Drugs from bugs:
Venomics of predatory Heteroptera

Dissertation

in Partial Fulfilment of the Requirements for the Degree of

“doctor rerum naturalium” (Dr. rer. nat.)

Submitted to the Council of the Faculty of Biological Sciences

of Friedrich Schiller University Jena

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Date of public defense: 06.09.2023

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1. INTRODUCTION

Insects are the outcome of the most successful evolutionary radiation, making up more than 60% of all eukaryotic organisms on earth (Misof et al., 2014; Mora et al., 2011). They are enormously important to our ecosystems, helping with pollination, seed dispersal, and the decomposition of organic matter, and representing important links in the food chain (Prather et al., 2013). The origin of insects dates back approximately 479 million years. Since then, they have undergone remarkable diversification, resulting in more than 5 million different species (Misof et al., 2014; Stork, 2018). The diversification of insects has been driven mainly by the exploitation of terrestrial habitats as well as the evolution of flight and complete metamorphosis (Misof et al., 2014). In addition, the evolution of venom has accelerated the diversification of insects because it enables adaptation to new ecological niches by facilitating dietary switches, expanding prey ranges, and improving defenses against predators (Arbuckle & Harris, 2021). Therefore, venomous insects are important as models to study adaptive evolution. Furthermore, their venoms provide large arsenals of diverse molecules that are active against a variety of animal targets, offering leads for the development of therapeutics and insecticides (Walker et al., 2016). Venom research has focused primarily on hymenopterans, resulting in a highly biased view of venom evolution and ecology in insects. Research that considers neglected venomous insects is therefore necessary to fully understand venom evolution and dynamics, while also offering a broad resource for drug discovery.

1.1. Venom evolution in insects

Venom is defined as a secretion that is injected into another animal, thereby affecting its physiology and biochemistry (Fry et al., 2009). In insects, venom use for predation, parasitoidism and defense has evolved independently at least 14 times in Hemiptera, Neuroptera, Hymenoptera, Diptera, Lepidoptera and Coleoptera (Walker et al., 2018c) (major venom evolution events shown in Figure 1). Secretions that facilitate parasitism, such as blood or hemolymph feeding, may also be defined as venoms and have evolved independently at least nine times in insects (Walker et al., 2018c). Although some insects use their venom exclusively to deter predators, most insect venoms are used primarily for

predation and only secondarily for defense (Schendel et al., 2019; Walker et al., 2018c). The venom delivery systems of insects vary greatly in structure, complexity and origin. The stinging apparatus in female hymenopterans, for example, derives from the ovipositor and is used to inject venom produced in modified sexual accessory glands (Robertson, 1968; Van Marle & Piek, 1986). Venomous caterpillars release their venom via hairs or needle-like spines that originate from evaginations on the body wall and their toxins are produced in specialized enlarged cells at the base of the hairs/spines (Gilmer, 1925; Veiga et al., 2001; Walker et al., 2021). In contrast, venomous species from Asilidae (Drukewitz et al., 2018; Owsley, 1946) and Heteroptera (Swart & Felgenhauer, 2003; Walker et al., 2018b; Walker et al., 2016) produce venom in specialized salivary glands and use their mouthparts to inject the secretions into target organisms.

The recruitment of venom proteins to secretory cells via the adaptation of non-venom proteins is a widely accepted paradigm (Casewell, 2012; Fry, 2005; Walker, 2020), but the underlying genetic mechanisms are not yet fully understood. Proteins may be recruited by the co-option of single-copy genes or they may undergo gene duplication and neofunctionalization events, increasing the functional diversity within a gene family (Drukewitz et al., 2019; Martinson et al., 2017; Pineda et al., 2014). Both mechanisms can result in highly complex venoms. In particular, animal venoms used primarily to overwhelm and predigest prey may contain more than 1000 different compounds and can trigger diverse physiological effects (Escoubas et al., 2006; Lavergne et al., 2015). In contrast, venoms used exclusively for defense are usually less complex (Casewell et al., 2013). Despite the diversity of venomous insects and convergently evolved venom systems, only few species have been studied to determine their venom composition, with most research concentrating on Hymenoptera. In this thesis, I focus on predatory true bugs (Heteroptera), which have been largely neglected in venom research.

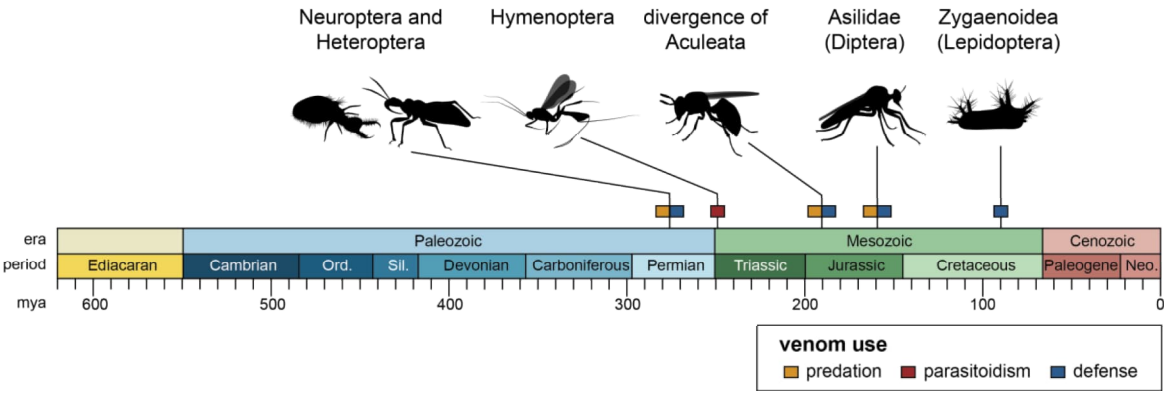

Figure 1: Venom evolution in insects, modified from Walker (2020). Estimated dates of major venom evolution events in Neuroptera (Winterton et al., 2018), Heteroptera (Wang et al., 2016), Hymenoptera (Peters et al., 2017), Diptera (Dikow et al., 2017) and Lepidoptera (Kawahara et al., 2019). Venoms used for predation, parasitoidism and defense are highlighted in yellow, red and blue, respectively.

1.2. Venom evolution in Heteroptera

The suborder Heteroptera is a monophyletic group of at least 40,000 species within the order Hemiptera and belongs to one of the most successful radiations among insects that have exploited a wide range of feeding styles and habitats (Weirauch & Schuh, 2011; Weirauch et al., 2019). Heteroptera are distributed worldwide in terrestrial and aquatic environments (Weirauch et al., 2019). Some species occur in uncommon habitats, such as sea skaters (Gerromorpha) that live on the open ocean (Andersen, 1999) or thread-legged bugs (Emesinae) that inhabit spider webs (Hickman, 1969). Like all hemipterans, true bugs are hemimetabolous and all developmental stages use their piercing-sucking mouthparts to inject saliva into their food and suck up the liquid or liquefied components (Johnson et al., 2018). Recent phylogenetic studies suggest the most recent common ancestor (MRCA) of true bugs was terrestrial (Weirauch et al., 2019). Moreover, the divergence of the Heteroptera from the remaining Hemiptera ~278 million years ago was accompanied by a trophic switch from phytophagy to zoophagy (Walker et al., 2016; Wang et al., 2016; Weirauch et al., 2019) (Figure 2). This led to the evolution of venomous saliva, which facilitates the overwhelming, killing and pre-digestion of animal prey. Although some clades such as Enicocephalomorpha, Dipsocoromorpha, Gerromorpha, Nepomorpha, Leptopodomorpha and Cimicomorpha mainly retained the predatory lifestyle, the divergence of the Pentatomomorpha was accompanied by a transition to phytophagy/mycophagy (Weirauch et al., 2019) (Figure 2). Over time, a wide variety of

feeding styles has evolved among true bugs, including hematophagy, mycophagy, phytophagy, zoophagy and mixed feeding styles such as zoophytophagy or phytozoophagy. Secondary switches to predation have occurred independently several times, for example in some pentatomomorphan groups (Walker et al., 2016; Weirauch et al., 2019) (Figure 2). The evolution of diverse dietary habits probably involved radical changes in salivary protein composition, including the recruitment or loss of toxins in response to trophic switches (Walker et al., 2016). However, the dynamics of the evolution of dietary shifts and the associated effector molecules are not yet known.

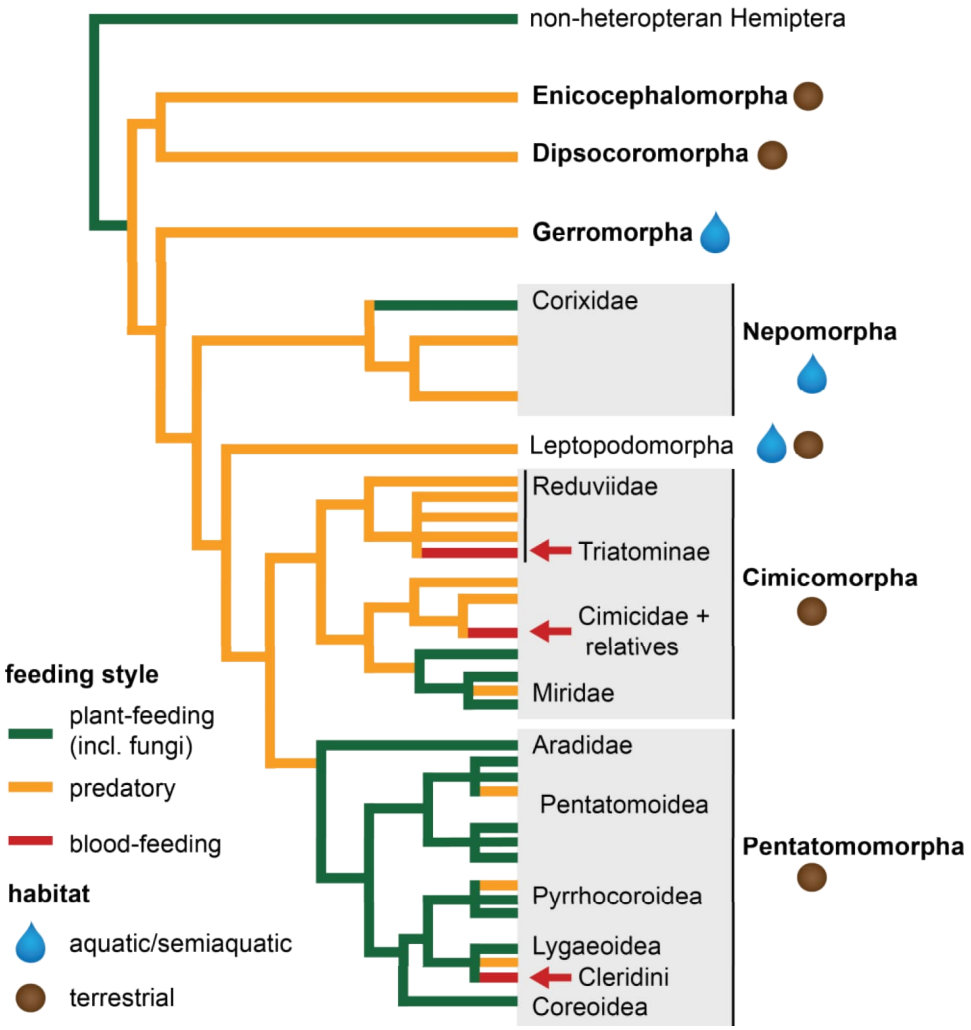

Figure 2: Simplified phylogenetic tree of Heteroptera, modified from Walker et al. (2016). The colors represent different trophic strategies (green = phytophagous/mycophagous, orange = zoophagous, red = hematophagous). The occupied habitats are represented by icons. Phylogenetic relationships are based on Wang et al. (2016), Schuh et al. (2009) and Hua et al. (2008).

1.2.1. Venom system in Heteroptera

The evolution of an adapted venom system was necessary for the divergence of the predatory Heteroptera. Their stinging apparatus comprises specialized piercing-sucking mouthparts as well as venom glands homologous to the salivary glands (Swart & Felgenhauer, 2003; Walker et al., 2018b; Walker et al., 2016) (Figure 3A). The venom gland complex includes two paired glands that usually consist of an anterior main gland (AMG), a posterior main gland (PMG) and an accessory gland (AG) (Baptist, 1941; Walker et al., 2018b). The AMG and PMG are connected at a junction called the hilus, from where the venom gland duct leads to a venom pump and into the proboscis. The AG is connected to the hilus via a separate duct (Baptist, 1941; Walker et al., 2018b) (Figure 3B). This structural complexity allows the secretion and storage of distinct venom mixtures in separate compartments (Walker et al., 2018a; Walker et al., 2018b; Walker et al., 2019). Although the glandular structure of most heteropterans follows the same scheme, the glands can vary greatly in their substructure, size and location. For example, the Nepidae and Gerridae have modified main glands consisting of several small lobules and an additional anterior lobe duct. Moreover, the glands of notonectids are located in the head capsule and not in the thorax as in most true bugs (Baptist, 1941).

The proboscis of heteropterans is a highly specialized elongated structure that allows the injection and uptake of liquids. The labium forms an elongated hollow sheath enclosing the mandibles and maxillae that form the so-called stylets (Smith et al., 1985). The paired, asymmetric maxillae interlock in such a way that two separate canals are formed: a food canal and a venom canal (Cohen, 1990; Smith et al., 1985; Walker et al., 2018b). For venom injection and food uptake, the stylets move forward to emerge from the labial tip and penetrate the prey (Smith et al., 1985; Walker et al., 2016). Digestive enzymes and effector molecules secreted by the glands are injected through the venom canal and distributed within the prey. The digested tissues are then taken up through the food canal into the foregut (Cohen, 1990; Walker et al., 2018b; Walker et al., 2016) (Figure 3C).

