mite (van Toor et al., 2015). The observed activity of its venom shows the potential of finding lead-compounds for pesticides, specifically targeting *Varoa*.

A major goal of this first publication was to confirm the presence and identify of peptide neurotoxins in the venom of the house pseudoscorpion. Such toxins are commonly discovered in arachnid venoms from spiders and scorpions (Langenegger et al., 2019; Quintero-Hernández et al., 2013). In our publication, several precursors were found that show moderate similarity to toxin sequences deposited in the Uniprotdatabase, all belonging to scorpions and spiders. These potential neurotoxins exhibit six to ten Cysteines, most likely forming three to five disulfide bridges. The best annotated BLAST-hits are potassium channel toxins from buthid scorpions found for CHTX-Cc1a and b. At least for one of these (Potassium channel toxin alpha-KTx 1.16), the inhibitory effect on potassium channels was demonstrated (Kuzmenkov et al., 2018). Hence, CHTX-Cc1a and b were considered the most likely candidates for causing the effects on the potassium channel observed for the crude venom. However, the similarity of these toxins to their BLAST-hits is relatively low and even slight sequence differences can be sufficient to alter the activity of a neurotoxin (Peigneur et al., 2012). In addition, both compounds lack the toxin signature described by Zhu et al. (2014) for potassium channel toxins. It is noteworthy, that all scorpion BLAST-hits come from the family Buthidae. This might be explained by the overrepresentation of this scorpion family in the Uniprotdatabase (von Reumont et al., 2022), though it could also indicate that pseudoscorpion venom is more similar to scorpion venom from the parvorder Buthida in contrast to that of Iurida. In this regard, it would be promising to investigate the venom of the most basally branching venomous pseudoscorpions and compare their venom composition with the most basal scorpion groups.

Besides finding potential neurotoxins, we mostly identified compounds classified as ‘novel’ with no significant matches to compounds from the database. This demonstrates the potential to find uncommon compounds in pseudoscorpion venom, which was also demonstrated for other neglected groups like remipedes, robber flies or blood worms (Drukewitz et al., 2018; von Reumont et al., 2017, 2014c).

In addition, also peptides with similarity to AMPs were discovered, which, except for one potential defensin, were mainly classified as additional checacins. These compounds were selected as candidates for further, more specific activity tests and will be discussed in the next subchapter.

With respect to precursor processing, most of the pseudoscorpion venom precursors are cleaved at dibasic sites or the quadruplet motif (Kozlov et al., 2005) as described for scorpion and spider venoms. In our first publication about the venom of the house pseudoscorpion, we described the 'LEAP'-motif, a novel unique cleavage site of pseudoscorpion venom compounds (Krämer et al., 2019). In the work presented in chapter 2.1, we found additional evidence supporting the presence of this site, though it is subject to some variations (e. g. LESP instead of LEAP). Most important for this cleavage site seems to be the C-terminal Proline, which was present in all cases. Considering post-translational modifications (PTMs), we mostly found amidations and disulfide bridges, which are also frequently described for scorpions and spiders (Delgado-Prudencio et al., 2019; King and Hardy, 2013). The only more exceptional PTM that we found for some of the compounds was the N-terminal pyroglutamate formation. In this context, Marchi et al. (2022) highlight the need for more thorough investigation of PTMs in arachnid venoms as at least for the venom compounds from *Tityus serrulatus* e. g. phosphorylation and N-linked glycosylation were described (Verano-Braga et al., 2013).

Another point left for discussion is the comprehensiveness of the venom analysis we performed for the house pseudoscorpion. Our study meets the state-of-the-art criteria of venom research, as we relied on a combination of proteomic and transcriptomic data to identify the venom compounds (von Reumont et al., 2022; Walker et al., 2020). To ensure the identification of a maximal number of venom compounds, our proteomic workflow comprised both a top-down and a bottom-up approach, which differ in suitability depending on protein size and properties (Slagboom et al., 2022). For the bottom-up analysis we applied the single-pot, solid-phase-enhanced procedure (Hughes et al., 2019) which is especially useful for small amounts. In addition, we performed the bottom-up analysis with three different amounts of venom, demonstrating that an increase did not allow for identifying additional compounds. Moreover, we allocated the venom compounds identified by Orbitrap MS to corresponding mass signals of MALDI-TOF MS mass fingerprints of the venom. This is an additional confirmation of the genuine venom compound masses, as MALDI-TOF MS especially well preserves the native compounds. By allocating most of the dominant MALDI-TOF MS masses to the identified compounds, we could demonstrate the comprehensiveness of our study, especially as the overlap between MALDI-TOF MS and Orbitrap MS was shown to be relatively small (Biass et al., 2009). Figure 3 visualizes the complete integrated proteomic and transcriptomic workflow applied for analyzing the venom of the house pseudoscorpion.

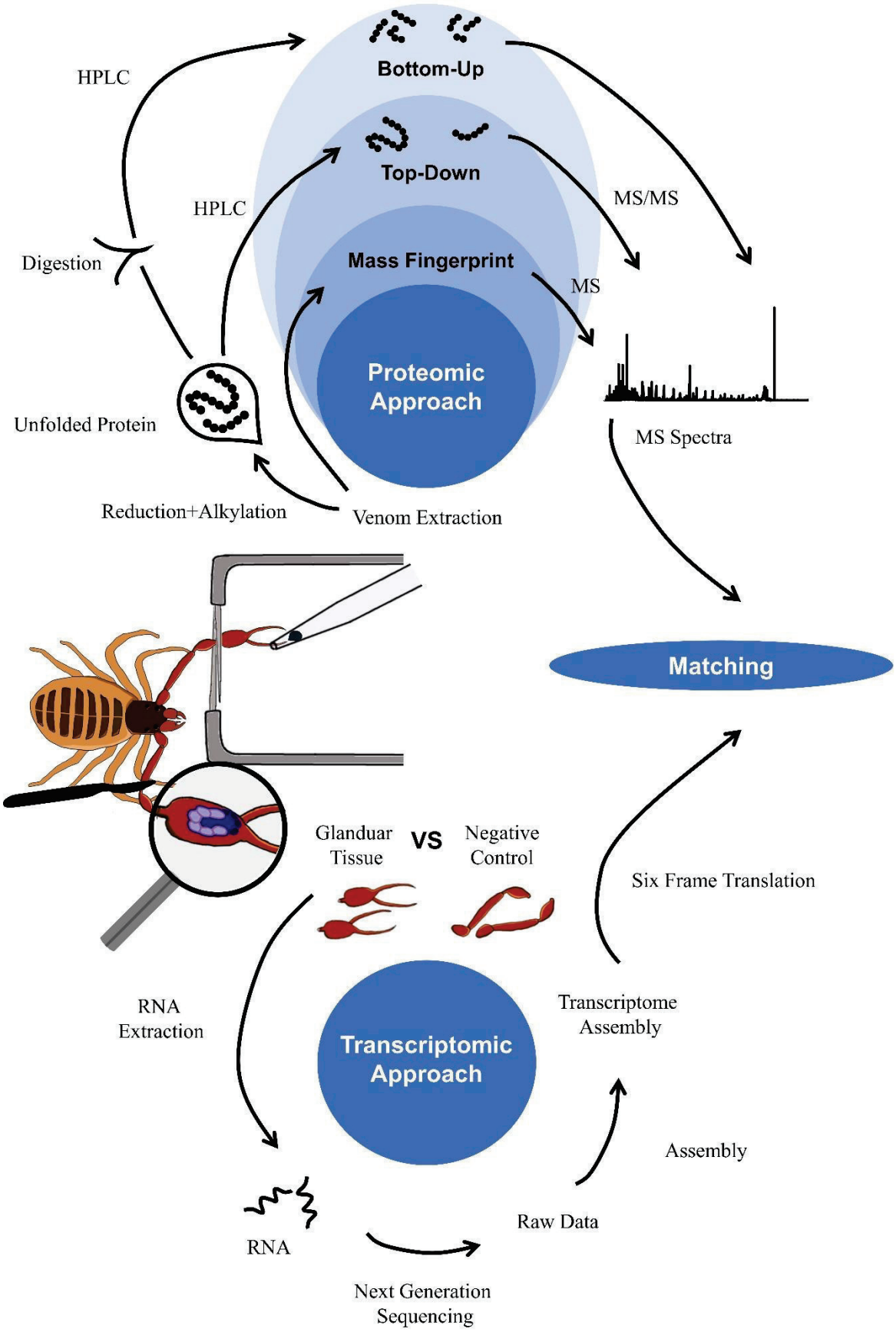

Figure 3. Combined proteomic and transcriptomic workflow to identify venom compounds from the pseudoscorpion *C. cancroides*. Bottom-up proteomics was performed for three samples with different venom amounts (venom from 24, 44 and 64 extractions).