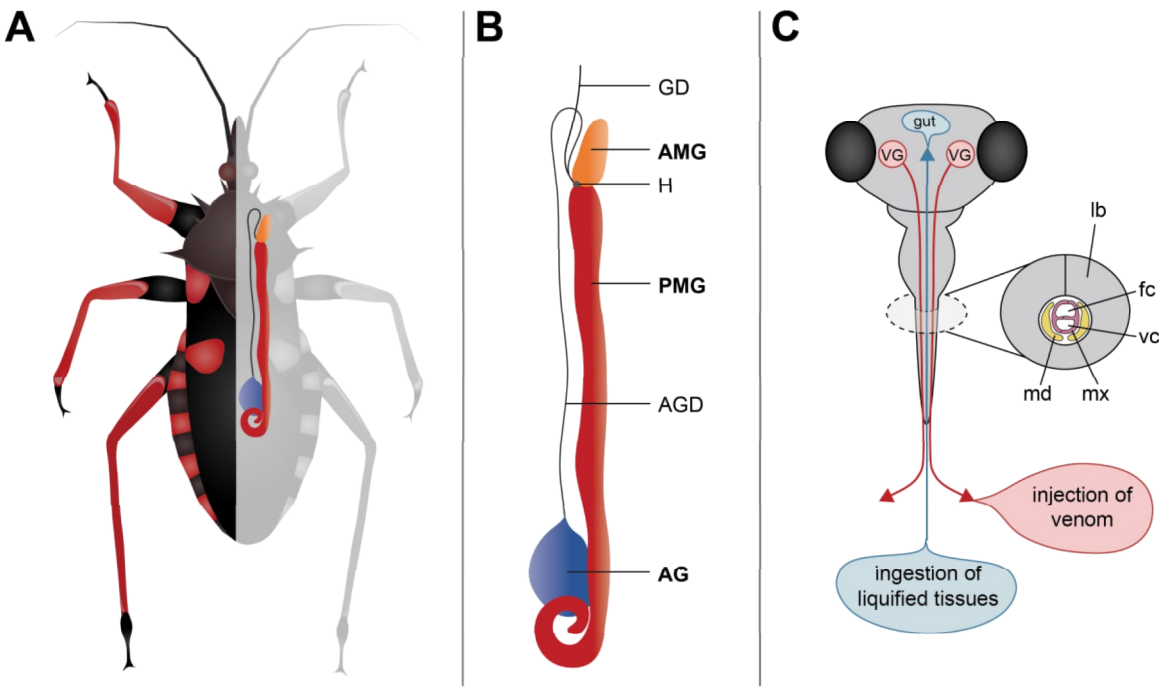

Figure 3: The venom apparatus in Heteroptera. (A) Typical location of the venom gland complex in true bugs. **(B)** Structure of a typical bilobed venom gland complex comprising an anterior main gland (AMG), a posterior main gland (PMG), an accessory gland (AG), a hilus (H), a gland duct (GD) and an accessory gland duct (AGD). **(C)** Schematic of non-refluxing extra-oral digestion through a specialized elongated proboscis consisting of a labium (lb), mandibles (md) and maxillae (mx). Venom is produced in the venom glands (VG) and injected into prey through the venom canal (vc). Pre-digested, liquefied tissue is ingested through the food canal (fc) into the gut.

1.2.2. Venom use and function

Predatory true bugs use their venom primarily for predation, which includes the immobilization, killing and extra-oral digestion (EOD) of prey animals. Some species use communal predatory behavior to overwhelm large prey (Inoue, 1985), but most heteropterans are solitary predators that rely on potent venom. Assassin bug (Reduviidae) species such as *Rhynocoris carmelita* (Stål) and *Platymeris rhadamanthus* Gerstaecker can paralyze and kill prey several hundred times larger and heavier than themselves within seconds (Edwards, 1961). The giant water bugs (Belostomatidae) have evolved strong venom and raptorial forelegs to subdue vertebrates, including amphibians, fish, birds, snakes and turtles (Ohba & Nakasuji, 2006; Ohba, 2011, 2019). Most predatory heteropterans can also inflict painful defensive bites to deter predators. Bites from belostomatids and reduviids have been described as particularly painful, causing numbness, edema, nausea, heart palpitations and hyperventilation (Haddad Jr et al.,

2010; Hartwig, 1977). Some of these symptoms can last up to several months (Hartwig, 1977).

The dual use of venom for predation and defense implies a broad spectrum of activity against different targets. Furthermore, the morphology of predatory true bug venom glands suggests that they secrete different venom mixtures in separate gland compartments. In phytophagous bugs, a functional compartmentalization of saliva used to form a so-called “stylet sheath” and saliva used for EOD has been reported (Miles, 1967). In some predatory bugs, AMG extracts cause paralysis in insects, whereas PMG venom has strong digestive effects, suggesting the spatial separation of neurotoxins and digestive enzymes (Haridass & Ananthakrishnan, 1981). In 2018, the harpactorine assassin bug *Pristhesancus plagipennis* Walker was shown to use distinct venoms in a context-dependent manner, which is thus far a unique trait in insects. AMG venom was only secreted in response to harassment and was therefore defined as defensive venom. In contrast, PMG venom was elicited by electrostimulation and had strong insecticidal and liquefying effects, thus suggesting that it is the main predation venom. The AG secreted only few proteins and presumably serves to recycle water from the gut and hemolymph (Walker et al., 2018b). *P. plagipennis* was the first reported case of stimulus-dependent venom deployment in insects, but its ecological relevance and distribution within the Heteroptera are unclear.

1.2.3. Biochemistry of venom

Enzymes

Animal venoms are complex cocktails of peptides, salts, polyamines, amino acids, neurotransmitters and other organic or inorganic molecules (Inceoglu et al., 2003; Jasys et al., 1990; Nelson & O'Connor, 1968; Weisel-Eichler et al., 1999). Heteropteran venoms are usually rich in proteins (Rügen et al., 2021; Walker et al., 2018a; Walker et al., 2018b; Walker et al., 2019; Yoon et al., 2022), but venom composition has been analyzed in only a few species. Most research has focused on large species such as assassin bugs and giant water bugs. The main components of heteropteran venoms are digestive enzymes that play an important role in the EOD of animal tissue (Cohen, 1998; Walker et al., 2017). Trypsin-like and chymotrypsin-like serine endoproteases are usually among the most

abundant and strongly expressed venom proteins, reflecting their importance in liquefying the protein-rich prey by cleaving internal peptide bonds (Cohen, 1995; Walker et al., 2016; Yoon et al., 2022; Zibaee et al., 2012). However, exopeptidases such as aminopeptidases and carboxypeptidases have also been identified in heteropteran venoms, albeit in much lower amounts (Ambrose & Maran, 2000; Walker et al., 2016; Yoon et al., 2022; Zibaee et al., 2012). Lipases are commonly found in snake, spider and insect venoms (Casewell et al., 2009; Cohen, 1998; Fernandes-Pedrosa et al., 2008; Walker et al., 2018a). They play important roles in tissue digestion but also in the depolarization of nerve and muscle cells by disrupting cell membranes (Cohen, 1995; Edwards, 1961). Triacylglycerol lipases and phospholipases have been detected in heteropteran venoms, but their role in envenomation and EOD has not been investigated (Sahayaraj et al., 2010; Walker et al., 2018a; Walker et al., 2017; Yoon et al., 2022). Other digestive enzymes found in heteropteran venoms, such as carboxylesterases, nucleases and carbohydrases, are probably involved in tissue liquefaction, spreading of venom and nutrient acquisition (Walker et al., 2017; Yoon et al., 2022).

Pore-forming proteins

Animal venoms often contain highly cytotoxic pore-forming proteins. In bee venom, the pore-forming protein melittin is the major pain-causing component, partly reflecting its lytic activity (which promotes the release of cytokines) and partly due to its interaction with nociceptors and sodium channel (Askari et al., 2021; Chen et al., 2016; Shin & Kim, 2004; Tosteson et al., 1985). Trialysin, a protein first found in the hematophagous bug *Triatoma infestans* (Klug), causes the lysis of bacterial, protozoan and mammalian cells by forming voltage-gated channels in lipid bilayers (Amino et al., 2002; Rocha et al., 2022). In the predacious reduviids *P. plagipennis* and *P. rhadamanthus*, several trialysin homologs were found to be strongly expressed in the venom glands (Walker et al., 2017; Walker et al., 2019). These so-called redulysins share the conserved cytolytic motif of trialysin and are thought to facilitate the overwhelming of prey, liquefaction and/or pain induction (Walker et al., 2017; Walker et al., 2019). Furthermore, they may also sterilize animal prey before ingestion to avoid the uptake of pathogens. However, the ecological role and activity range of redulysins are unknown.

Neurotoxins

The paralytic effects of heteropteran venoms were initially thought to reflect the EOD and lysis of muscle and nerve tissue rather than the activity of neurotoxins. Edwards (1961) described the strong liquefying activity of assassin bug venom and argued that the disruption of cell membranes by lytic proteins was responsible for the quick paralysis of prey insects. However, several cysteine-rich peptides with an inhibitor cystine knot (ICK) motif and homology to calcium channel blockers from cone snails (conotoxins) were subsequently isolated from assassin bug venoms (Bernard et al., 2001). One of these peptides (Ptu1) from the reduviid bug *Peirates turpis* Walker was shown to reversibly block calcium channels in cell culture, thus indicating paralytic activity. However, Ptu1 showed no toxicity when injected into vertebrates and invertebrates, thus leaving its ecological role unclear (Bernard et al., 2001). Peptides homologous to Ptu1 have been detected in several other heteropteran venoms, including *P. plagipennis* and *P. rhadamanthus* (Walker et al., 2017; Walker et al., 2019).

Non-peptide small molecules can also cause neurotoxic effects. Venom from the predatory pentatomid bug *Podisus nigrispinus* (Dallas) retained most of its insecticidal activity after treatment with protease inhibitors or proteinase K, showing that molecules other than peptides confer toxicity. Two small molecules – *N,N*-dimethylaniline and 1,2,5-trithiepane – were identified in the insecticidal ether extracts of *P. nigrispinus* venom and are assumed to be responsible for the paralysis and death of prey insects (Campos et al., 2022; Martínez et al., 2016). Overall, few studies have described neurotoxins in heteropteran venoms and not much is known about their distribution, biochemistry and mode of action.

Uncharacterized polypeptides

The activity of some heteropteran venom peptides can be inferred from their structure and homology, but the function and mode of action of many peptides remain uncharacterized. Walker et al. (2017) analyzed the venom composition of *P. plagipennis* and identified numerous polypeptides with unknown functions. These included CUB domain proteins, odorant-binding proteins, hemolysin-like proteins, disulfide-rich and glycine-rich peptides, and several uncharacterized proteins that were grouped into 23 heteropteran venom protein families. Some families showed homology to other insect

proteins, whereas others were novel. In the giant water bug *Lethocerus distinctifemur* Menke subsequent analysis of venom revealed 23 novel polypeptides that showed no homology to any known sequences. Even so, most of the detected proteins were homologous to known assassin bug proteins (Walker et al., 2018a). The complexity and plasticity of venom protein composition in true bugs hinder the identification of the principal toxic agents and their ecological functions.

1.3. Ecological implications

The modification of the salivary/venom protein composition was probably a major evolutionary driver facilitating the diversification of heteropteran trophic strategies (Walker et al., 2018a; Walker et al., 2016). Some salivary protein families, such as S1 peptidases, are found in all true bugs analyzed thus far, regardless of their lineage, feeding style or habitat (Agustí & Cohen, 2000; Walker et al., 2016; Yoon et al., 2022). In contrast, other proteins are only present in particular species/lineages and may reflect adaptations to certain ecological niches, including diet, habitat and microhabitat. For example, venom from hematophagous bugs contains compounds that have anesthetic and anticoagulant effects on vertebrates, an adaptation that enables them to remain undetected and undeterred by immune defenses during blood feeding (Andersen et al., 2005; Dan et al., 1999; Pereira et al., 1996). In contrast, venom from predatory assassin bugs evolved to induce intense pain in vertebrates in order to deter predators (Hartwig, 1977; Walker et al., 2019). Researchers have attempted to link salivary/venom protein composition and activity to feeding strategies. For example, high amylase, α-glucosidase and pectinase activity indicate phytophagy because these proteins break down plant polysaccharides. In contrast, high protease and hyaluronidase activity are associated with a predatory lifestyle because they digest animal protein and the extracellular matrix (Agustí & Cohen, 2000; Zeng & Cohen, 2000). Most predatory bugs are generalists, but some species have specialized and their venom systems are likely to have adapted to their needs. The millipede assassin bug *Haematoloecha nigrorufa* (Stål) has specialized mouthparts differing considerably from those of other assassin bugs, suggesting that these morphological changes represent an adaptation to feeding on millipedes (Wang et al., 2020). Changes in the venom protein composition have not been investigated,

although venom composition is also likely to reflect prey preferences. For example, the venom of the giant water bug *L. distinctifemur* contains a venom 5' nucleotidase, which is assumed to inhibit platelet aggregation, thus indicating an adaptation to vertebrate feeding (Walker et al., 2018a). Only a few true bug species from a small number of families have been studied in detail, so the dynamics of the salivary/venom protein composition in response to trophic switches are unclear. Research on a broad range of species that utilize different habitats. microhabitats and feeding strategies will provide new insights into venom plasticity and may allow researchers to infer the ecology of species based on the composition of salivary proteins.

1.4. From venoms to drugs

Venomous animals are valuable sources of bioactive molecules with potential applications in medicine, agriculture and biotechnology. Candidates for drug or pesticide development must be potent, stable, target-specific, and easy to produce in large quantities (King, 2013; Lewis & Garcia, 2003). Only a few venom compounds have been used to develop commercial products thus far. The first venom-based therapeutic was developed in the 1970s: the angiotensin-converting enzyme inhibitor captopril from the Brazilian viper *Bothrops jaracaca* (Wied-Neuwied), which is indicated for hypertension (Rubin et al., 1978). Additional venom-derived drugs have been approved or progressed to clinical trials, mainly peptides from venomous snakes, spiders and cone snails (King, 2013; Pennington et al., 2018). In 2014, the first venom-derived bioinsecticide was approved in the US: Spear® is based on the spider peptide ω/κ-hexatoxin-Hv1a, which is neurotoxic in a broad range of pest insects but does not affect vertebrates or beneficial insects such as bees (Fletcher et al., 1997; Nakasu et al., 2016; Nakasu et al., 2014). Insect venoms have been largely overlooked for drug development, mainly because they are difficult to source sufficient amounts for testing. However, advances in next-generation sequencing, mass spectrometry and small-scale bioactivity assays have made it much easier to analyze small biological samples. Because heteropteran venoms show diverse activities against invertebrates and vertebrates, they are promising candidates for the discovery of new drugs and pesticides. The diverse trophic strategies of heteropteran species, combined with the complexity and plasticity of their venoms, are likely to offer a rich source of

currently unknown active substances. Therefore, research focusing on the ecology and evolution of venomous true bugs contributes to our knowledge of venom diversity, function and activity and provides a basis for the discovery of novel bioactive substances.