This first publication was the foundation for further investigations. Here we provided an overview of the identified pseudoscorpion venom compounds. The following studies focused on functional aspects of pseudoscorpion venom, in which we applied different strategies to characterize pseudoscorpion venom fractions and compounds with respect to their specific activity.

3.2 Antimicrobial, Insecticidal and Cytotoxic Activity of Linear Venom Peptides from the Pseudoscorpion *Chelifer cancroides*

In the second publication included into this dissertation, we performed the first activity tests for pseudoscorpion venom compounds on organismic level utilizing bioassays with microbes, mammalian cells and aphids. For these tests, we selected Checacin1, the first compound we identified in the venom of *C. cancroides* (Krämer et al., 2019). The checacins are linear cationic peptides with no Cysteines and, by now, we have described seven in total (chapter 2.1, Krämer et al., 2021). Based on a similarity of Checacin1 to Megicin-18, an AMP characterized for the venom of the scorpion *Mesobuthus gibbosus* (Diego-García et al., 2014), we preliminarily classified the checacins as AMPs. For Megicin-18 the antimicrobial activity had been confirmed based on activity tests against several gram-positive bacteria including multi-resistant strains (Liu et al., 2018). In addition, the therapeutic potential of Megicin-18 had been demonstrated based an effective treatment of mice infected with methicillin-resistant *Staphylococcus aureus* (Liu et al., 2018). In general, such peptides often take effect by acting on cell membranes, inducing pore formation, which results in leakage of intercellular components (Hancock et al., 1995). This mode of action was also demonstrated for Megicin-18 by documenting the disruption of bacterial cells using transmission electron microscopy (Liu et al., 2018). The selectivity of membrane-lytic peptides depends on properties like their charge state (Hancock et al., 1995). Cationic linear peptides show a higher affinity for bacterial cell membranes. These are generally more negatively charged compared to membranes of animal cells (Yeaman and Yount, 2003). Checacin1 exhibits another feature relevant for a membrane-lytic activity, a hydrophobic N-terminus, which is inserted into the membrane causing the pore formation (Langenegger et al., 2019). In figure 4, the proposed mechanism of action assumed for Checacin1 is visualized. For testing the activity of Checacin1, peptides were produced by chemical synthesis which is a fast and cost-efficient way for producing linear peptides without complex PTMs. Besides the complete Checacin1 sequence, also naturally occurring (Checacin$^{1-11}$, Checacin$^{12-25}$) and one artificial fragment (Checacin$^{1-21}$) of the Checacin1 sequence were synthetized. This was done for two reasons. First, for the naturally occurring fragments, which show corresponding signals in MALDI-TOF MS mass fingerprints of the venom, we wanted to investigate, if these fulfill an actual function in the venom. Second, testing the activity of the fragments provided insights on which part of the sequence is crucial for the activity of the complete Checacin1.

Regarding the activity on microbes, only the complete Checacin1 and the artificial fragment Checacin$^{1-21}$ showed an effect on the tested strains. Checacin1 inhibited the growth of all tested bacterial strains also including the gram-negative strains with MIC comparable to that of commercially available antibiotics and also showed inhibiting activity against one of the tested fungi. Checacin$^{1-21}$ only affected one of the tested bacterial strains. This highlights the

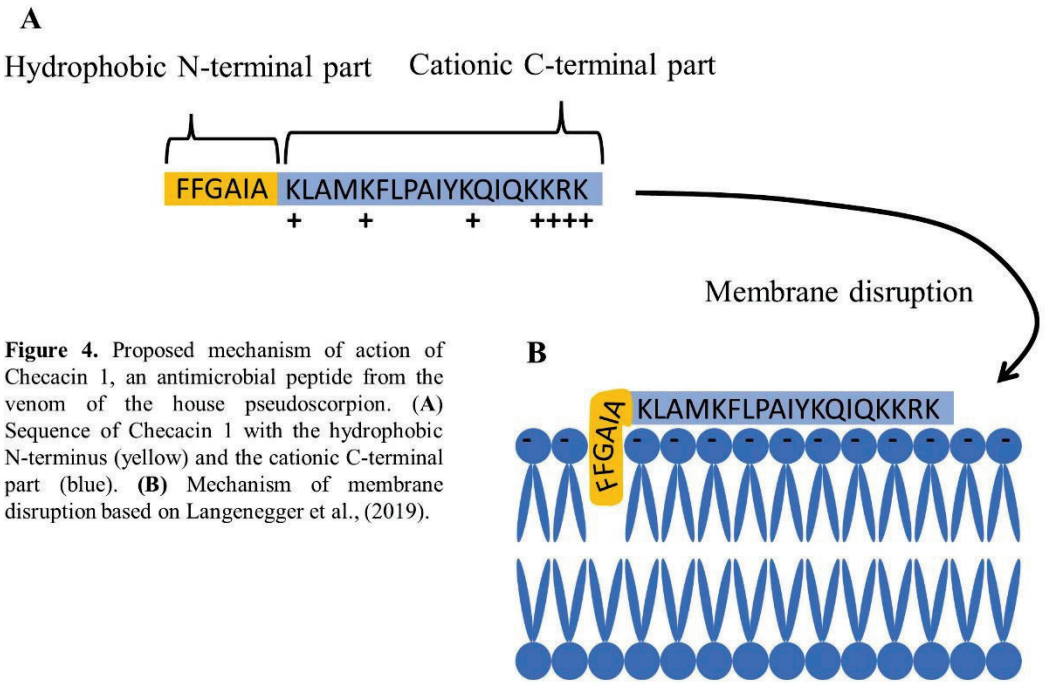

Figure 4. Proposed mechanism of action of Checacin 1, an antimicrobial peptide from the venom of the house pseudoscorpion. **(A)** Sequence of Checacin 1 with the hydrophobic N-terminus (yellow) and the cationic C-terminal part (blue). **(B)** Mechanism of membrane disruption based on Langenegger et al., (2019).

importance of the cationic C-terminus of the complete Checacin1, that is missing in this fragment. Both peptides also showed cytotoxic effects after application to a mammalian cell culture, and also in this case Checacin1 was substantially more potent. Due to these cytotoxic effects, the potential of both peptides for application as drug leads is limited, though the concentration causing an inhibitory effect on bacteria was clearly lower. All the tested peptides were shown to reduce the survival rate of aphids, but the complete Checacin1 and Checacin$^{1-21}$ killed aphids much more efficient, on a comparable level as a commercial insecticide. In previous studies it was demonstrated that decreased aphid survival after application of AMPs can be caused by the loss of the aphid's bacterial symbionts (Luna-Ramírez et al., 2014). As Checacin1 and Checacin$^{1-21}$ showed an activity on bacteria, these might as well affect the symbionts present in the gut biome of the aphids. Nevertheless, Checacin1 seems to act on a wide variety of membranes and hence, a cytotoxic effect also on insect cells can be assumed. It is noteworthy that the peptides were tested in a feeding assay and were stable enough to pass the insects digestive system. In this regard, two of the tested peptides (Checacin1 and Cheacin$^{12-15}$) were produced with N-terminal amidation, which is a PTM protecting the peptide against proteolytic cleavage. Interestingly, also the peptides without amidation were effective against the aphids.

As some linear venom peptides were shown to be highly specific to bacteria, initially these peptides were classified as AMPs and an antibacterial activity can be confirmed in many cases (e. g. Harrison et al., 2014). Regarding the function in venom, it was speculated that AMPs protect the venom against bacteria. However, in many cases such peptides are not selective to bacteria but also cytotoxic to eucaryotic cells (Almaaytah and Albalas, 2014), which indicates that they play a more active role in envenomation of the prey. This highlights the importance of performing activity tests on a broad spectrum of organisms.

With the second included publication we demonstrated that Checacin1, a peptide we had previously classified as AMP, indeed is highly effective in killing bacteria, though this peptide

also showed cytotoxic and insecticidal activities. Based on the sequence properties of this peptide, it is likely that it acts by pore formation which is confirmed for Megicin-18 that shares nearly half of the sequence. Besides investigating pseudoscorpion AMPs, another focus of this dissertation was to further characterize the potential neurotoxins that we had identified for the house pseudoscorpion (chapter 2.1). Our progress in this regard will be discussed in the next subchapter.

3.3 Fractionation of Pseudoscorpion Venom for Activity Tests on Specific Ion Channels

Chapter 2.3 of this dissertation describes our advances in fractionating the venom of the house pseudoscorpion with HPLC and measuring the effect of the resulting fractions on specific ion channels. Our aim was to narrow down which venom compounds cause the previously described effects on potassium and sodium channels and the research is still ongoing. By using HPLC coupled to a highly sensitive mass spectrometer (MaXis), we could demonstrate an effective fractionation of the house pseudoscorpion's venom, as most of the previously identified venom compounds could be allocated to different chromatogram peaks. However, activity tests with selected fractions of this HPLC run did not cause any effect on neither the sodium nor the potassium channel. As the activity tests were performed in a manual set-up, it was not possible to test all fractions resulting from the HPLC-run. Hence, the bioactive peptides we were looking for might not have been part of the tested fractions. It is also likely that the concentration of toxins after reconstitution was too low to inflict any effect on the tested ion channels. For tackling the latter issue, it might be promising to test the resulting fractions in a more comprehensive way that allows to localize the bioactive compounds in a test with the whole sample plate. One option would be to apply an automated test system for the two-microelectrode patch clamp method as described in Baburin et al. (2006). However, systems like this are not easily adaptable for smaller volumes, which is a precondition for testing the pseudoscorpion venom fractions. Hence, another option might be to perform a ligand-binding assay (Kool et al., 2011; Yu et al., 2016) with the fractionated pseudoscorpion venom. A screening like this would allow to identify the fractions containing the neurotoxins binding to selected channels, without providing further information on the kind of modulation. These binding assays can be applied automated, on-line to HPLC and MS, allowing for an efficient high throughput screening of venom compounds as demonstrated by Heus et al. (2013). The fractions identified by the ligand-binding assay could then be used to estimate the specific effect using the two-microelectrode patch clamp to measure the modulation of the ion channels expressed in oocytes. However, these screening systems are configured to certain ion channels like the nicotinic acetylcholine receptor and cannot easily be modified for other ion channels. The reason for this is that binding assays require a soluble protein that can be taken as a surrogate for the complete ion channel (Heus et al., 2013).

In case pseudoscorpion venom cannot be obtained in sufficient quantities for these approaches, another option is to produce selected toxins using synthesizers or recombinant production (von Reumont et al., 2022).

In summary, chapter 2.3 of this thesis shows our progress on identifying pseudoscorpion neurotoxins and characterizing their activities, which is mostly complicated by the tiny venom

amounts obtainable from pseudoscorpions and the high degree of novelty of their compounds that makes it hard to identify the best candidates for recombinant production.

3.4 Equipped for Sexual Stings? Male-Specific Venom Peptides in *Euscorpius italicus*

With the third publication included in this dissertation, we focused on another understudied aspect of Panscorpion venom research, which concerns the sex-specific expression of venom compounds. This phenomenon has superficially been observed for many scorpion venoms (e. g. Miller et al., 2016; Olguín-Pérez et al., 2021; Sousa et al., 2010), though only a few studies have identified the corresponding compounds or compared the expression levels between the sexes (Cid Uribe et al., 2017; Rodríguez-Ravelo et al., 2015; Ward et al., 2018; Yang et al., 2022). Also, the specific function these compounds might fulfill in a mating-related context remains unknown. Within scorpions, sexual dimorphisms occur, but these differ greatly in their expression. For some subgroups this mostly comprises slight differences in size and proportions barely noticeable (e. g. Lira et al., 2018), whereas other subgroups can exhibit pronounced differences of the telson morphology that are clearly visible (e. g. Sentenská et al., 2017). Another relevant aspect is the performance of a sexual sting during mating which was observed at least for seven scorpion families (Lira et al., 2018; Sentenská et al., 2017). Depending on the species, the duration of the sexual sting can be relatively short or, in extreme cases, take up to 50 minutes (Olguín-Pérez et al., 2021). At least for one species, evidence of actual venom transfer during this behavior was provided (Olguín-Pérez et al., 2021). It is likely that the different degree of sex-specific differences in the venom often has evolved alongside the tendency to perform a sexual sting. A mating related function of such compounds is highly reasonable for subgroups performing the sexual sting and exhibiting pronounced sexual dimorphisms. In this regard, it was shown for a buthid scorpion which occasionally performs the sexual sting during mating, that this behavior increases the mating success (Lira et al., 2018). Figure 1 (Introduction) shows the most recent scorpion phylogeny, in which those families are marked that are known to perform a sexual sting. This demonstrates that this behavior occurs throughout the scorpion tree, which might be an indication for a plesiomorphic trait.

To advance the knowledge on sex-specific venom compositions for scorpions, we performed a comprehensive analysis for the venom of *E. italicus*. For the genus *Euscorpius* a distinctive sexual dimorphism of the telson morphology was described, which also affects the architecture of the venom gland (Sentenská et al., 2017). In addition, these scorpions are known to perform extensive sexual stings (Braunwalder, 2005). In personal observations I could document that females trying to fend off approaching males by pedipalp beating and stinging, ceased all aggressive behavior after the first successful sexual sting placed by the male. Hereafter, the female completely followed the male's guidance in the promenade a deux. It seems evident that this effect is caused by compounds injected during the sexual sting. The behavioral alteration might be comparable to that of cockroaches after being stung by *Ampulex compressa*, which lose all movement initiative but can still be guided by the wasp towards the borrow (Fouad et al., 1996). However, in case of the female scorpions the effect is highly reversible.

In chapter 2.4 of this dissertation, we demonstrated that MALDI-TOF MS mass fingerprints generated for both sexes of *E. italicus* in the mass range from 3,000-10,000 Da clearly deviate. Based on these spectra, these differences would be classified as absolute. However, our comprehensive integrated transcriptomic and proteomic investigation revealed that the observed differences, result from a different level of gene expression and are indeed not absolute. As an outcome of this analysis, we obtained the sequence of the most prominent compound upregulated in male venom. This compound shows a 50% sequence similarity to a potential sodium channel toxin from the venom of the non- buthid scorpion *Superstitionia donensis* (Santibáñez-López et al., 2016). For this species, forming the monotypic family Superstitioniidae, the performance of a sexual sting during mating has not been described. As the potential effect on sodium channels was only assessed based on similarity, the actual effect caused by both compounds still has to be demonstrated.

In our analysis, we also found toxins that are much less expressed in male venom than in female and juvenile venom. Many of these are similar to neurotoxins from other non-buthid scorpions and we assume that these compounds might be harmful for females and were downregulated in male venom for this reason. For many of the compounds down-regulated in male venom, BLAST-hits were found with toxins from *Megacormus gertschi*, a scorpion also belonging to the Euscorpiidae family that performs a sexual sting. For the main compound up-regulated in male venom though, the bets BLAST-hit belongs to a much more distantly related scorpion. In the venomics study on *M. gertschi*, it was not stated how many specimens were used for venom and RNA extraction and their sex was not specified (Santibáñez-López et al., 2017). Hence, it might be that only females were used in that study, which would explain that we found BLAST-hits for those compounds up-regulated in female venom, but none for those up-regulated in male venom. Moreover, we found that the expression of venom compounds indeed is not sex-specific but male-specific. We concluded this based on the high similarity of venom mass fingerprints from females and juveniles. We could also demonstrate the manifestation of the male-specific morphology after the adult molt which is accompanied by the transition towards the typical male-specific expression of venom compounds.