1.5. Aim of the thesis

Heteropterans form a hyper-diverse group of insects that successfully utilize a wide range of habitats and feeding styles. The ecology and evolution of venomous true bugs is largely unexplored. Most studies thus far have focused on individual species from the families Reduviidae or Belostomatidae, and the comparative analysis of closely-related species with different trophic strategies is rare. Furthermore, many of the compounds identified in heteropteran venoms are uncharacterized, so we know little about their functions and modes of action. Therefore, the aim of this thesis was to characterize and compare venom composition and deployment in different true bug species and identify molecular adaptations to their ecological niche (Figure 4).

The true water bugs (Nepomorpha) are one of the oldest groups of Heteroptera and have largely retained the ancestral predatory lifestyle. Water bugs occupy a broad range of aquatic habitats and can feed on insects, crustaceans and small vertebrates. However, the impact of their ecological niche on venom composition is unknown. In **manuscript I**, we used a proteo-transcriptomic approach to compare the venom composition of four water bug species occupying the same habitat. We aimed to identify species-depedent protein-level adaptations to particular microhabitats, diets or foraging strategies, and discussed their role in EOD and defense.

The venom composition of the assassin bug *P. plagipennis* was analyzed by Walker et al. (2018b) revealing that different venom mixtures are used in a context-dependent manner. In **manuscript II,** we considered whether stimulus-dependent venom deployment is a widely distributed trait among Reduviidae and how much the venom composition and deployment differs between species. We used a proteotranscriptomic approach and bioassays to analyze the venom composition of two closely related assassin bug species occupying the same habitats – *Psytalla horrida* (Stål) and *Platymeris biguttatus* (L.). Furthermore, we established a venom collection method using a prey dummy, which enabled us to collect secretions that reliably represent predation venom.

Assassin bug venom can have tremendous effects on vertebrates as well as invertebrates, but most of the venom compounds are uncharacterized. In **manuscript III,** we aimed to identify the major cytotoxic compounds of the PMG venom in *P. horrida*. We fractionated PMG venom extracts and analyzed the protein composition of the fractions that exhibited strong cytotoxic activity. We then screened synthetic and recombinant peptides from different families that were abundant in the toxic fractions and discussed their function and modes of action.

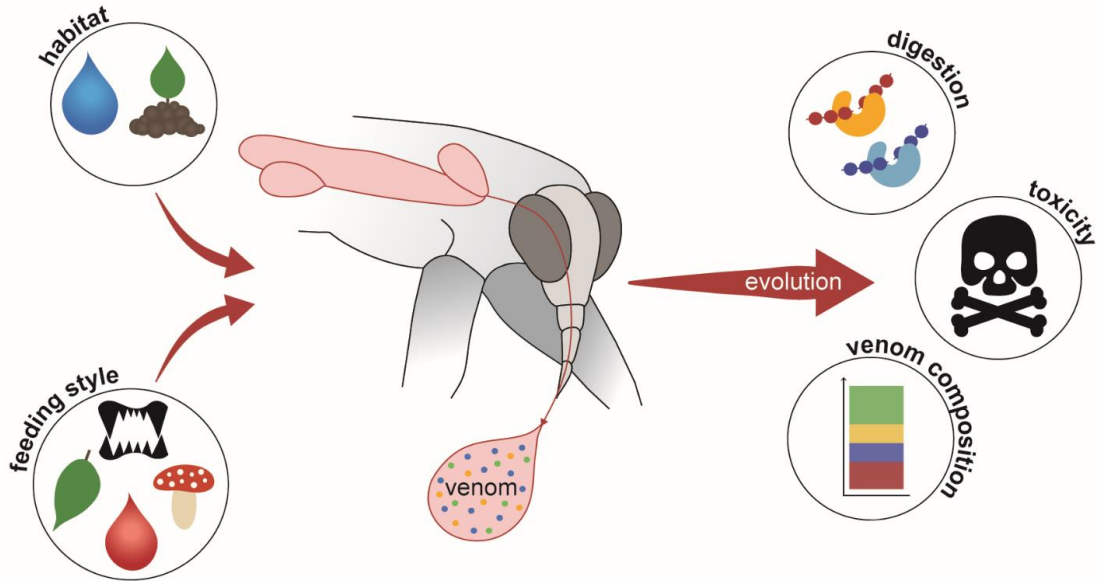

Figure 4: Schematic overview of the main research question addressed in this thesis. We studied the impact of the ecological niche (including habitat and feeding style) on venom evolution, function and deployment in Heteroptera. We analyzed and compared the venom composition and activity in different heteropteran species to identify species-dependent adaptations particularly with respect to extra-oral digestion and toxicity.

2. MANUSCRIPT OVERVIEW

2.1. Manuscript I

You are what you eat – ecological niche and microhabitat influence venom activity and composition in aquatic bugs

Maike L. Fischer, Sol A. Yepes Vivas, Natalie Wielsch, Roy Kirsch, Andreas Vilcinskas, Heiko Vogel

Accepted for publication in *Proceedings of the Royal Society B: Biological Sciences*

Summary

In **manuscript I**, we compared the venom protein composition and activity of four closely related water bug species (*Ilyocoris cimicoides*, *Notonecta glauca*, *Nepa cinerea* and *Corixa punctata*) occupying different ecological niches. Using an integrated proteomic and transcriptomic approach, we identified interspecific differences in the venom composition, particularly in the digestive enzyme repertoire, and found proteins probably associated with vertebrate feeding, prey overwhelming and antipredator defense. In addition, we detected significant differences in antimicrobial, insecticidal and hemolytic activity of the crude venom extracts and found evidence that amylases play a critical role in both phytophagous and zoophagous species by exhibiting dual activity against starch and glycogen. These results provide evidence that the ecological niche strongly influences venom composition and activity in water bugs.

Author contributions		
	Conceptualization	MF (25%), HV, AV
	Designed experiments	MF (30%), HV, AV, RK
	Performed experiments	MF (65%), HV, SYV, NW
	Data analysis and statistics	MF (70%), HV, NW, SYV
	Data visualization	MF (100%)
	Writing – original draft	MF (90%), HV, NW
	Writing – review and editing	MF (60%), HV, RK, NW, AV

Angaben zum Eigenanteil

(gemäß der Durchführungsbestimmung zu § 8 Abs. 2 der Promotionsordnung vom 23.09.2019 der Fakultät für Biowissenschaften der FSU Jena, Anlage 2, Formular 1)

Manuskript Nr. 1

Titel des Manuskriptes: You are what you eat – ecological niche and microhabitat influence venom activity and composition in aquatic bugs

Autoren: Maike L. Fischer, Sol A. Yepes Vivas, Natalie Wielsch, Roy Kirsch, Andreas Vilcinskas, Heiko Vogel

Bibliographische Informationen: Akzeptiert zur Publikation in *Proceeding of the Royal Society B: Biological Sciences*

Die Kandidatin ist

X Erstautor/-in, ☐ Ko-Erstautor/-in, ☐ Korresp. Autor/-in, ☐ Koautor/-in.

Status: Akzeptiert zur Publikation

Anteile (in %) der Autoren / der Autorinnen an den vorgegebenen Kategorien der Publikation

Autor/-in	Konzeptionell	Daten-analyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Maike L. Fischer	25%	70%	70%	80%	0%
Sol A. Yepes Vivas	0%	10%	10%	0%	0%
Natalie Wielsch	5%	10%	10%	3%	10%
Roy Kirsch	5%	0%	0%	5%	10%
Andreas Vilcinskas	20%	0%	0%	5%	0%
Heiko Vogel	45%	10%	10%	7%	80%
Summe:	100 %	100 %	100 %	100 %	100 %

_____ _____

Unterschrift Kandidat/-in Unterschrift Betreuer/-in (Mitglied der Fakultät)

2.2. Manuscript II

Context-dependent venom deployment and protein composition in two assassin bugs

Maike L. Fischer, Natalie Wielsch, David G. Heckel, Andreas Vilcinskas, Heiko Vogel

Published August 17 2020 in *Ecology and Evolution*

Ecology and Evolution 10.18 (2020): 9932-9947, doi: 10.1002/ece3.6652

Summary

Manuscript II describes the comparative analysis of venom deployment and protein composition in the two closely related African assassin bug species *Psytalla horrida* and *Platymeris biguttatus*. Using a comparative proteotranscriptomic approach combined with bioassays, we found that the anterior main gland (AMG) and posterior main gland (PMG) of both species secrete distinct protein mixtures that facilitate the immobilization, killing and EOD of prey. In particular PMG venom had strong hemolytic, antibacterial, insecticidal and digestive effects. By collecting venom using an artificial prey dummy, we showed that the predatory venom in both species originated exclusively from the PMG, as reported for other predatory heteropterans. Whereas *P. biguttatus* also used PMG venom alone for defense, *P. horrida* secreted different types of venom depending on the context: AMG venom was released in response to strong harassment, whereas PMG venom was elicited by mild harassment without fixation. This is the second case of context-dependent venom deployment in Heteroptera and contributes to our understanding of venom deployment and activity in predatory true bugs.

Author contributions		
	Conceptualization	MF (10%), HV, AV
	Designed experiments	MF (20%), HV, AV
	Performed experiments	MF (70%), HV, NW
	Data analysis and statistics	MF (70%), HV, NW
	Data visualization	MF (100%)
	Writing – original draft	MF (90%), HV, NW
	Writing – review and editing	MF (60%), HV, NW, AV, DH

Angaben zum Eigenanteil

(gemäß der Durchführungsbestimmung zu § 8 Abs. 2 der Promotionsordnung vom 23.09.2019 der Fakultät für Biowissenschaften der FSU Jena, Anlage 2, Formular 1)

Manuskript Nr. 2

Titel des Manuskriptes: Context-dependent venom deployment and protein composition in two assassin bugs

Autoren: Maike L. Fischer, Natalie Wielsch, David G. Heckel, Andreas Vilcinskas, Heiko Vogel

Bibliographische Informationen: Fischer, M. L., Wielsch, N., Heckel, D. G., Vilcinskas, A., & Vogel, H. (2020). Context-dependent venom deployment and protein composition in two assassin bugs. *Ecology and evolution*, 10(18), 9932-9947, doi: 10.1002/ece3.6652.

Die Kandidatin ist

X Erstautor/-in, ☐ Ko-Erstautor/-in, ☐ Korresp. Autor/-in, ☐ Koautor/-in.

Status: veröffentlicht am 17. August 2020 in „Ecology and Evolution“

Anteile (in %) der Autoren / der Autorinnen an den vorgegebenen Kategorien der Publikation

Autor/-in	Konzeptionell	Daten-analyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Maike L. Fischer	10%	70%	70%	85%	0%
Natalie Wielsch	5%	10%	10%	3%	10%
David G. Heckel	5%	0%	0%	2%	20%
Andreas Vilcinskas	30%	0%	0%	3%	0%
Heiko Vogel	50%	20%	20%	7%	70%
Summe:	100 %	100 %	100 %	100 %	100 %

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Unterschrift Kandidat/-in Unterschrift Betreuer/-in (Mitglied der Fakultät)

2.3. Manuscript III

An assassin's secret: multifunctional cytotoxic compounds in the predation venom of the assassin bug *Psytalla horrida*

Maike L. Fischer, Benjamin Fabian, Yannick Pauchet, Natalie Wielsch, David G. Heckel, Silke Sachse, Andreas Vilcinskas, Heiko Vogel

In preparation to be submitted to *Toxins*

Summary

Manuscript III describes the identification and characterization of cytotoxic compounds in the PMG venom of the African assassin bug *Psytalla horrida*. Using cation-exchange chromatography, PMG venom extracts were separated into 43 fractions that were screened for cytotoxic activity. Two fractions had strong antimicrobial, insecticidal and hemolytic activity. Liquid chromatography tandem mass spectrometry revealed that both fractions contained gelsolin, S1 family peptidases, redulysins and uncharacterized venom protein family 2 proteins. Synthetic redulysin peptides based on the predicted lytic domain of *P. horrida* redulysins showed strong antimicrobial activity against *Escherichia coli* and/or *Bacillus subtilis* but only weak effects on insect cell viability and erythrocyte integrity. In contrast, a recombinant venom protein family 2 protein significantly reduced insect cell viability but had no effects on microbial growth or red blood cells. The results show that *P. horrida* secretes several cytotoxic compounds from different gene families that target different organisms and contribute to antimicrobial defense as well as prey overwhelming, killing and liquefaction.

Author contributions		
	Conceptualization	MF (30%), HV, AV
	Designed experiments	MF (30%), HV, AV, YP, BF, SS
	Performed experiments	MF (70%), BF, HV, NW
	Data analysis and statistics	MF (70%), BF, HV, NW
	Data visualization	MF (100%)
	Writing – original draft	MF (75%), BF, HV, NW
	Writing – review and editing	MF (50%), HV, BF, NW, SS, AV

Angaben zum Eigenanteil

(gemäß der Durchführungsbestimmung zu § 8 Abs. 2 der Promotionsordnung vom 23.09.2019 der Fakultät für Biowissenschaften der FSU Jena, Anlage 2, Formular 1)

Manuskript Nr. 3

Titel des Manuskriptes: An assassin's secret: multifunctional cytotoxic compounds in the predation venom of the assassin bug *Psytalla horrida* (Reduviidae, Hemiptera)

Autoren: Maike L. Fischer, Benjamin Fabian, Yannick Pauchet, Natalie Wielsch, Silke Sachse, Andreas Vilcinskas, Heiko Vogel

Bibliographische Informationen: In Vorbereitung zur Einreichung bei *Toxins*

Die Kandidatin ist

X Erstautor/-in, ☐ Ko-Erstautor/-in, ☐ Korresp. Autor/-in, ☐ Koautor/-in.