Apart from the medically relevant Buthidae, the venom of the remaining scorpion families was much less investigated. Only a few reviews on the coverage of scorpion venomics exist. For example, Santibáñez-López et al. (2015) provided a comprehensive overview of the Mexican scorpion families investigated in this regard. Furthermore, Smith and Alewood (2015) stress that in case of the non-buthid scorpion families often solely transcriptomic approaches were applied without confirmation by proteomic data. By performing an up-to-date literature search as part of this thesis, I could show that most non-buthid families are still very underrepresented in venom research, as either only single compounds are identified, or proteomic data is still lacking (figure 1, SM 1.1). For some families, not a single venom study was identified in my review and for most of those covered, venom research was performed for single representatives. Besides our interest in their venom's sex specificity, we selected a representative of the *Euscorpius* group for a comprehensive venom analysis as this scorpion genus, that is distributed throughout much of Europe, had so far been completely neglected by venom research. Moreover, *M. gertschi* is the only other representative of the Euscorpiidae family investigated in this regard (Santibáñez-López et al., 2017). The coverage of this family used to be larger when Scorpiops still was classified into Euscorpiidae, but this genus was reclassified to the reinstated family Scorpiopidae (Sharma et al., 2015). With the publication on the venom

composition of *E. italicus*, we could add to the scarce knowledge of venom compounds from the scorpion family of Euscorpiidae. Our publication focused on the sex-specific compounds, though we did perform a comprehensive investigation of the whole venom identifying much more compounds than highlighted in the paper (SM 4.1).

More comprehensive investigations of the venom composition of non-buthid scorpion venoms can be provide a promising source of compounds with potential for therapeutic application. One reason is that most of these venoms are relatively harmless for humans, but still many compounds with beneficial activities can be identified as demonstrated for the genus *Heterometrus* (Gomes and Gomes, 2015). In addition, due to the bias of venom research towards Buthidae the non-buthid families have still a higher potential for identifying novel compounds as shown by the high number of venom compounds with no significant BLAST-hits, we identified for *E. italicus*. Likewise, with the identification of an alkaloid in scorpion venom, Banerjee et al. (2018) demonstrated the high potential of finding uncommon compounds in non-buthid venoms.

In the context of this thesis, a yet unanswered question remains: Do pseudoscorpion venoms also contain sex-specific compounds, and do they perform a behavior similar to that of the sexual sting? Comparing mass fingerprints of male and female house pseudoscorpions of repeated venom extractions did not reveal any considerable differences (Supplementary material SM 5.1), at least within the observed mass range. For this reason, we did not differentiate between sexes for performing the comprehensive analysis described in the first chapter of this dissertation. With respect to a sexual dimorphism, there are differences between the sexes in case of house pseudoscorpions, though this does not affect the external morphology of the venom delivery system. The house pseudoscorpion belongs to the Cheliferoidea superfamily, the only pseudoscorpions which retained the promenade a deux (Ontano et al., 2021). Although I could not find any evidence for venom transfer during mating, pseudoscorpions belonging to Cheliferidae indeed perform a unique mating behavior utilizing ram's horn organs (Kirchmair and Raspotnig, 2021). These paired evaginations of the posterior ventral diverticulum are only extruded during courtship. It was shown that females react with decreased resistance after initial extrusion of these organs, which is comparable to the female reaction after a sexual sting in case of scorpions. For the ram's horn organs, a release of pheromones was proposed (Weygoldt, 1969), though this remains to be proven. In case of the sexual sting applied by *E. italicus*, we are now one step ahead in understanding their mating-related use of venom by identifying the male-specifically expressed compounds that are promising candidates for activity tests to better understand their effects on female scorpions.

4 Conclusion and Outlook

The overall aim of this dissertation was to advance knowledge about understudied aspects of Panscorpion venom research. To do so, I focused on investigating pseudoscorpion venom which was only scarcely considered by previous studies. Moreover, I added to the limited research on sex-specific characteristics of Panscorpion venoms. In chapter 2.1, I describe the application of our previously developed pseudoscorpion venom extraction procedure that allowed to obtain sufficient venom amounts for state-of-the-art proteomic workflows. This enabled the first comprehensive analysis of a pseudoscorpion's venom integrating transcriptomic and proteomic data. Hereby, we described the first potential pseudoscorpion neurotoxins whose presence in the venom is confirmed and we identified numerous novel compounds with no significant similarities to previously described substances. Another achievement was the performance of activity tests with pseudoscorpion crude venom on specific ion channels. These findings provide insights on how pseudoscorpions are capable to rapidly paralyze their prey. With our studies on the venom composition of the house pseudoscorpion, we also identified the checacins, which were investigated in chapter 2.2 in more depth. For Checacin1, which corresponds to the most prominent signal in MALDI-TOF MS mass fingerprints of the venom, we revealed potent activities against several microbes including gram-negative and gram-positive strains. In addition, an aphid feeding assay with this peptide demonstrated insecticidal activities and a sufficient stability to pass an insect's digestive system. However, the applicability as a lead compound of Checacin1 is limited due to the also discovered cytotoxic activity on mammalian cells. Based on the sequence properties of Checacin1, this peptide most likely takes effect by disrupting cell membranes which was also demonstrated for Megicin-18, a similar AMP from scorpion venom. Besides Checacin1, we have identified additional checacins in the venom of the house pseudoscorpion, which all vary in their relative abundance. Our ongoing research builds up on this by investigating how the abundance of checacins in the venom affects their efficiency. For this reason, additional synthetized checacins are tested in the same set-up described in chapter 2.2 of this thesis. With respect to the neurotoxic activity caused by the venom, we aimed to narrow down the toxic activity to fractions obtained with an HPLC run. The current status of this approach is summarized in chapter 2.3 of this thesis. So far, the first fractions were tested in a manual set-up. Due to the high number of novel compounds in the venom of the house pseudoscorpion, it is hard to identify the actual toxins causing the previously observed activities on ion channels by crude venom. Hence, it might be more convenient to test the activity of venom fractions with a more comprehensive automated strategy like a binding assay established e. g. for the Acetylcholine receptor. However, establishing a binding assay for new receptors is laborious. As it could be that the venom amounts we can obtain from pseudoscorpions are insufficient for this approach, I also plan to utilize recombinant production to generate selected neurotoxins and novel compounds. Despite the contribution of the work summarized in this dissertation, pseudoscorpion venom remains poorly investigated as existing advances on pseudoscorpion venom research only cover three species from different families. The remaining 18 families of venomous pseudoscorpions remain to be studied in this regard. In addition, the low similarity of the venom compositions between *S. apimelus* and *C. cancroides* is conspicuous. In ongoing research, we are investigating the venom of another garypid pseudoscorpion with the aim to identify homologs to the previously identified pseudoscorpion venom compounds. Future research should

furthermore perform a proteomic analysis for the venom of *S. apimelus* to see which of the described compounds can be retrieved as part of the venom composition. Another finding of this thesis was the identification of the male-specifically expressed venom compounds for the scorpion *E. italicus*. Our progress on characterizing the venom composition of a representative of this understudied scorpion group was described in chapter 2.4 of this dissertation. A mating related function of the male-specifically expressed compounds in the venom of the genus *Euscorpius* is likely as this group in known to perform an extensive sexual sting. In the course of conducting this research, I observed that females stopped all reluctant behavior towards male mating attempts and became completely passive after receiving a sexual sting. Now, we have obtained the sequence of the compound that likely causes this effect. As a next step, recombinant production can be used for generating it. Then, an application to female scorpions will show if the potential effect caused by the sexual sting is reproducible. In case of pseudoscorpions, at least for the investigated house pseudoscorpion, no differences between the sexes were observed in the venom composition. There is also no evidence for venom transfer in a mating-related context. However, cheliferid pseudoscorpions evolved a behavior which might fulfill a similar function as the sexual sting in scorpions. During the promenade a deux, ram's horn organs are extruded apparently reducing female resistance against male mating attempts. Consequently, it seems that, as with their venom delivery system, pseudoscorpions have again evolved their very own solution.

To sum up, with the current dissertation, I could provide fascinating insights into the venom of pseudoscorpions, one of the smallest venomous creatures ever studied. Additionally, I advanced the understanding of venom usage within Panscorpiones by getting one step closer to confirming a mating-related function of venom compounds during the sexual sting performed by various scorpions.