Status: In Vorbereitung zur Einreichung

Anteile (in %) der Autoren / der Autorinnen an den vorgegebenen Kategorien der Publikation

Autor/-in	Konzeptionell	Daten-analyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Maike L. Fischer	30%	70%	80%	75%	0%
Benjamin Fabian	5%	10%	10%	10%	0%
Yannick Pauchet	10%	0	0%	2%	8%
Natalie Wielsch	2%	5%	5%	3%	5%
Silke Sachse	3%	0%	0%	2%	15%
Andreas Vilcinskas	20%	0%	0%	3%	22%
Heiko Vogel	30%	15%	5%	5%	50%
Summe:	100 %	100 %	100 %	100 %	100 %

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Unterschrift Kandidat/-in Unterschrift Betreuer/-in (Mitglied der Fakultät)

3. MANUSCRIPTS

3.1. Manuscript I

You are what you eat – ecological niche and microhabitat influence venom activity and composition in aquatic bugs

Maike L. Fischer, Sol A. Yepes Vivas, Natalie Wielsch, Roy Kirsch, Andreas Vilcinskas, Heiko Vogel

Accepted for publication in *Proceedings of the Royal Society B: Biological Sciences*

Shown here is the accepted version of the article

You are what you eat – ecological niche and microhabitat influence venom activity and composition in aquatic bugs

Maike L. Fischer, Sol A. Yepes Vivas, Natalie Wielsch, Roy Kirsch, Andreas Vilcinskas, Heiko Vogel

Abstract

True water bugs (Nepomorpha) are mostly predacious insects that live in aquatic habitats. They use their piercing–sucking mouthparts to inject venomous saliva that facilitates the capture and extra-oral digestion of prey animals, but their venom can also be deployed for defence. In Central Europe, nepomorph species representing different families co-exist in the same habitat. However, their feeding ecology, including venom composition and deployment, has not been investigated in detail. We used an integrated proteotranscriptomic and bioactivity-based approach to test whether venom composition and activity differs between four water bug species sharing the same habitat but occupying different ecological niches. We found considerable species-dependent differences in the composition of digestive enzymes and venom components that probably evolved as adaptations to particular food sources, foraging strategies, and/or microhabitats. The venom of *Corixa punctata* differed substantially from that of the three strictly predatory species (*Ilyocoris cimicoides*, *Notonecta glauca* and *Nepa cinerea*), and the abundance of herbivory-associated proteins confirms a mostly plant-based diet. Our findings reveal independent adaptations of the digestive and defensive enzyme repertoires accompanied by the evolution of distinct feeding strategies in aquatic bugs.

Keywords: ecological niche, diet, feeding style, venom, water bugs, proteotranscriptomics

Introduction

True bugs (Heteroptera) are a diverse group of hemimetabolous insects that exploit a wide range of habitats and food sources around the world (1). Although most true bugs feed on plants, recent phylogenetic studies suggest that heteropterans shifted to a predatory lifestyle when they diverged from the remaining phytophagous Hemiptera (2). The infraorder Nepomorpha (true water bugs) comprises 11–13 families of almost exclusively predacious species that spend most of their lives underwater (1). Specific adaptations to their predatory lifestyle include the evolution of a strong but short rostrum (1), raptorial legs (3, 4), and the secretion of venomous saliva that is used to paralyse, kill and pre-digest animal prey, but also in defence against enemies (1, 5-8). Some water bug venoms have remarkable effects on animals, including changes in contractile force and coronary flow in guinea pigs (9), paralysis in fish (10), and systolic arrest in cockroach heart–dorsum preparations (11).

The salivary/venom glands of true bugs usually feature three spatially separated parts: an anterior main gland (AMG), a posterior main gland (PMG), and an accessory gland (AG) (12). The context-dependent deployment of AMG and PMG venom has been proven only for the terrestrial predacious assassin bugs *Pristhesancus plagipennis* and *Psytalla horrida* (7, 8). Differential venom deployment is not known among water bugs, although the analysis of AMG and PMG venom from the giant water bug *Lethocerus distinctifemur* (Nepomorpha, Belostomatidae) showed that the glands secrete distinct sets of proteins, suggesting different functional roles (13). Proteins identified in water bug venoms include proteases,

haemolysin-like proteins, protease inhibitors, hyaluronidases, phospholipases, amylases and numerous uncharacterised peptides (10, 13-15). However, most studies thus far have focused on belostomatid venoms so the dynamics of venom composition across different nepomorph families remain unclear, particularly when considering adaptation to microhabitats, differences in prey (or even shifts to a non-predacious lifestyle), and changes in predatory selection pressure.

Common European water bugs include the saucer bug *Ilyocoris cimicoides* (Naucoridae), the backswimmer *Notonecta glauca* (Notonectidae), the water scorpion *Nepa cinerea* (Nepidae), and the lesser water boatman *Corixa punctata* (Corixidae). These species co-exist in the same type of habitat but differ in terms of microhabitat preferences, food spectra and foraging strategies (Figure 1). *Ilyocoris cimicoides*, *N. glauca* and *N. cinerea* are strict predators of insects (16-20), crustaceans (16, 21-23), tadpoles (24, 25) and fish (26-28), but the dietary habits of *C. punctata* are not yet fully understood. Different studies have reported inconsistent feeding styles for *C. punctata*, ranging from strict zoophagy (29) to saprophagy (30) and omnivorous behaviour, including the unusual ability to ingest solid food (31). Furthermore, foraging strategies differ among the predatory species. *Nepa cinerea* is a slow-moving ambush predator that hides in vegetation close to the water surface and quickly grabs approaching prey using its highly specialised predatory forelegs (3, 20, 29, 32). In contrast, *N. glauca* and *I. cimicoides* are good swimmers and actively hunt their prey (16, 32, 33). *Notonecta glauca* usually searches for prey on the water surface or swimming in the open water (18, 34, 35), whereas *I. cimicoides* remains on the sediment or in vegetation to hunt and feed on prey (32, 33). These microhabitat preferences may be associated with different food spectra given that the composition of the prey community differs between microhabitats (34).

The effects of ecological niches on saliva composition and activity in heteropterans are poorly understood and little is known about the consistency of adaptations to specific conditions. Previous studies have focused on terrestrial bugs and mainly investigated differences in the activity of digestive enzymes to draw conclusions about dietary habits (36-39). Recently, the salivary protein composition was analysed in several terrestrial and two aquatic bugs from different suborders, revealing patterns that may indicate specific dietary habits (40). The proteins characteristic of predatory bugs included CUB domain proteins, haemolysins, Ptu1-like peptides, redulysins, and several uncharacterised peptides. In contrast, typical herbivore-associated proteins included amylases, glucosidases, vitellogenins and cathepsins. However, few studies have considered species in the same infraorder with different ecological adaptations. We hypothesised that aquatic bug species sharing the same habitat but occupying different ecological niches (including microhabitat, food spectrum, and foraging strategy) would have different venom compositions and activities. Our main objectives were therefore to collect and analyse gland-specific gene expression data and venom gland protein compositions using an integrated proteotranscriptomic and bioassay-based approach, followed by the comparison of venom composition and activity in *I. cimicoides*, *N. glauca*, *N. cinerea* and *C. punctata* to identify interspecific differences and relationships with each ecological niche. We discuss the ecological insights that can be drawn from these results in order to determine whether it is possible to infer the ecology of a given heteropteran species from the composition of its venom, or *vice versa* (Figure 1).

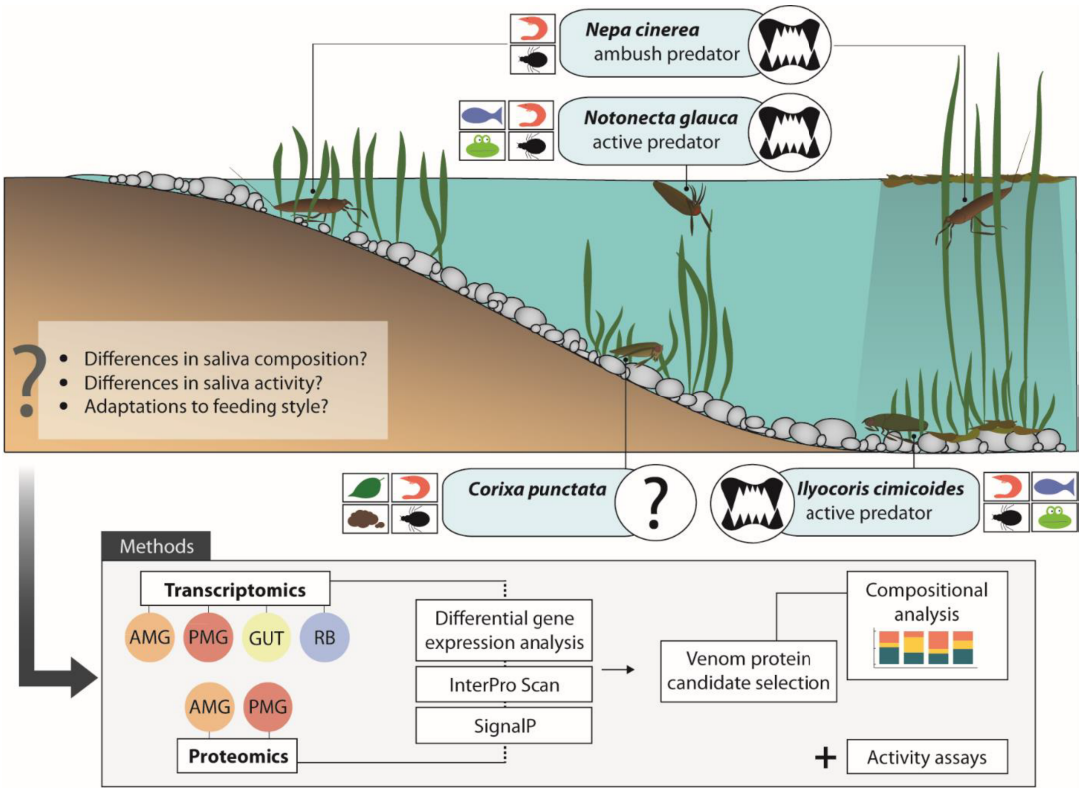


Figure 1. Schematic overview of the microhabitat preferences, food spectra (insects, crustaceans, fish, tadpoles, plants and detritus), feeding styles and foraging strategies of the water bugs *I. cimicoides*, *N. glauca*, *N. cinerea* and *C. punctata*, based on our experimental observations and the literature. We applied a proteotranscriptomic and bioassay-based approach to identify salivary proteins and saliva activity. AMG = anterior main gland; PMG = posterior main gland; RB = remaining body tissue.

Materials and methods

Insects and venom collection

Specimens of *I. cimicoides*, *N. glauca*, *N. cinerea* and *C. punctata* were collected in Thuringia, Germany and kept in water-filled containers before dissection. Venom likely to have a defensive function was obtained by harassment. Specimens were captured with forceps and held above the water, which elicited defensive behaviour. An artificial prey dummy was built by enclosing a droplet of phosphate-buffered saline (PBS) within a piece of stretched Parafilm (Figure S1) and was offered to the insects for venom injection (7). The artificial prey dummy was moved on the water surface to simulate moving prey and induce hunting behaviour. When a bug attacked the artificial prey, it was allowed to inject saliva for 1.5 min before removing the dummy and recovering the venom. It was not possible to collect defensive venom from *N. cinerea* or defensive and prey-killing venom from *C. punctata*. In addition to non-invasive collection, venom was also extracted directly from the venom glands of fifth-instar or adult bugs. The specimens were anaesthetised at –20 °C for 5 min before dissection in PBS. The AG, AMG and PMG (only

AMG and PMG for *C. punctata*) were separated and placed in pre-cooled tubes containing 10–20 µL PBS on ice. The samples were briefly vortexed and centrifuged (4000 g, 3.5 min) and the supernatant was transferred to a fresh tube. The venom of several individuals was pooled and stored at –20 °C for analysis. The total protein concentration in the samples was measured using an N60 nanophotometer (Implen).

Proteomic analysis

The proteins in the venom samples were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–12% Criterion XT gradient gels (Bio-Rad) with XT MES running buffer at 125 V for 1.5 h, alongside protein molecular weight markers. The gels were stained for 1.5 h using a 1:1 mixture of Coomassie Brilliant Blue R-250 and colloidal Coomassie Brilliant Blue G-250 (Thermo Fisher Scientific), washed in Millipore water overnight and then scanned. For liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis, protein bands from each gel lane were excised and digested with trypsin (41). Further details of LC-MS sample processing, data acquisition and data processing are presented in Methods S1, Section 1.

Venom gland collection and RNA isolation

AMG, PMG, gut, and remaining body tissue (fat body, muscle tissue and integument) were carefully removed and placed in separate ceramic bead tubes containing 500 µL TRI Reagent (Sigma-Aldrich). The tissues of several individuals were pooled and homogenised using a TissueLyser LT (Qiagen). Total RNA was extracted using the Direct-zol RNA Miniprep Kit (Zymo Research). The quantity of RNA was measured using the Implen N60 nanophotometer and RNA integrity was confirmed using an Agilent 2100 Bioanalyzer and RNA Nanochip (Agilent Technologies).

RNA-Seq and de novo transcriptome assembly

For all species, transcriptome sequencing of AMG, PMG, gut, and remaining body tissue was performed by the Max-Planck Genome Center Cologne (http://mpgc.mpipz.mpg.de/home/) using an Illumina HiSeq3000 Genome Analyzer platform. Poly(A) mRNA was extracted from 1 µg total RNA using oligo-dT attached to magnetic beads, and was fragmented to an average length of 250 bp. Sequencing libraries were generated using the TruSeq RNA library preparation kit (Illumina) and paired-end (2 × 150 bp) read technology was used for sequencing. All generated reads were processed using an in-house assembly and annotation pipeline as previously described (42). Details of assemblies, annotations and RNA-Seq mapping are presented in Methods S1, Section 2.

Venom activity bioassays

Haemolytic activity was determined on defibrinated horse blood (Thermo Fisher Scientific). Erythrocytes were harvested by centrifugation (1500 g, 3 min), washed three times with PBS, and prepared as a 1:10 erythrocyte suspension in PBS. We mixed 20 µL venom extract in PBS (concentrations shown in Figure 2) with 180 µL of the cell suspension (n = 3) in a 96-well plate and incubated the cells at 37 °C for 1 h. We used 1% Triton X-100 and PBS as positive and negative controls, respectively (n = 3). The cells were centrifuged (2000 g, 10 min) and the supernatants were transferred to a clear 96-well plate. The absorbance at 440 nm was measured using an Infinite m200 plate reader (Tecan). Relative haemolysis was calculated in relation to the positive control (defined as 100%).

Bacterial growth inhibition was tested using a liquid growth antibacterial assay with *Escherichia coli*. We inoculated 50 µL of an *E. coli* overnight culture into 5 mL lysogeny broth (LB) and incubated the cells at 37 °C for 2–3 h. The culture was diluted with LB to an optical density at 600 nm (OD_{600}) of 0.003 and 90 µL of the bacterial suspension was mixed with 10 µL venom extract in PBS (concentrations shown in Figure 2) in a clear, sterile 96-well plate. We used 0.05 mg/mL gentamycin and PBS as the positive and negative controls, respectively. The absorbance at 595 nm was measured using the Tecan Infinite m200 plate reader over a period of 24 h at 5-min intervals. The temperature was held constant at 30 °C. Relative growth inhibition was calculated in relation to the positive control (defined as 100%) at the time the growth control reached OD_{595} = 0.35.

Cytotoxic effects against *Spodoptera frugiperda* (Sf9) cells were tested using an MTT assay based on thiazolyl blue tetrazolium bromide. The cells were cultured in Sf-900 II SFM medium (Gibco) containing 0.05 mg/mL gentamycin in a sterile 96-well plate (Thermo Fisher Scientific). After 24 h, the culture medium was replaced with 100 µL venom extract in culture medium (concentrations shown in Figure 2). We used 100 µL 0.1% Triton X-100 and 100 µL culture medium as positive and negative controls, respectively. The cells were incubated at 27 °C for 4 h. The culture medium was then replaced with 100 µL 0.5 mg/mL MTT solution in culture medium and incubated at 27 °C for 2 h. The MTT solution was removed and replaced with 50 µL DMSO per well. After incubation at 27 °C for 10 min, the plate was briefly vortexed, and the absorbance at 540 nm was measured using the Tecan Infinite m200 plate reader. The relative cell viability was calculated in relation to the negative control (defined as 100%).

Carbohydrase activity assay

The degradation of starch and glycogen by venom extracts was measured using the 3,5-dinitrosalicylic acid (DNS) method as previously described (43). Briefly, crude venom extracts in PBS (concentrations shown in Figure 2) were mixed with either 1% (w/v) starch or 1% (w/v) glycogen in water at a ratio of 3:1 (v/v) and incubated at 25 °C for 2 h. An equal volume of a 99:1 (v/v) mixture of solution 1 (44 mM DNS, 21 mM phenol, 250 mM sodium hydroxide) and solution 2 (400 mM sodium sulfite) was added to each reaction and incubated at 99 °C for 5 min. We then added 200 mM potassium sodium tartrate at a ratio of 1:6 (v/v) and measured the absorbance at 575 nm using the Tecan Infinite m200 plate reader.

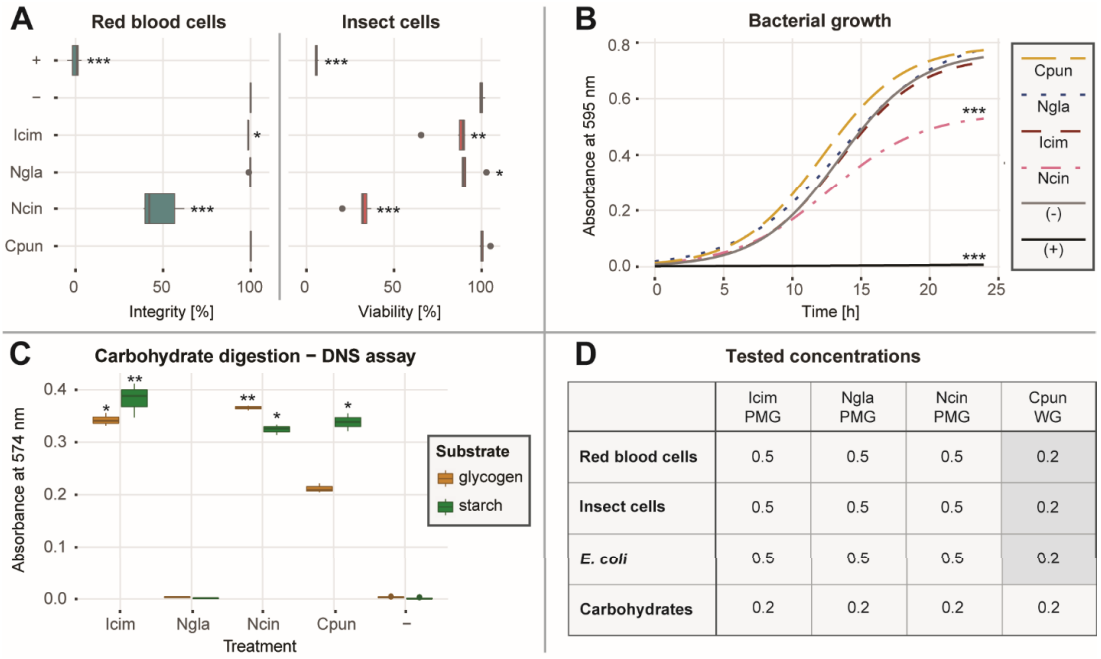
Statistical analysis

Statistical analysis was conducted using R v4.0.3 and the integrated development environment RStudio v1.2.1335 (http://www.R-project.org/). For the haemolysis, cytotoxicity and carbohydrase activity assays, we performed Kruskal-Wallis tests with subsequent Dunn's test for multiple comparisons using the FSA package (44) in order to identify significant differences compared to the negative controls. For the analysis of bacterial growth curves, the data were fitted to a logistic model using the growthcurver package (45). Permutation tests for pairwise comparisons of growth curves were performed using the statmod package (46).

Results

Venom activity toward different substrates

We carried out bioactivity assays on different cells and substrates to identify species-dependent differences in venom activity. The *N. cinerea* PMG extract showed strong toxicity toward horse erythrocytes and Sf9 cells, and inhibited the growth of *E. coli*. In contrast, the *I. cimicoides* and *N. glauca* PMG extracts showed little and no activity, respectively, against erythrocytes, and only mild toxicity toward Sf9 cells. Whole gland extracts (AMG + PMG) from *C. punctata* showed no toxicity toward any of the cells (Figure 2A,B). The ability of extracts to degrade the polysaccharides starch and glycogen was determined using the DNS method by quantifying the reducing groups released during substrate hydrolysis. The extracts from *I. cimicoides*, *N. cinerea* and *C. punctata* were able to degrade both substrates, whereas the PMG extract from *N. glauca* did not digest either of them (Figure 2C).



	Icim PMG	Ngla PMG	Ncin PMG	Cpun WG
Red blood cells	0.5	0.5	0.5	0.2
Insect cells	0.5	0.5	0.5	0.2
E. coli	0.5	0.5	0.5	0.2
Carbohydrates	0.2	0.2	0.2	0.2

Figure 2: Effects of PMG extracts or whole gland (WG = AMG + PMG) extracts on different substrates. Significant differences compared to the negative control are highlighted with asterisks (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). Box plots present the median (line), interquartile range (box) and data range (whiskers). **A** Effects on horse erythrocyte integrity and insect cell viability: (-) = PBS; (+) = 0.1% Triton-x-100. Statistical analysis: Dunn's test, n = 3 (haemolysis), n = 6 (cell viability). **B** Effects on *E. coli* growth: (-) = PBS; (+) = 0.05 mg/mL gentamycin. The data were fitted to a logistic model and plotted as growth curves. Statistical analysis: permutation test, n = 3. **C** Digestion of amylose and glycogen: (-) = PBS. Statistical analysis: Dunn's test, n = 3. **D** Summary of the final venom concentrations [mg/mL] tested in the different bioassays.

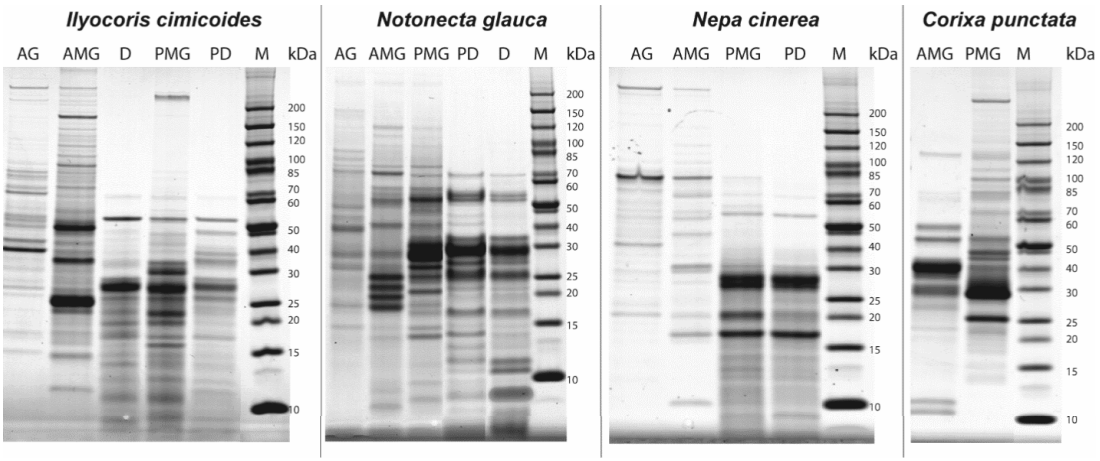


Figure 2: SDS-PAGE analysis of venom gland extracts and venom collected non-invasively from *I. cimicoides*, *N. glauca*, *N. cinerea* and *C. punctata*. AG = accessory gland extract; AMG = anterior main gland extract; PMG = posterior main gland extract; PD = prey-dummy venom; D = defensive venom; M = protein marker (kDa).

Glandular origin of defence and predation venom

AG, AMG and PMG extracts, as well as non-invasively collected venom samples, were fractionated by SDS-PAGE to visually compare their banding patterns. The protein bands of prey dummy and defensive venoms resembled the PMG extracts of the corresponding species, suggesting that the PMG is the glandular origin of both venom types (Figure 3). Proteomic analysis of excised bands showed that the AMG and PMG secrete distinct sets of proteins, but the proportion of gland-specific proteins differed between species. In *N. glauca*, 45% of the proteins were secreted by both glands, whereas in *N. cinerea* almost all proteins (95%) were specific to either the AMG or PMG (Figure 4). Furthermore, most proteins in the prey-dummy and defensive venoms were PMG-specific or produced by both lobes. Very few proteins were AMG-specific (Figure S2), confirming that both the prey-dummy and defensive venoms originate from the PMG.

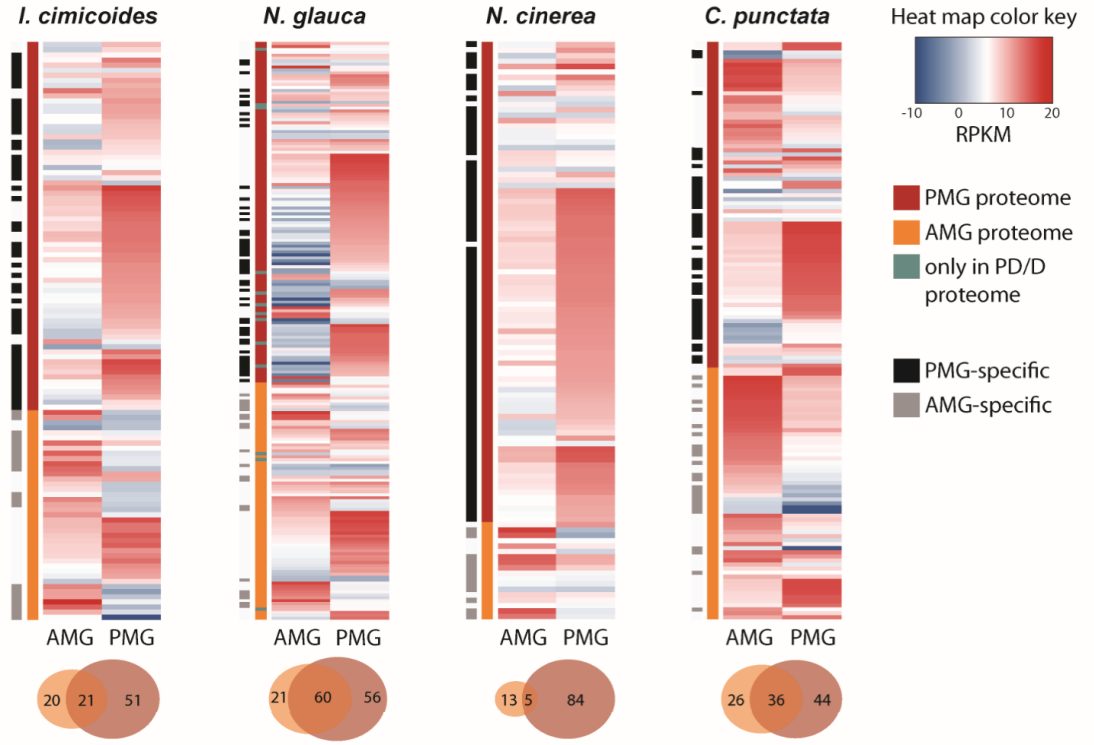


Figure 3: AMG-specific, PMG-specific and common gene expression in *I. cimicoides*, *N. glauca*, *N. cinerea* and *C. punctata*. The gene expression levels (RPKM) of the identified venom proteins in the AMG (highlighted in orange), PMG (highlighted in red) or only PD/D proteomes (highlighted in green) are shown in the heat maps. PMG-specific and AMG-specific proteins are shown using black and grey bars, respectively. The number of proteins unique to and common to the AMG and PMG in each species are visualised using Venn diagrams.

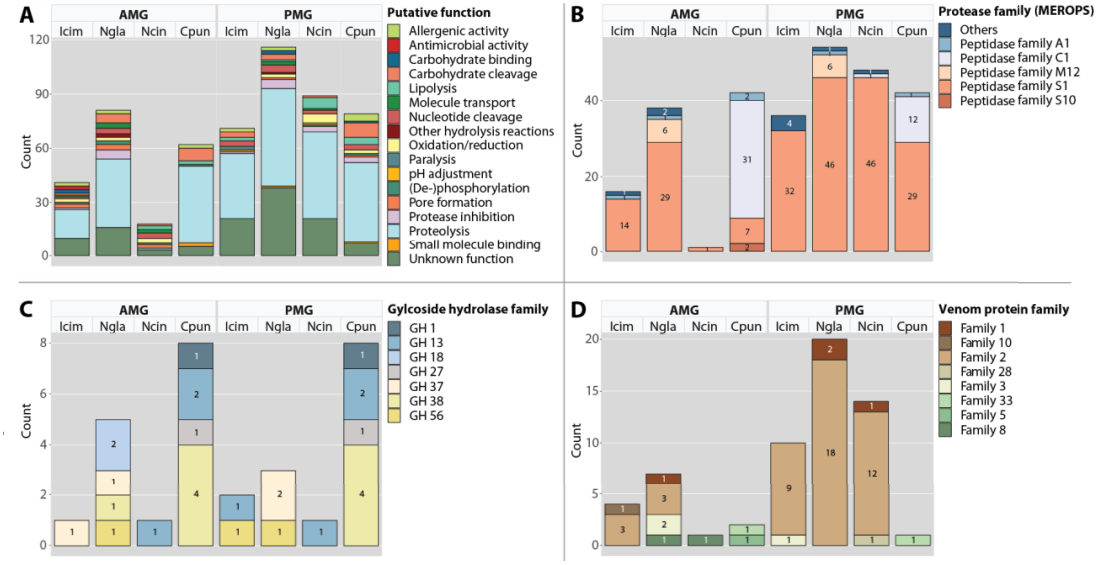


Figure 5: Protein composition of AMG/PMG venom in *I. cimicoides* (Icim), *N. glauca* (Ngla), *N. cinerea* (Ncin) and *C. punctata* (Cpun) based on proteomics analysis and shown as relative transcript abundance. (**A**) Colour-coded blocks show the number of contigs identified by proteotranscriptomic analysis encoding specific classes of functional proteins. Categories are further subdivided into (**B**) protease families (MEROPS), (**C**) glycoside hydrolase families, and (**D**) venom protein families.