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I also want to express my gratitude towards the whole Predel lab for many wonderful Mensa breaks and some memorable conference trips and barbecues. I am especially grateful to Dr. Lapo Ragionieri for his support with data analysis, mass spectrometry and lab work, always helping with any problem. I would like to thank Stephan Schaffrath for introducing me to scorpion venom extraction and MALDI-TOF MS. I thank Ricardo Pommerening for contributing substantially to the scorpion venom research presented. Thanks to Anja Nonn and Daniela Mardones Agurto for taking care of my animals while I was finishing this dissertation. Last but not least, I am grateful to Laura Beische and Dr. Marcel Bläser for many nice distractions from the everyday work routine in the lab.

I am grateful to my family for their enduring positive enforcement during all my studies and to my friends for their sustained interest in my pseudoscorpion stories.

Finally, I want to thank Catharina for proofreading this thesis and especially for patiently supporting me throughout all the good and bad times.

7 Supplementary Material

SM Table of Contents

SM 1: Introduction

SM 1.1 SM 1.1: Table listing the extant scorpion families with the respective progress in venom research. Here, only progress in identification of venom compounds is included, not considering functional characterization.

Scorpion family	Venom composition research	Publications
Chaerilidae	Transcriptomics Single toxins Uniprotentries	He et al. (2013) e. g. Xie et al. (2012) von Reumont et al. (2022)
Pseudochactidae	NA	NA
Buthidae	Transcriptomics + Proteomics Uniprotentries	e. g. Marchi et al. (2022); Rodríguez de la Vega et al. (2010) von Reumont et al. (2022)
Iuridae	Transcriptomics	Grashof et al. (2019)
Bothriuridae	Single toxin/Biochemical characterization	Ramos and Escobar (2006)
Caraboctonidae	Single toxin Biochemical characterization	Escobar et al. (2008) Costal-Oliveira et al. (2015, 2012)
Superstitioniidae	Transcriptomics + Proteomics	Santibáñez-López et al. (2016)
Euscorpiidae	Transcriptomics + Proteomics, Uniprotentries	Santibáñez-López et al. (2017) von Reumont et al. (2022)
Chactidae	Proteomics (partial) Single toxins Uniprotentries	Estrada-Gómez et al. (2021) e. g. Valdez-Cruz et al. (2004) von Reumont et al. (2022)
Scorpiopidae	Transcriptomics Single toxins	Ma et al. (2012, 2009) Yuan et al. (2010); Zhang et al. (2019)
Typhlochactidae	NA	NA
Troglotayosicidae	NA	NA
Belisariidae	NA	NA
Hadruridae	Transcriptomics+Proteomics	Rokyta and Ward (2017)
Vaejovidae	Transcriptomics+Proteomics	Cid-Uribe et al. (2018)

Hormuridae	Proteomics (partial)	Estrada-Gómez et al. (2021); Schwartz et al. (2008)
Scorpionidae	Transcriptomics + Proteomics	Bringans et al. (2008); e. g. Deng et al. (2018); Diego-García et al. (2012); Ma et al. (2010)
	Uniprotentries	von Reumont et al. (2022)
Diplocentridae	Transcriptomics	Grashof et al. (2019)
Urodacidae	Transcriptomics+Proteomics	Luna-Ramírez et al. (2015, 2013)
Hemiscorpiidae	Transcriptomics Uniprotentries	Kazemi-Lomedasht et al. (2017), von Reumont et al. (2022)
Heteroscorpionidae	NA	NA

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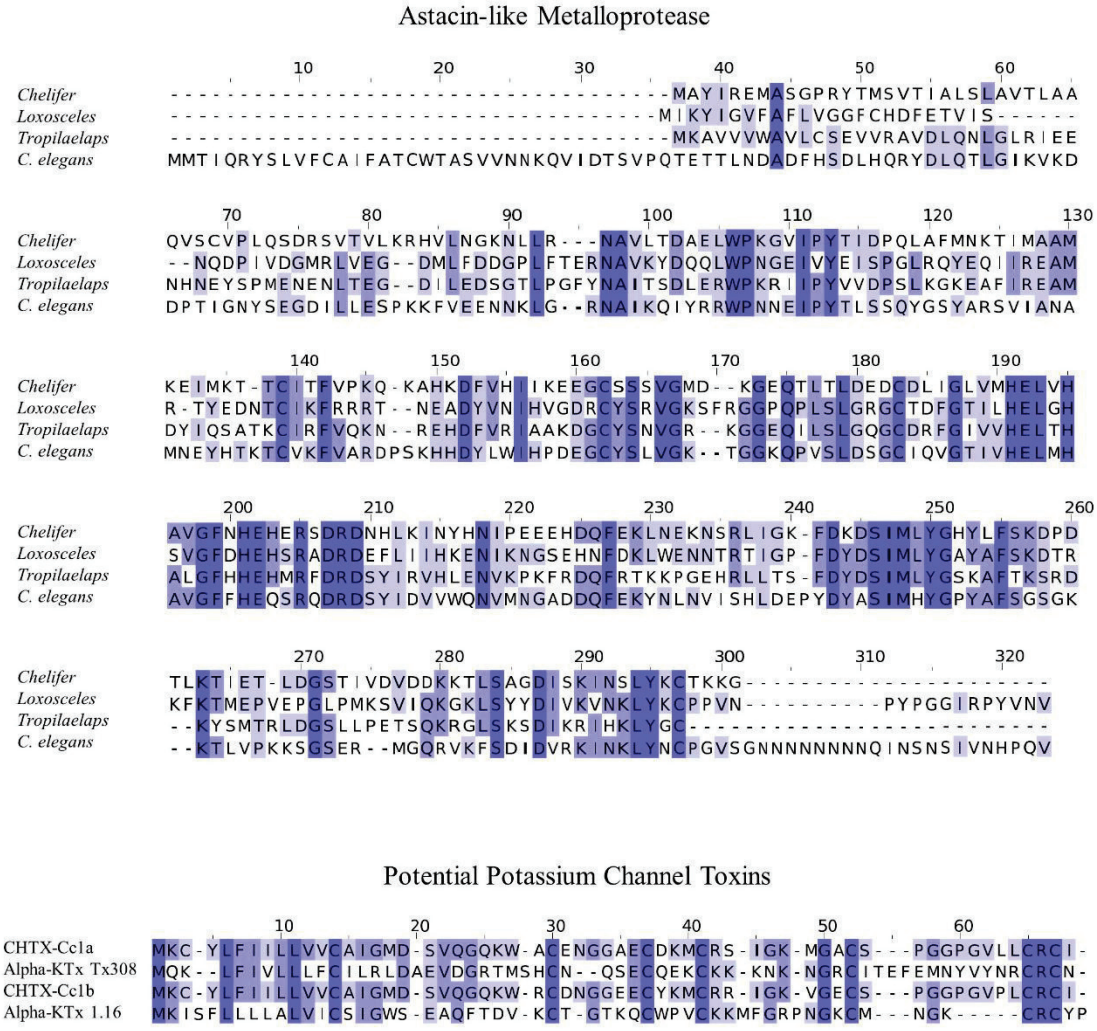
SM 2: A Pseudoscorpion's Promising Pinch: The venom of *Chelifer cancroides* contains a rich source of novel compounds

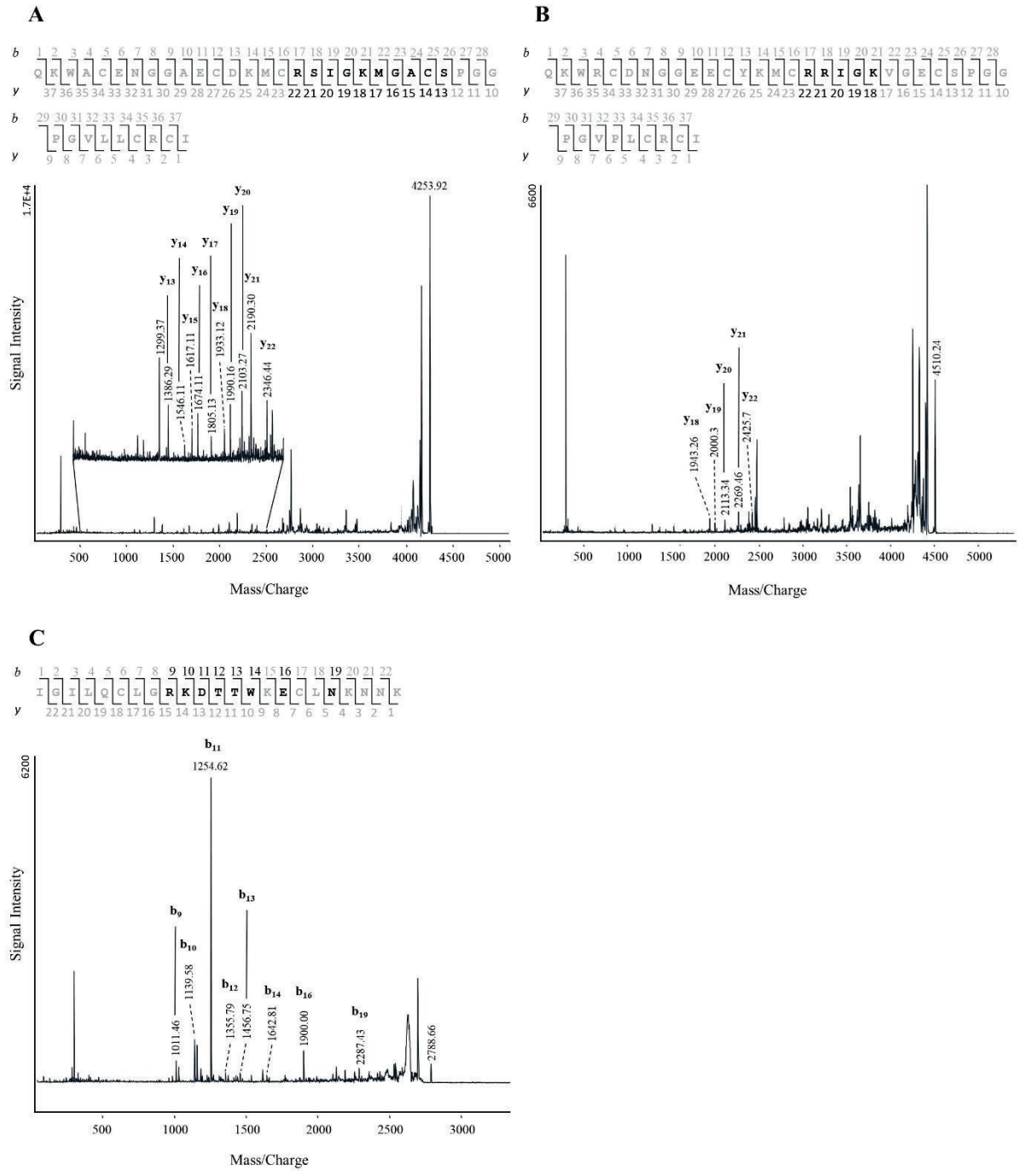
SM 2.1 Supplementary Material 1: Table of all venom compounds identified in *C. cancroides* by a combined transcriptomic and proteomic approach.

The online version of this supplementary table can be found at:
https://doi.org/10.1016/j.toxicon.2021.08.012

SM 2.2 Supplementary Material 2: Alignments of selected venom compounds (Chelifertoxin Cc1, Putative *Chelifer* Metalloendopeptidase Precursor 1) identified in *C. cancroides* with their corresponding BLAST hits (BLAST search against Toxprot database from Uniprot).



SM 2.3 Supplementary Material 3: MALDI-TOF MSMS spectra of U-Chelifertoxin-Cc1a (A), U-Chelifertoxin-Cc1b (B) and Novel Chelifer Venom Compound 2 (C).



SM 2.4 Supplementary Material 4: Table of venom compounds identified for *S. apimelus* and corresponding BLAST hits from the chelal hand transcriptome and potential venom compounds of *C. cancroides*.

The online version of this supplementary table can be found at:
https://doi.org/10.1016/j.toxicon.2021.08.012

SM 3: Antimicrobial, Insecticidal and Cytotoxic Activity of Linear Venom Peptides from the Pseudoscorpion *Chelifer cancroides*

SM 3.1 Table S1: Aphid Raw Data

The online version of this supplementary table can be found at:
https://doi.org/10.3390/toxins14010058

SM 3.2 Table S2: Cytotox Raw Data

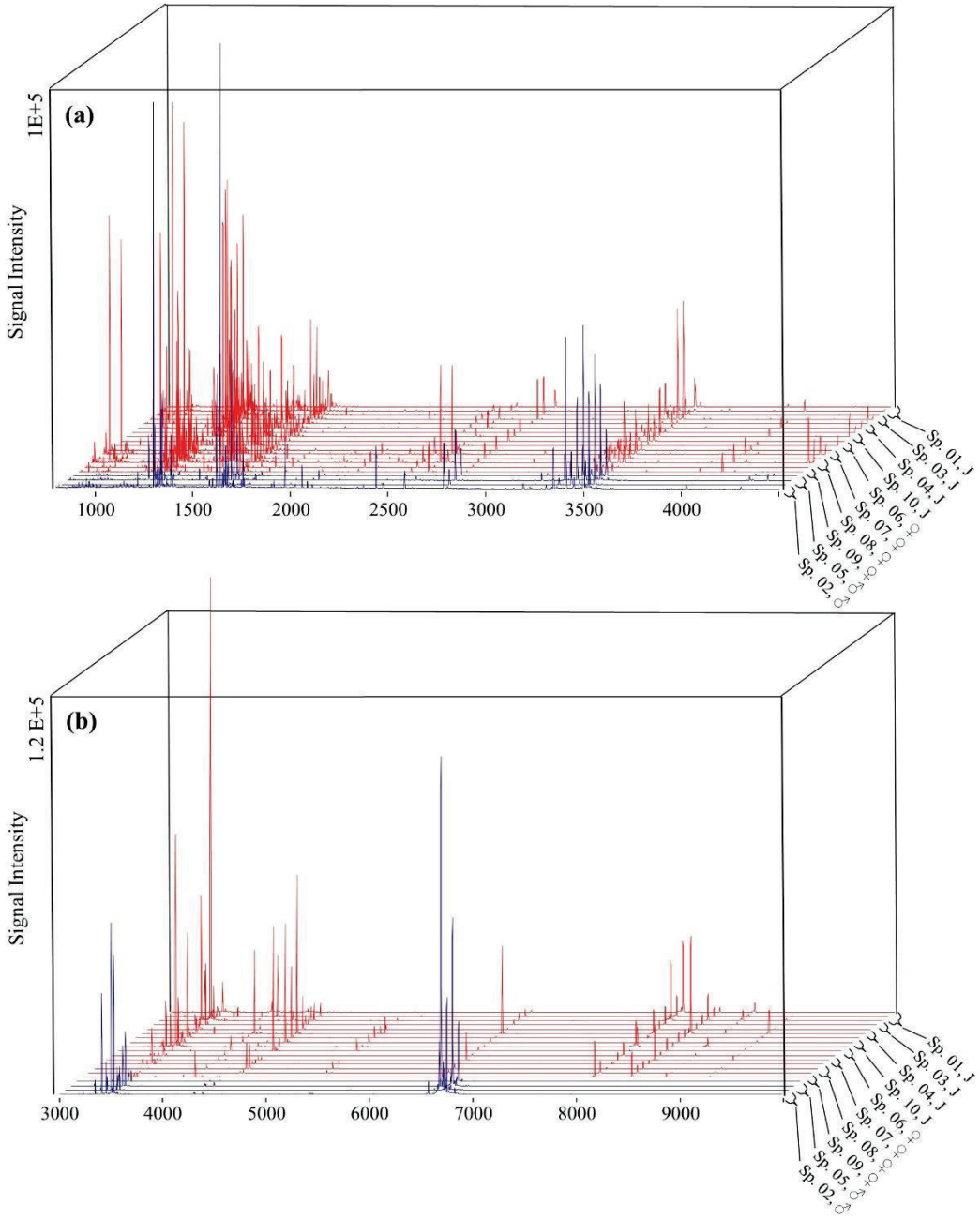
The online version of this supplementary table can be found at:
https://doi.org/10.3390/toxins14010058