Protein composition of AMG and PMG venoms

Next-generation sequencing (RNA-Seq) was carried out to identify and quantify venom-associated transcripts, thus providing more insight into the protein composition of AMG and PMG venom. RNA isolated from the AMG, PMG, gut, and remaining body tissue was used for Illumina sequencing, which yielded 30–40 million reads per sample. Information on the *de novo* reference transcriptome assemblies is provided in Table S1. Most of the identified proteins were proteases, followed by uncharacterised heteropteran venom proteins assigned to various families. Digestive enzymes such as lipases, carbohydrases, nucleases and nucleotidases were also detected (Figure 5A). Tissue-specific RPKM levels showed that most venom proteins were associated with highly gland-specific gene expression patterns (Figure 4). The most abundant transcripts in the PMG encoded S1 family peptidases and members of venom protein family 2 (as well as a CUB domain protein in *N. cinerea*), whereas the most abundant transcripts in the AMG encoded haemolysins, venom protein family 2 members, and other uncharacterised proteins. In *C. punctata*, the most abundant AMG transcripts encoded C1 family peptidases. Ptu1-like peptides, which are common channel modulators in heteropteran venoms, were detected only in *I. cimicoides*. Remarkable interspecific differences were found among the digestive enzymes. In the three predatory species, most of the proteases were S1 family peptidases, whereas more than half of the proteases in *C. punctata* were C1 family peptidases, most of which were expressed in the AMG. Furthermore, several M12 family metallopeptidases were identified solely in *N. glauca* (Figure 5B). In addition to proteases, several carbohydrases from different glycoside hydrolase (GH) families were also identified. The largest number of GHs was detected in *C. punctata* and included families GH1, GH13, GH27

and GH38. In the predatory species, we identified carbohydrases from families GH13, GH18, GH37, GH38 and GH56 (Figure 5C). We also observed remarkable differences in the expression of uncharacterised heteropteran venom protein families. In *I. cimicoides*, *N. glauca* and *N. cinerea*, most such proteins belonged to venom protein family 2 and were strongly expressed in the PMG. In contrast, *C. punctata* expressed no venom protein family 2 proteins but did express two venom proteins from families 5 and 33, which were not present in the other species. We also identified venom proteins from families 1, 3, 8, 10 and 28 (Figure 5D).

Discussion

We used an integrated proteotranscriptomic and bioactivity-based approach to investigate how ecological niches shape the venom/saliva protein composition and activity in four water bug species that co-exist in the same aquatic habitats. The two main salivary glands (AMG and PMG) secreted distinct and complex sets of proteins, although only the PMG was found to be involved in envenomation. The composition and biological activity of the venoms differed between the four species, so we considered the impact of the microhabitat, food spectrum and foraging strategy in more detail.

Water bugs are well-adapted to an aquatic lifestyle and many different species co-exist in the same habitat, but even so the microhabitat preferences of individual species can differ considerably. The behaviour of each species reflects whether it spends its time primarily near the water surface, on vegetation, or on the sediment at the bottom of the pond, and this is likely to result in microhabitat-specific adaptations (17, 35). *Notonecta glauca* is an active predator, searching for prey on the water surface or in the water column, and is therefore exposed to predators (16, 18, 34, 35). In contrast, *N. cinerea* usually hides in vegetation to wait for prey, but this low mobility may increase its susceptibility to predation (29, 32). Both species therefore rely on their potent venom to deter predators. Defensive venom generally induces pain, which motivates predators to quickly release their prey and also deters future attacks through avoidance behaviour (47-49). For example, the haemolytic polypeptide melittin in bee venom induces intense pain in vertebrates (50, 51), mainly by acting directly on primary nociceptive cells (52, 53) but possibly also by disrupting mast cell membranes and causing tissue damage, thus triggering the release of pain-inducing compounds (53-55). Pore-forming peptides from ants (56), spiders (57), fish (58), and bacteria (59) also have nociceptive effects on vertebrates. In our experiments, *N. cinerea* PMG venom had strong haemolytic, cytotoxic and antimicrobial effects, indicating the presence of lytic proteins that may be responsible for severe pain following envenomation (20). In contrast, PMG venom from *N. glauca* did not show strong lytic activity, although the Notonectidae are known for their painful stings (60). In snake venoms, metalloproteases induce potent hyperalgesia possibly by triggering mast cell activation (61-63). Similarly, the role of metalloproteases in pain induction, myotoxicity, and inflammation by centipede venom has been discussed (64, 65). We identified several M12 family metalloproteases solely in *N. glauca* venom, which suggests that such proteins are species-dependent adaptations to a microhabitat with higher risks of predation. However, a detailed characterisation of *N. glauca* M12 family metalloproteases is necessary to clarify their function and putative role in pain induction.

Microhabitat preferences are also likely to affect feeding habits because the abundance and availability of food vary within a water body. *Notonecta glauca*, *I. cimicoides* and *N. cinerea* are generalist predators

that feed on insects and crustaceans (16-19, 21-23, 29, 30), and also vertebrates such as fish and tadpoles in the case of *N. glauca* and *I. cimicoides* (24-28). We identified a protein in *N. glauca* and *I. cimicoides* PMG venom that is homologous to venom 5' nucleotidase 1 from the belastomatid *L. distinctifemur* (13). Such enzymes are often found in snake, spider and true bug venoms, and they inhibit platelet aggregation in vertebrate prey (13, 66-69). This indicates that *N. glauca* and *I. cimicoides* have adapted their venom composition for vertebrate prey, similarly to water bugs of the family Belastomatidae. Predatory species that feed on large, mobile animals require adaptations to quickly overwhelm their prey. This is facilitated by morphological structures such as raptorial forelegs or venom components that induce paralysis. A common channel modulator associated with paralytic activity in heteropteran venoms is Ptu1, an inhibitor cystine knot (ICK) family peptide first isolated from the assassin bug *Peirates turpis* (70, 71). Only *I. cimicoides* venom contained Ptu1-like peptides, and the transcripts were most abundant in the PMG. *Nepa cinerea* may not require paralytic venom because it uses its specialised predatory forelegs to prevent the escape of its prey (4). However, the Notonectidae do not have well-developed raptorial forelegs and instead quickly paralyze their prey (72-74). We found many uncharacterised proteins in *N. glauca* venom, including several peptides with no known homologs, suggesting that prey immobilisation by this species is facilitated by other proteins with distinct mechanisms of action. One protein family that was particularly abundant and strongly expressed in the venom glands of the three predatory species was heteropteran venom protein family 2, a group also present in other zoophagous and hematophagous bugs from various families but not in the phytophagous species investigated thus far (7, 8, 13, 40, 75, 76). The role of these proteins is unclear, but their strict gland-specific expression and abundance in predatory species suggest a key role in predation. Their complete absence in *C. punctata* indicates a non-predatory lifestyle. Further research, including the heterologous expression and characterisation of different venom protein family 2 members, is needed to determine their specific function in venom activity.

Unlike most water bugs, *C. punctata* reportedly feeds on detritus, algae, small insects, and crustaceans (29-31). In addition to this diet, its mouthpart morphology has also puzzled scientists because it differs considerably from the elongated rostrum typical of true bugs (Figure S3). These highly specialised mouthpart structures allow the Corixidae to ingest both liquid and solid food, a unique feature among heteropterans (31, 74). Mouthpart morphology and digestive enzymes play a key role in extra-oral digestion, and changes in enzyme composition may facilitate adaptation to different food sources. The ratio of amylase to protease activity has been used to predict heteropteran feeding habits, assuming that high protease and low amylase activity represent zoophagy whereas low protease and high amylase activity represent phytophagy (39). The structural similarity between starch and glycogen – the main carbohydrate storage products in plants and animals, respectively – may allow amylases to digest both substrates, as described for the midgut amylases of *Andralus spinidens* (77). We found that *I. cimicoides*, *N. cinerea* and *C. punctata* venoms can digest both starch and glycogen, indicating that the presence of salivary amylases and amylase activity does not necessarily imply phytophagy. Predatory insects rely heavily on proteases to digest protein-rich animal prey. In true bugs, most salivary proteases are serine endopeptidases, which have optimal activity at basic or neutral pH (78-81). In contrast, cysteine or aspartic endopeptidases (cathepsins) are typically found in gut secretions and are most active at acidic pH (78, 79, 81, 82). Surprisingly, most of the proteases detected in *C. punctata* saliva were cysteine-type C1 family peptidases. Salivary cysteine proteases have been found primarily in phytophagous hemipterans, where they digest plant-derived proteins and play a role in immunity against bacterial pathogens (83-86). In addition, salivary cathepsins from aphids elicit plant defences during feeding (87). The strong association between salivary cysteine proteases and herbivory suggests that *C. punctata* secretes C1 peptidase-rich

saliva as an adaptation to a mainly plant-based diet. Similarly, the composition of polysaccharide-degrading GH families partially reflects feeding type differences between water bug species. Whereas some GH families (e.g., GH56 hyaluronidases, GH37 trehalases) are found exclusively in predacious species, targeting abundant polysaccharides in prey, bi-functional amylases (GH13) occur in predacious and herbivorous species. Predacious species could benefit from dual-function enzymes because the amylase/glycogenase GH13 enzyme could allow easier access to the major dietary polysaccharides, enabling (among other factors) potential host shifts from herbivory to carnivory.

Our study shows that the venom composition and activity differ among four aquatic bug species occupying different ecological niches within the same habitat. We found remarkable interspecific differences and identified venom proteins that probably facilitated adaptations to particular food sources, foraging strategies and/or microhabitats. Many different factors influence salivary composition and assumptions about the dietary habits of true bugs should not rely solely on compositional analysis. Furthermore, research covering a wide range of heteropteran infraorders and families, feeding habits, and ecological niches is needed to derive general patterns and adaptations. Even so, it is clear that the compositional analysis of salivary venom provides strong evidence for the ecological adaptations of water bugs, and that the unique venom composition of *C. punctata*, including the abundance of herbivory-associated enzymes, is sufficient to hypothesise a predominantly plant-based diet in this species. Hypotheses based on venom protein profiles can therefore be used as the basis for additional experiments to determine the precise ecological niches occupied by hemipteran species.

Acknowledgements

M.L.F., S.A.Y.V., H.V., N.W. and D.G.H. acknowledge funding from the Max Planck Society, Germany. We thank Henriette Ringys-Beckstein and Bianca Wurlitzer for technical assistance in the laboratory. The work is part of the Animal Venomics project embedded into the LOEWE Centre for Translational Biodiversity Genomics (LOEWE–TBG) and was granted to A.V. under the program “Landes-Offensive zur Entwicklung Wissenschaftlich-Ökonomischer Exzellenz” of the Hessian Ministry of Higher Education, Research and the Arts. The authors acknowledge Richard M. Twyman for manuscript editing.

Author contributions

M.L.F.: data curation, formal analysis, investigation, methodology, visualisation, writing - original draft; **S.A.Y.V.:** formal analysis, investigation; **N.W.:** data curation, methodology, writing - review & editing; **R.K.:** formal analysis, methodology, writing - review & editing; **A.V.:** conceptualisation, funding acquisition, supervision, writing - review & editing; **H.V.:** conceptualisation, data curation, formal analysis, funding acquisition, supervision, writing - review & editing

All authors gave final approval for the publication and agreed to be held accountable for the work described therein.

Competing interests

We declare we have no competing interests.

Data accessibility

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB58831 (https://www.ebi.ac.uk/ena/browser/view/PRJEB58831). The Illumina short read data can be found with the following sample accession numbers: ERS14412810-ERS14412813 (*Corixa punctata*), ERS14412814-ERS14412818 (*Ilyocoris cimicoides*), ERS14412819-ERS14412823 (*Nepa cinerea*), ERS14412824-ERS14412827 (*Notonecta glauca*). The sequence and transcriptome assembly data have also been deposited in the Edmond Data Repository and are directly accessible via the following weblinks: https://doi.org/10.17617/3.VOQQRJ (*Corixa punctata*), https://doi.org/10.17617/3.OSCGGN (*Ilyocoris cimicoides*), https://doi.org/10.17617/3.7FEIDD (*Nepa cinerea*), https://doi.org/10.17617/3.B0WMNP (*Notonecta glauca*).

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3.2. Manuscript II

Context-dependent venom deployment and protein composition in two assassin bugs

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Published August 17 2020 in *Ecology and Evolution*

Ecology and Evolution 10.18 (2020): 9932-9947, doi: 10.1002/ece3.6652

Received: 3 April 2020 | Revised: 7 July 2020 | Accepted: 16 July 2020

DOI: 10.1002/ece3.6652

Ecology and Evolution Open Access WILEY

ORIGINAL RESEARCH

Context-dependent venom deployment and protein composition in two assassin bugs

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Abstract

The Heteroptera are a diverse suborder of phytophagous, hematophagous, and zoophagous insects. The shift to zoophagy can be traced back to the transformation of salivary glands into venom glands, but the venom is used not only to kill and digest invertebrate prey but also as a defense strategy, mainly against vertebrates. In this study, we used an integrated transcriptomics and proteomics approach to compare the composition of venoms from the anterior main gland (AMG) and posterior main gland (PMG) of the reduviid bugs *Platymeris biguttatus* L. and *Psytalla horrida* Stål. In both species, the AMG and PMG secreted distinct protein mixtures with few interspecific differences. PMG venom consisted mostly of S1 proteases, redulysins, Ptu1-like peptides, and uncharacterized proteins, whereas AMG venom contained hemolysins and cystatins. There was a remarkable difference in biological activity between the AMG and PMG venoms, with only PMG venom conferring digestive, neurotoxic, hemolytic, antibacterial, and cytotoxic effects. Proteomic analysis of venom samples revealed the context-dependent use of AMG and PMG venom. Although both species secreted PMG venom alone to overwhelm their prey and facilitate digestion, the deployment of defensive venom was species-dependent. *P. biguttatus* almost exclusively used PMG venom for defense, whereas *P. horrida* secreted PMG venom in response to mild harassment but AMG venom in response to more intense harassment. This intriguing context-dependent use of defensive venom indicates that future research should focus on species-dependent differences in venom composition and defense strategies among predatory Heteroptera.

KEYWORDS

assassin bug, defense venom, prey-killing venom, proteomics, transcriptomics, zoophagy

1 | INTRODUCTION

The use of venom for predation and defense is common in the animal kingdom (Case well, Wüster, Vonk, Harrison, & Fry, 2013). Venoms produced by snakes, spiders, scorpions, sea anemones, and cone snails have been investigated in detail because they are toxic toward vertebrates and thus medically relevant (King, 2015). Among insects, research has focused mainly on Hymenoptera because their

venoms are often allergenic and pose a risk of fatal anaphylaxis in humans (Bonifazi et al., 2005; Müller, 2010). In contrast, the suborder Heteroptera has been largely overlooked, although some species can inflict severe defensive bites on humans when disturbed (Haddad, Schwartz, Schwartz, & Carvalho, 2010; dos Santos, de Souza, Zanette, da Silva, & Strussmann, 2019). The Heteroptera are a diverse group of phytophagous, zoophagous, and hematophagous species that have adapted to exploit many terrestrial, aquatic, and semiaquatic habitats (Henry, 2009; Schuh & Weirauch, 2020). It is likely that the divergence of Heteroptera from more basal Hemiptera was accompanied by a shift to zoophagy, although some groups later shifted back to phytophagy (Johnson et al., 2018). The composition and evolution of Heteropteran venoms was recently reviewed in detail by Walker, Weirauch, Fry, and King (2016).

All heteropteran species feature piercing/sucking mouthparts, allowing them to inject salivary secretions into their food and suck up the liquid components (Cohen, 1998; Panfilio & Angelini, 2018). The saliva facilitates the extra-oral digestion of solid tissues and therefore improves access to nutrients (Cohen, 1998). Proteases have an essential role in nonrefluxing extra-oral digestion (Cohen, 1993, 1998) and are abundant in the saliva of numerous heteropteran species (Boyd, Cohen, & Alverson, 2002; Swart, Deaton, & Felgenhauer, 2006; Walker, Hernández-Vargas, Corzo, Fry, & King, 2018). For example, in the salivary secretions of the Australian assassin bug *Pristhesancus plagipennis*, 69 of 127 enzymes are associated with proteolysis, whereas only three have putative functions in lipid catabolism, one in nucleic acid catabolism, and 10 proteins are associated with cytolysis (Walker et al., 2017).

In zoophagous Heteroptera, the salivary glands (also called venom glands) not only secrete enzymes for the digestion of animal tissue (Cohen, 1995, 1998) but also proteins and peptides that facilitate the capture of prey (Edwards, 1961; Walker et al., 2017, 2019). The rapid paralysis of insects attacked by predatory assassin bugs such as *Rhinocoris carmelita* Stål and *Platymeris rhadamanthus* Gerstaecker was initially attributed to the disruption of cell membranes by digestive enzymes rather than the action of neurotoxins (Edwards, 1961). However, the subsequent analysis of assassin bug salivary peptides revealed similarities to the neurotoxic peptide ω-conotoxin from cone snails (Corzo, Adachi-Akahane, Nagao, Kusui, & Nakajima, 2001). Further characterization of Ptu1, a peptide from the reduviid species *Peirates turpis* Walker, revealed the presence of an inhibitor cystine knot (ICK) motif that causes the reversible inhibition of Cav2.2 voltage-gated calcium channels (Bernard, Corzo, Mosbah, Nakajima, & Darbon, 2001), thus refuting the hypothesis put forward by Edwards (1961). Recently, several Ptu1-like peptides were identified in the salivary secretions of *P. plagipennis* Walker and *P. rhadamanthus* (Walker et al., 2017, 2019). The venoms from both reduviid species induced rapid paralysis when injected into insects (Walker, Mayhew, et al., 2018; Walker et al., 2019).

Many predatory bugs use venom not only to attack and digest prey but also defensively when they are disturbed. Backswimmers (Notonectidae), also called water bees, occasionally inflict painful bites on humans during swimming (Diaz, 2016). Bites inflicted by assassin bugs and belostomatids are extremely painful, and can trigger various symptoms including edema (Haddad et al., 2010; Hartwig, 1977; dos Santos et al., 2019), paresthesia and pruritus (dos Santos et al., 2019), and pseudoparalysis (Haddad et al., 2010). Such defensive bites mainly target vertebrates and probably fulfill different functions compared to bites administered when killing and digesting invertebrate prey. Predatory bugs may therefore produce distinct venom components that are specifically active against invertebrates and vertebrates, or even different types of venom for each purpose (Haridass & Ananthakrishnan, 1981; Walker et al., 2017).

Hemipteran salivary glands comprise two accessory glands and a pair of principal glands that typically feature an anterior main gland (AMG) and a larger posterior main gland (PMG) as distinct lobes (Baptist, 1941). The effects of reduviid gland homogenates on arthropods were shown to depend on the source, with AMG extracts causing paralysis and PMG extracts failing to induce paralysis but leading to death after a few hours, suggesting that AMG venom is used for prey immobilization whereas the PMG secretes digestive enzymes. In contrast, accessory gland homogenates did not show any effects when injected into prey (Haridass & Ananthakrishnan, 1981). The analysis of venom collected from *P. plagipennis* by electrostimulation revealed the presence of both neurotoxic peptides and digestive enzymes in the secretions (Walker et al., 2017), but more detailed analysis showed that the AMG and PMG secretions differ substantially, and are used for defense and prey killing/digestion, respectively (Walker, Mayhew, et al., 2018). The deployment of functionally distinct venoms has also been reported in scorpions (Inceoglu et al., 2003) and cone snails (Dutertre et al., 2014).

The defensive venom of *P. plagipennis* mainly consists of hemolysin-like proteins, protease inhibitors, and several novel and uncharacterized proteins (Walker, Mayhew, et al., 2018). However, the mode of action of these secretions is largely unknown and requires further investigation. Furthermore, it remains unclear how the differential use of AMG and PMG venom is regulated in the insects and which ecological stimuli trigger the release of the specific venom types. In contrast to *P. plagipennis*, a recent study of the red spot assassin bug *P. rhadamanthus* showed that it uses PMG venom for both prey killing and defense (Walker et al., 2019). However, the authors only analyzed the defense spray and not the venom that is injected defensively by *P. rhadamanthus*. Thus, it is unclear whether the defensive use of AMG venom is unique to *P. plagipennis* or if there are more species with this remarkable adaptation. Furthermore, the hypothesis that *P. plagipennis* AMG venom is specialized for defense remains to be tested.

To gain insight into the context-dependent deployment of venom by reduviid bugs and corresponding differences in venom composition, we conducted an integrated transcriptomics and proteomics analysis (Figure 1) to identify and compare the venom components of the reduvine species *Platymeris biguttatus* L. and *Psytalla horrida* Stål. Both species are native to western Africa, with overlapping habitats and a similar prey range (Chłond, Bugaj-Nawrocka, & Junkiert, 2015; Gordon, 2017; Guilbert & Chłond, 2009). Ecological niche modeling revealed that *P. biguttatus* prefers tropical savanna as well as open areas with tree

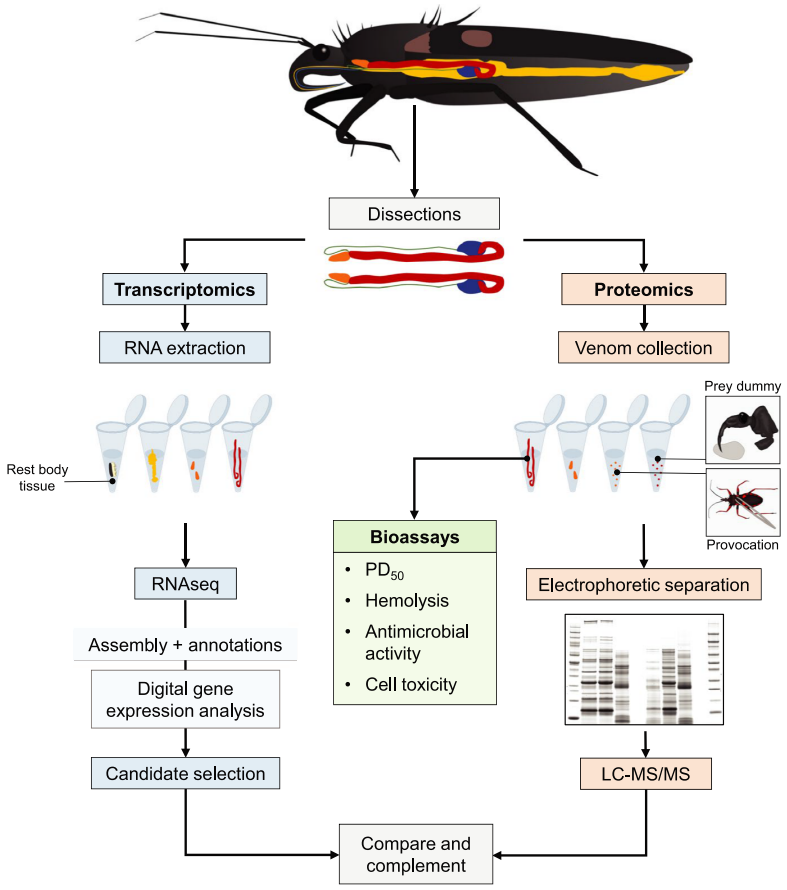


FIGURE 1 Schematic workflow of an integrated transcriptomic, proteomic and assay-based approach to identify the venom-specific proteins and venom activity of *P. biguttatus* and *P. horrida*

vegetation and shares potentially suitable niches with *P. rhadamanthus* (Chłond et al., 2015). We compared AMG and PMG extracts from the two species and confirmed that the PMG is the glandular origin of prey-killing venom in both species. However, the analysis of venoms secreted in response to different stress stimuli revealed that *P. horrida* secretes both PMG and AMG venoms defensively in a context-dependent manner, whereas *P. biguttatus* defensive secretions originate mostly from the PMG. We carried out a comprehensive analysis of venom components from both species and also conducted in vitro and in vivo bioactivity assays to investigate their effects. Our results contribute to a better understanding of venom deployment and function and provide a basis for further studies that will unravel the ecology of predatory Heteroptera and identify venom components with potential applications in medicine and agricultural pest control.

2 | METHODS

2.1 | Insects

Psytalla horrida and *P. biguttatus* specimens were obtained from an insectarium breeding source (Jörg Bernhardt, personal communication) and kept at room temperature in terraria laid out with sand, coconut fibers, and pieces of bark as hiding places. The bugs were fed once per week with *Acheta domesticus* L. or *Galleria mellonella* L. larvae, both of which were obtained from Tropic Shop (Nordhorn, Germany). Venom injection assays were carried out using *G. mellonella* obtained from BioSystems Technology (Exeter, UK).

2.2 | Venom collection

Venom likely to have a defensive function was obtained by exposing the insects to different forms of stress, including mild harassment, cold stress, and the more intense harassment of restrained bugs. For mild harassment, the insects were separated in plastic boxes and prodded with forceps, but were not restrained and were allowed to escape. In most cases, individual bugs did not attack the forceps but a small droplet of saliva emerged at the proboscis tip, which could be collected using a pipette tip and transferred to a precooled 1.5-ml Eppendorf tube. For cold stress, the bugs were exposed to −20°C for 3 min, which induced salivation. The droplet that emerged at the proboscis tip was also collected and transferred to a separate precooled tube. For strong harassment stress, a cold-anaesthetized

assassin bug was fixed on a foam cuboid and the proboscis was inserted into a pipette tip. When the insect was fully awake, it was tapped and gently squeezed with forceps, which induced salivation. The collected venom was transferred to a precooled tube.

In order to collect the saliva that *P. horrida* and *P. biguttatus* inject into their prey, an artificial prey dummy was prepared by enclosing a droplet of phosphate-buffered saline (PBS), typically 20–60 μl, in a piece of stretched Parafilm. The prey dummy was held in front of the bugs to simulate moving prey insects and induce hunting behavior. When the bug attacked the artificial prey, it was allowed to inject saliva for 1.5 min before removing the dummy. The PBS-venom mixture was recovered from the dummy and transferred to a precooled tube.

In addition to the noninvasive collection of saliva, venom was also extracted directly from the venom glands of fifth-instar or adult assassin bugs that were separated and anaesthetized at −20°C for 5 min before dissection in PBS. The posterior and anterior lobes were separated and immediately placed in precooled tubes containing 10–40 μl PBS on ice. The samples were briefly vortexed and centrifuged (4,000 *g* for 3.5 min at 4°C), and the supernatant was transferred to a fresh tube. The venom of several individuals was pooled and stored at −20°C for analysis. The total protein concentration in the venom samples was measured using an N60 nanophotometer (Implen).

2.3 | Proteomic analysis

The venom proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4%–12% Criterion XT gradient gels (BioRad) with XT MES running buffer at 125 V for 1.5 hr. Prestained and unstained high-mass-precision protein markers were used to determine the molecular weight (kDa) of the venom proteins. Gels were stained with a 1:1 mixture of Coomassie Brilliant Blue R-250 and colloidal Coomassie Brilliant Blue G-250 (Thermo Fisher Scientific) for 1.5 hr. Excess dye was removed by washing in Millipore water overnight, and the stained gel was then scanned and analyzed.

For LC-MS/MS analysis, protein bands from each gel lane were excised as 29 molecular weight fractions for tryptic digestion (Shevchenko, Tomas, Havli, Olsen, & Mann, 2006). Further details of LC-MS sample processing, data acquisition and data processing, such as search parameters specifying mass measurement accuracy, minimum number of product ion matches per peptide, minimum number of product ion matches per protein, minimum number of peptide matches, and maximum number of missed tryptic cleavage sites can be found in Methods S1, Section 1.

2.4 | Venom gland collection and RNA isolation

The anterior and posterior lobes of the venom gland complex from fifth-instar or adult assassin bugs, dissected as described above for venom collection, were placed in separate ceramic bead tubes containing 500 μl of TRI Reagent (Sigma-Aldrich). The alimentary canal was carefully removed and also transferred into 500 μl of TRI Reagent. Finally, the fat body, muscle tissue, and integument were combined as the "remaining body tissue" and placed in a separate tube with TRI Reagent. The tissues of two individuals were pooled and homogenized using a TissueLyser LT (Qiagen). Total RNA was extracted using the Direct-zol RNA Miniprep Kit according to the manufacturer's instructions (Zymo Research). The quantity of RNA was measured using a N60 nanophotometer, and its integrity was confirmed using an Agilent 2100 Bioanalyzer and RNA Nanochip (Agilent Technologies).