SM 4: Equipped for Sexual Stings? Male-Specific Venom Peptides in *Euscorpius italicus*

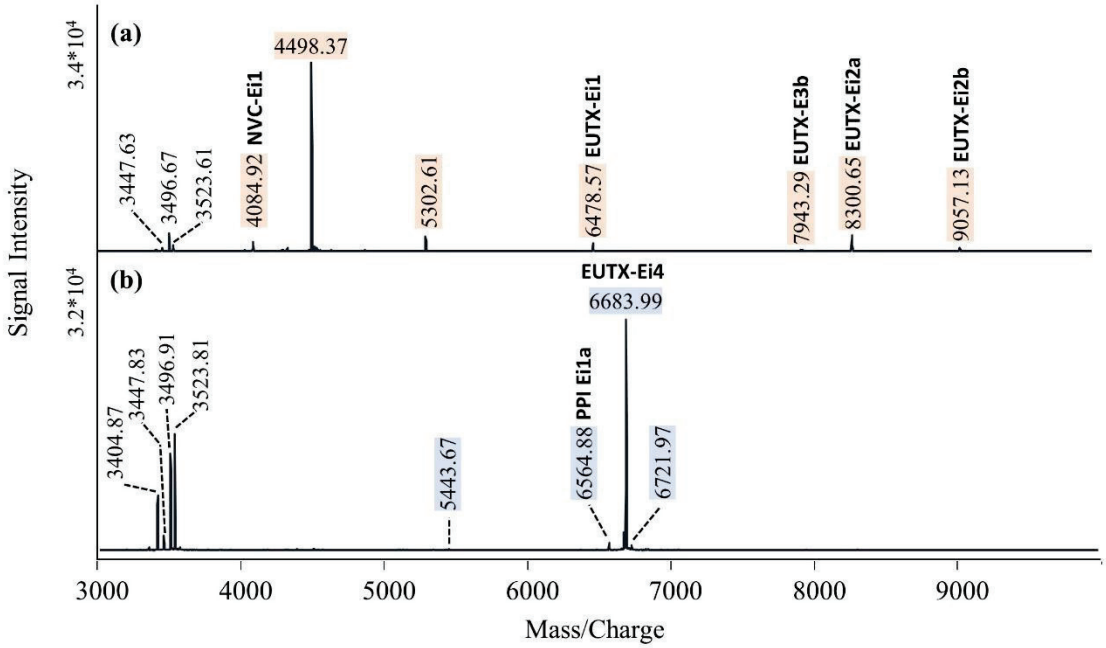
SM 4.1 Supplementary Material 1: Table of all venom compounds identified in *E. italicus* by a combined transcriptomic and proteomic approach.

The online version of this supplementary table can be found at:
https://doi.org/10.3390/ijms231911020

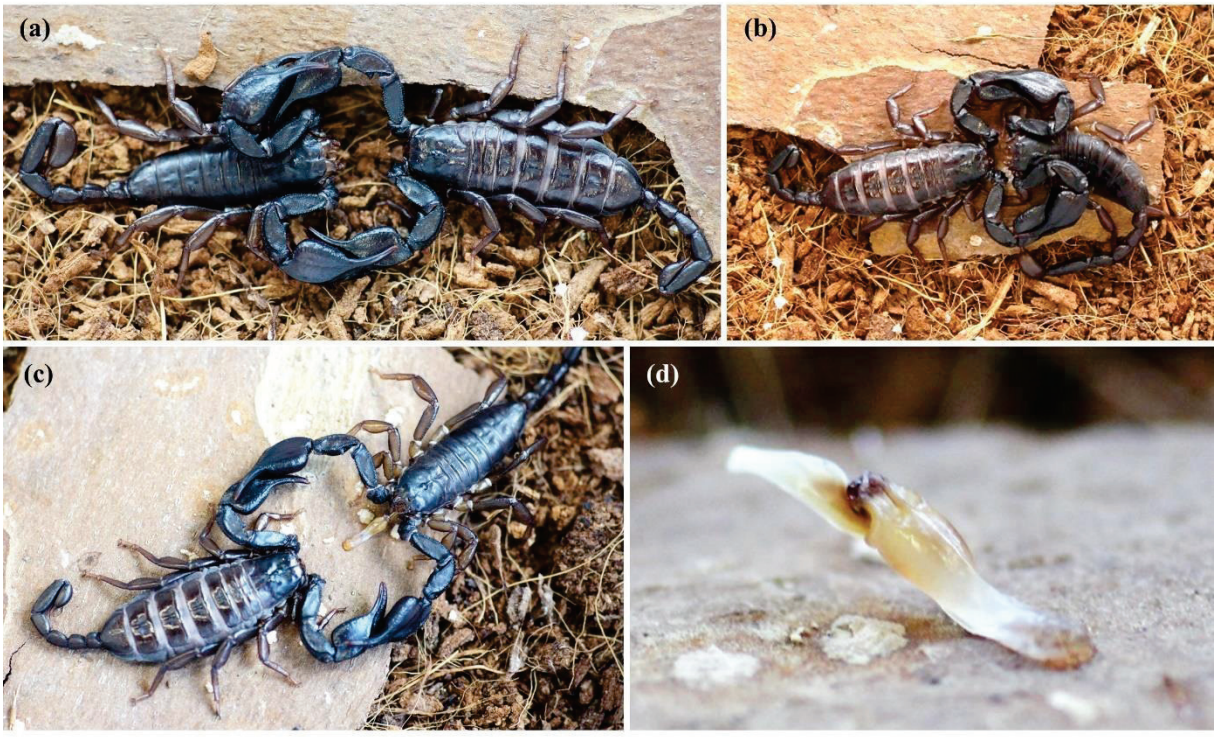
SM 4.2 Supplementary Material 2: Compilation of MALDI-TOF MS mass spectra of 20 venom samples from 10 individuals of *E. italicus* demonstrating a male specific expression of venom compounds. (**a**) Mass range from m/z 800 to 4500. (**b**) Mass range from m/z 3000 10 000.



SM 4.3 Supplementary Material 3: Comparison of MALDI-TOF MS mass fingerprints (m/z 3000 - 10,000) of venom extracted from one specimen of *E. italicus* before (**a**) and after (**b**) transition to an adult male. Male-specific ion signals are highlighted in blue; ion signals that are missing in mass spectra of venom from males are highlighted in beige.



SM 4.4 Supplementary Material 4: Documentation of mating behavior for *E. italicus*. (**a**) Promenade a deux (**b**) Sexual sting (**c**) Placement of the spermatophore (**d**) Close up of the spermatophore.



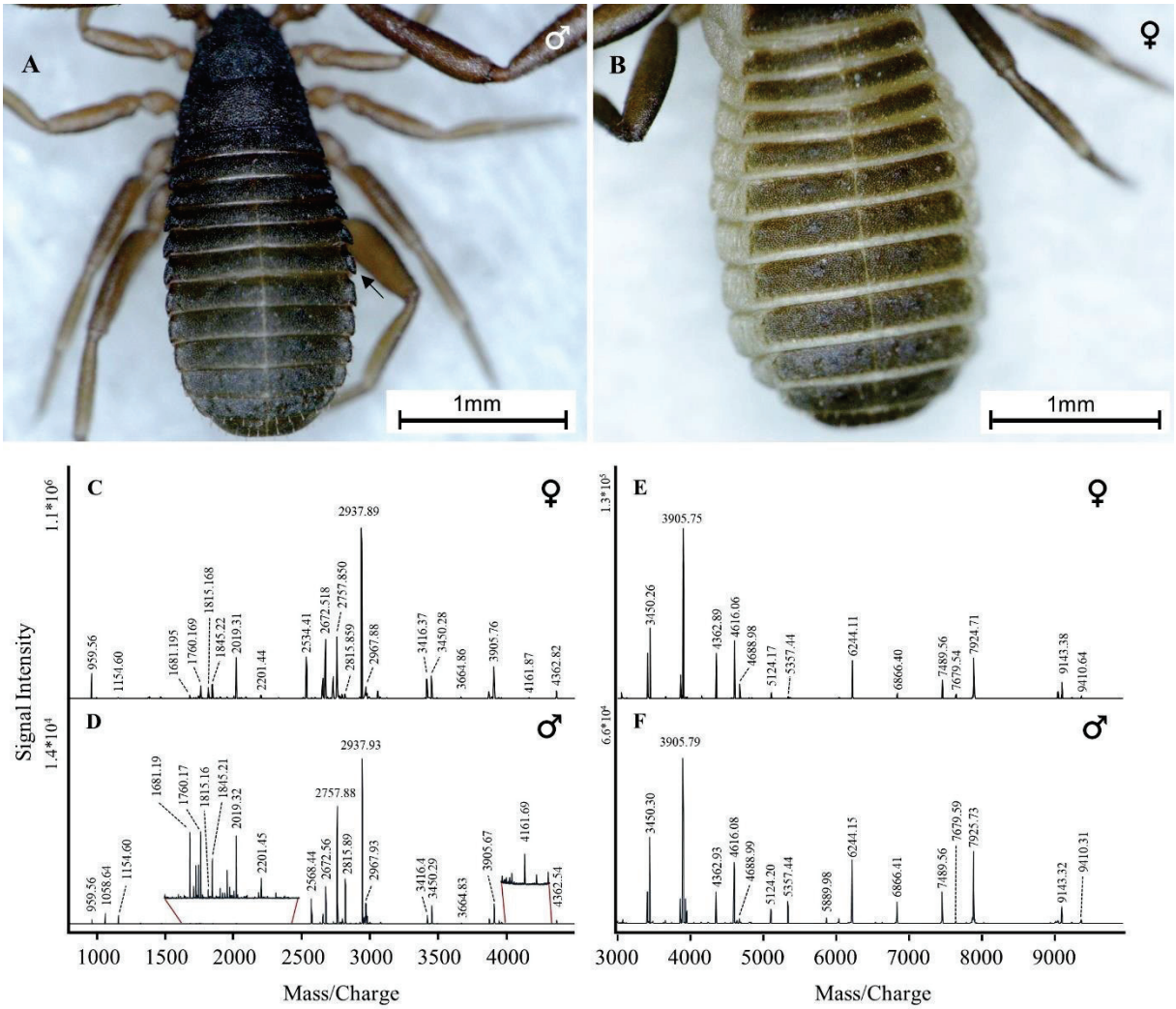
SM 4.5 Supplementary Material 5: To assess whether sex has a significant impact on the released venom amount from *E. italicus*, a linear model including specimens as fixed effects was applied, utilizing the software R (R Core Team (2022)). The venom amount was set as dependent variable with sex and maturity as explanatory variables. Venom was extracted from adult males (6 extractions; 2 specimens), adult females (12 extractions; 4 specimens), male juveniles (6 extractions; 2 specimens) and female juveniles (6 extractions; 2 specimens).

Observations	**30**
Dependent variable	Volume[µl]
Type	OLS linear regression
F(10,20)	**34.30**
R^2	0.94
Adj. R^2	0.92

	Est.	S.E.	t val.	p
Sex	0.50	0.24	2.12	0.05
Maturity	0.67	0.24	2.83	0.01
No. of groups: Specimen	10			
Standard errors	OLS			

SM 5: Discussion

SM 5.1 SM 5.1: Comparison of MALDI-TOF MS mass fingerprints of venom from both sexes of *C. cancroides*. Sex determination was done based on the presence of distinct lateral keels at the tergites of the male carapace (**A,** arrow) which are lacking in females (**B**) (Harvey, 2014). Mass fingerprints of female and male venom are shown in the mass range from 800 – 4,500 Da (**C**, **D**) and for the mass range from 3000-10,000 Da (**E**, **F**).



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8 Curriculum vitae

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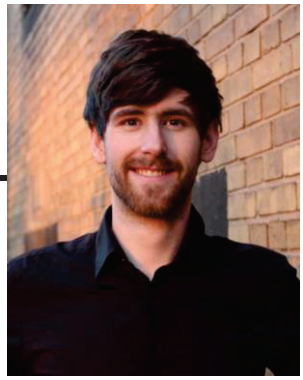

❖ Personal Information

Date of Birth:	16.04.1991
Place of Birth	Ahlen (North-Rhine Westphalia), Germany
Nationality:	German

❖ University Educational History

2019 - 2023	PhD in the working group of Prof. Dr. Predel, University of Cologne PhD thesis: “Novel Insights into Arachnid Venomics: Studies on Pseudoscorpion and Scorpion Venom”
2015 - 2018	Master of Science in “Organismic Biology Evolutionary Biology and Palaeobiology” at the Rheinische Friedrich-Wilhelms-University, Bonn Master thesis: “Analysis of the Venom Composition of the Pseudoscorpion *Chelifer cancroides* and the Role of the Lamina Defensor in Releasing the Venom”
2011 - 2014	Bachelor of Science in „Biowissenschaften“ at the Westfälische Wilhelms-University, Münster Bachelor thesis: “Study on the segregation of *Gammarus pulex* populations along a salinity gradient”

❖ Work and Research History

10/2018 – 10/2022	Scientific assistant, Lab of R. Predel, University of Cologne
04/2015 – 08/2015	Internship at the Biologische Station Rieselfelder, Münster
10/2014 – 03/2015	Internship at ASVO in a project for sea turtle conservation, Costa Rica

❖ Peer-Reviewed Publication

Krämer, J., Pommerening, R., Predel, R., 2022b. Equipped for Sexual Stings? Male-Specific Venom Peptides in *Euscorpius italicus*. Int. J. Mol. Sci. 23, 11020. https://doi.org/10.3390/ijms231911020

Krämer, J., Lüddecke, T., Marner, M., Maiworm, E., Eichberg, J., Hardes, K., Schäberle, T.F., Vilcinskas, A., Predel, R., 2022a. Antimicrobial, Insecticidal and Cytotoxic Activity of Linear Venom Peptides from the Pseudoscorpion *Chelifer cancroides*. Toxins 14, 58. https://doi.org/10.3390/toxins14010058

Krämer, J., Peigneur, S., Tytgat, J., Jenner, R.A., van Toor, R., Predel, R., 2021. A Pseudoscorpion's Promising Pinch: The venom of *Chelifer cancroides* contains a rich source of novel compounds. Toxicon 201, 92–104. https://doi.org/10.1016/j.toxicon.2021.08.012

Krämer, J., Pohl, H., Predel, R., 2019. Venom collection and analysis in the pseudoscorpion *Chelifer cancroides* (Pseudoscorpiones: Cheliferidae). Toxicon 162, 15–23. https://doi.org/10.1016/j.toxicon.2019.02.009

❖ Scientific Meetings and Conferences

09/2022	114th Annual Meeting of the German Zoological Society (DZG) Oral Presentation
09/2021	First European Venom Network International Congress Oral Presentation
09/2019	112th Annual Meeting of the German Zoological Society (DZG) Poster Presentation
08/2019	6th International Toxinology Meeting Venoms and Toxins 2019 Oral Presentation
09/2018	111th Annual Meeting of the German Zoological Society (DZG) Poster Presentation

❖ Teaching History

02/2021 - 03/2022	Internship/Bachelor thesis supervision, Lab of R. Predel, University of Cologne. Area: Venomics
10/2019 - 11/2022	Scientific assistant: Student course supervision, University of Cologne Area: Zoology

❖ **Courses and Career Development Workshops**

11/2022	„Großes Promovierenden-Forum ‚Kulturen des Entscheidens'" of the German Academic Scholarship Foundation
01/2023	Seminar in Good Scientific Practice – Scientific Integrity, University of Cologne

❖ **Funding Acquisition**

11/2019 - 04/2023	Scholarship for doctoral studies from the German Academic Scholarship Foundation

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Köln, 02.05.2023 

Jonas Krämer

9 Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

Teilpublikationen:

Krämer, J., Pommerening, R., Predel, R., 2022b. Equipped for Sexual Stings? Male-Specific Venom Peptides in *Euscorpius italicus*. Int. J. Mol. Sci. 23, 11020. https://doi.org/10.3390/ijms231911020

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