2.5 | RNA-SEQ and de novo transcriptome assembly

For both species, the AMG, PMG, gut, and remaining body tissue transcriptomes were sequenced by the Max-Planck Genome Center Cologne (http://mpgc.mpipz.mpg.de/home/) using an Illumina HiSeq3000 Genome Analyzer platform. Poly-A mRNA was isolated from 1 μg of total RNA using oligo-dT attached to magnetic beads and fragmented to an average of 250 bp before sequencing libraries were generated using the TruSeq RNA Library Preparation Kit v2 (Illumina). Paired-end (2 × 150 bp) read technology was used for sequencing, resulting in the following numbers of reads: *P. biguttatus* AMG = 50 million, PMG = 58 million, gut = 45 million, and remaining body tissue = 46 million; *P. horrida* AMG = 65 million, PMG = 68 million, gut = 51 million, and remaining body tissue = 60 million. All reads generated by the sequencing provider were processed using an in-house assembly and annotation pipeline. The presumed optimal consensus transcriptome for each species was then selected, as previously described (Vogel, Badapanda, Knorr, & Vilcinskas, 2014). Details of the transcriptome assemblies, transcript annotation, and RNA-Seq mapping can be found in Methods S1, Section 2.

2.6 | Venom activity bioassays

To investigate the effects of *P. horrida* and *P. biguttatus* venom on prey insects, *G. mellonella* larvae were injected with AMG or PMG venom in preliminary tests, which showed that only PMG venom had any effect. Only PMG venom was therefore used in further experiments. We injected 5 μl of various concentrated PMG venom samples into the first proleg of *G. mellonella* larvae using a DMP microsyringe pump (World Precision Instruments). For *P. horrida* venom, we tested protein concentrations of 0.8, 1.2, 1.6, and 2.0 μg/μl, and for *P. biguttatus* venom, we tested protein concentrations of 0.6, 1.0, 1.4, and 2.0 μg/μl. We injected 5 μl of PBS as a negative control. Treated insects were observed 1 min, 1 hr, and 24 hr postinjection, and their behavior was recorded. We differentiated between normal larval behavior, partial paralysis, complete paralysis, and death. PD_{50} values were calculated with a logistic model in R v3.6.0

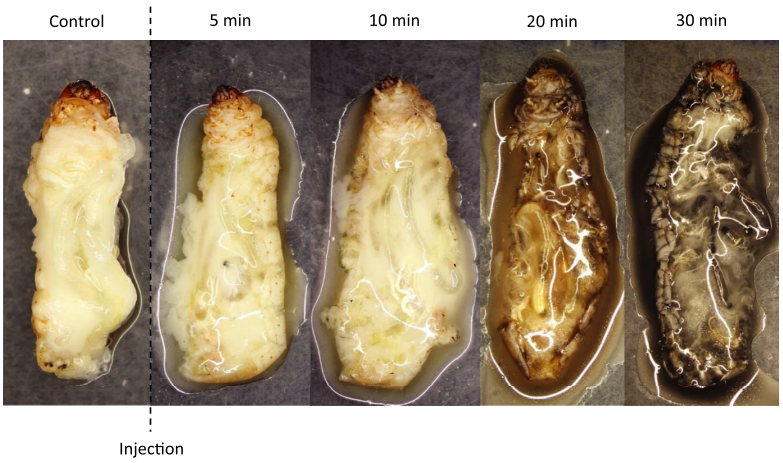


FIGURE 2 Digestive effects of *P. biguttatus* PMG venom on *G. mellonella* larvae at different time points after venom injection

using the HelpersMG package and were based on the observation of larvae that were completely paralyzed or only able to move their legs and/or mandibles.

Hemolytic activity was tested on blood agar plates. On each plate, seven holes were punched out using a sterile 5-mL pipette tip and the wells were filled with 2 μl of various concentrated venom extracts (PMG venom = 100, 20 or 1 μg/μl; AMG: venom = 20 or 1 μg/μl), 2 μl PBS as a negative control, or 2 μl 1% Triton-X-100 in water as a positive control. Hemolysis was tested on human blood agar, horse blood agar, and sheep blood agar plates. For each blood type and reduviid species, we prepared triplicates. The plates were incubated at 37°C for 24 hr and then photographed and inspected for hemolytic zones.

Bacterial growth inhibition was tested using a bacterial inhibition zone assay with *Escherichia coli*. Overnight cultures in lysogeny broth (LB) medium were prepared by inoculating 5 ml of fresh medium with one colony of *E. coli* and incubating overnight at 37°C. We transferred 100 μl of the overnight culture into 100 ml of warm LB agar, and plates were poured using 10 ml per Petri dish. On each plate, seven holes were punched out using a sterile 5 ml pipette tip and the wells were filled with 2.5 μl of various concentrated venom extracts (PMG venom = 80, 16 or 0.8 μg/μl; AMG venom = 16 or 0.8 μg/μl), 2.5 μl sterile PBS as a negative control, or 2.5 μl gentamycin (50 mg/ml) as a positive control. For each reduviid species, we prepared triplicates. The plates were incubated at 37°C for 24 hr and then photographed and inspected for bacterial growth inhibition zones.

Potential cytotoxic effects were tested on *Spodoptera frugiperda Sf*9 cells. The cells were cultured in Sf-900 II SFM medium (Gibco) and seeded in Petri dishes with a diameter of 6 cm. After 24 hr, the culture medium was replaced with fresh medium containing 50 μl diluted venom (5 μg/μl) or 50 μl sterile PBS (negative control) and the plates were incubated at 27°C for 24 hr. The cells were then examined by phase-contrast microscopy for cytotoxic effects.

3 | RESULTS

3.1 | Differential effects of AMG and PMG venom on *G. mellonella* larvae

Venom was extracted from the separated anterior and posterior lobes of *P. biguttatus* and *P. horrida* salivary glands by low-speed centrifugation. Injections of AMG venom had no effect on *G. mellonella* larvae (data not shown) so we focused on the effects of PMG venom, which caused rapid paralysis and death. With an estimated PD_{50} of 6.2 μg per larva (23.4 μg/g) after 1 hr, the *P. biguttatus* PMG secretions were more potent than those from *P. horrida*, with an estimated PD_{50} of 9.8 μg per larva (37.0 μg/g). The digestive effects of PMG venom were confirmed by allowing *P. biguttatus* to inject venom into *G. mellonella* larvae, removing the prey after 1.5 min, and examining the condition of inner structures at different time points. The larvae began to melanize and liquefy after 20 min, and most structures were almost fully digested after 30 min (Figure 2). These effects clearly indicated the presence of strong paralytic components and digestive enzymes in the PMG secretions.

Hemolysis, cytotoxicity, and antibacterial assays were carried out to characterize the activity of the AMG and PMG venoms in more detail. We found that the AMG venom (from both species) had no effect in any of the assays (data not shown). Hemolysis was tested on blood agar plates containing human, horse, or sheep erythrocytes. We found that 200 μg of *P. biguttatus* PMG venom generated large hemolytic zones on human and horse blood and also showed weak hemolytic activity against sheep blood. The same amount of PMG venom from *P. horrida* also showed strong hemolytic activity against human blood, but weaker effects against horse blood, and no activity against sheep blood (Figure 3a). These results indicated the presence of proteins with strong hemolytic activity in PMG venom. The application of 200 μg PMG venom from either species caused the significant inhibition of bacterial growth in an *E. coli* inhibition zone assay (Figure 3b). Finally, we tested for cytotoxic activity by exposing *Sf*9 cells to diluted venom extracts. We found that a

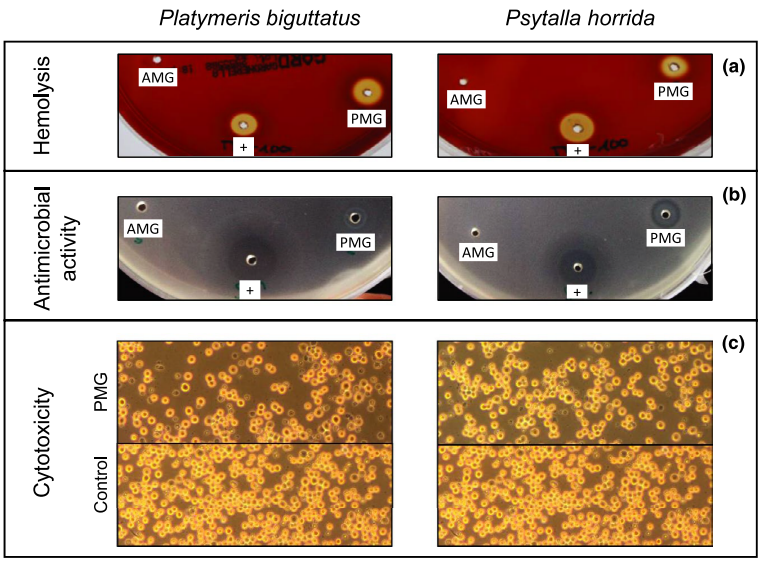


FIGURE 3 Hemolytic (a), antimicrobial (b), and cytotoxic (c) effects of PMG venom extracted from *P. biguttatus* and *P. horrida*. (a) AMG = 20 μg/μl anterior main gland venom; PMG = 100 μg/μl posterior main gland venom; + = 1% Triton X-100. PMG venom generated large hemolytic zones in blood agar plates, indicating the presence of proteins with strong hemolytic activity. (b) AMG/PMG as above; + = 0.5 μg/μl gentamycin. PMG venom from either species caused the significant inhibition of bacterial growth in an *E. coli* inhibition zone assay. (c) Diluted PMG venom displayed cytotoxic activity against Sf9 cells, reducing the cell density and causing extensive cell death

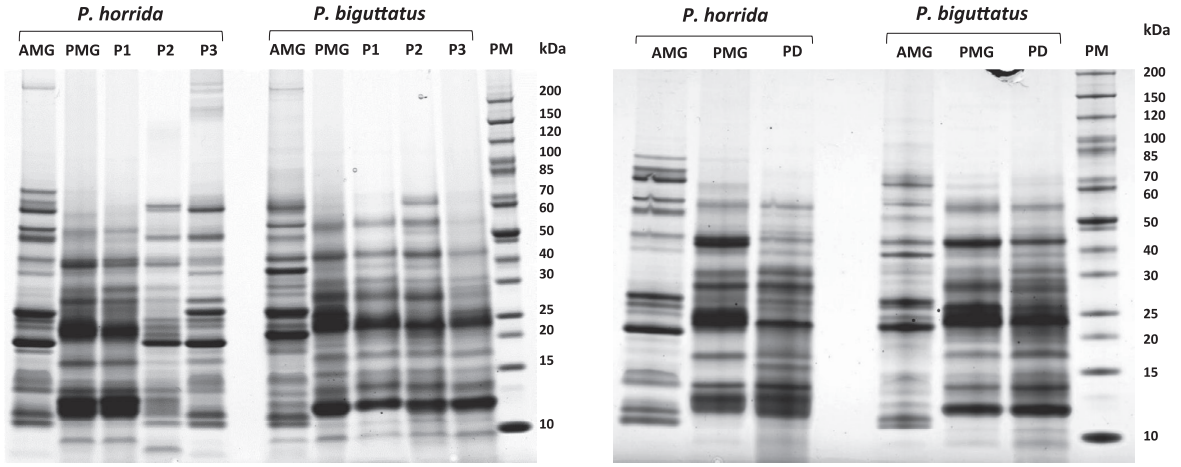


FIGURE 4 SDS-PAGE analysis of venom extracts from homogenized glandular tissue and venom collected without dissection from *P. horrida* and *P. biguttatus*. AMG = anterior main gland extract; PMG = posterior main gland extract; P1 = provocation venom (mild harassment); P2 = provocation venom (cold stress); P3 = provocation venom (strong harassment); I1 = injection venom (prey dummy) after 1.5 min; and PM = protein marker

concentration of 0.005 μg/μl of PMG venom from either species was cytotoxic, reducing the cell density and causing extensive cell death (Figure 3c). Taken together, these results indicate that PMG (but not AMG) secretions from *P. horrida* and *P. biguttatus* display neurotoxic, digestive, hemolytic, antibacterial, and cytotoxic effects, indicating different functional adaptations of the two types of venom.

3.2 | Spatial separation of venoms for defense and prey killing

In *P. plagipennis*, which produces defensive venom in the AMG and prey-killing venom in the PMG (Walker, Mayhew, et al., 2018), defensive venom can be collected by harassment and prey-killing venom by electrostimulation (Walker et al., 2017; Walker, Rosenthal, Undheim, & King, 2018). However, electrostimulation is an artificial situation, and the resulting venom may differ from that injected into prey. We therefore established a new method for the collection of saliva using a Parafilm prey dummy filled with PBS, allowing the isolation and further analysis of prey-killing venom. Defensive venom was collected by exposing insects to different forms of stress: mild harassment, cold stress, and the more intense harassment of restrained bugs.

In our initial experiments, we compared the protein content of AMG and PMG extracts in each species to the content of the venoms collected from the prey dummies and stress-induced secretions

(Figure 4). In both species, the protein bands in the prey-killing venom were similar to those in the PMG extracts, indicating that the posterior lobe is the glandular origin of the venom used to paralyze and digest prey. In *P. biguttatus*, the defensive venom samples were also similar to the PMG extract, whereas the composition of *P. horrida* defensive venom was context-dependent. Venom obtained by mild harassment was similar to the PMG extract, whereas venom collected during cold stress or strong harassment clearly resembled the AMG extract. The results confirmed that *P. horrida* and *P. biguttatus* produce different types of venom in the AMG and PMG, which to a certain extent fulfill the roles of defensive and prey-killing venoms, respectively.

Protein bands from the AMG and PMG extracts, the strong harassment venom, and the prey-killing venom were excised from the gel, digested with trypsin, and analyzed by LC-MS/MS. The predicted peptide sequences were searched against translated ORFs from the *P. horrida* and *P. biguttatus* transcriptome datasets described below (Figure 5). In both species, the prey-killing venom proteome was highly similar to the PMG proteome (Figure 6, Figure S1). In *P. horrida*, the defensive (strong harassment) venom proteome was more similar to the AMG proteome (Figure 7), whereas the defensive venom proteome of *P. biguttatus* mainly contained proteins from the PMG, along with some AMG proteins (Figure S2). Proteomic analysis therefore supported the theory that PMG secretions in both *P. horrida* and *P. biguttatus* are used mainly for prey killing, whereas AMG secretions in *P. horrida* serve as defensive venom in response to strong harassment.

3.3 | Gene expression and protein composition of PMG and AMG venom glands

Further evidence for the protein composition of PMG and AMG venom was obtained by next-generation sequencing (RNA-Seq),

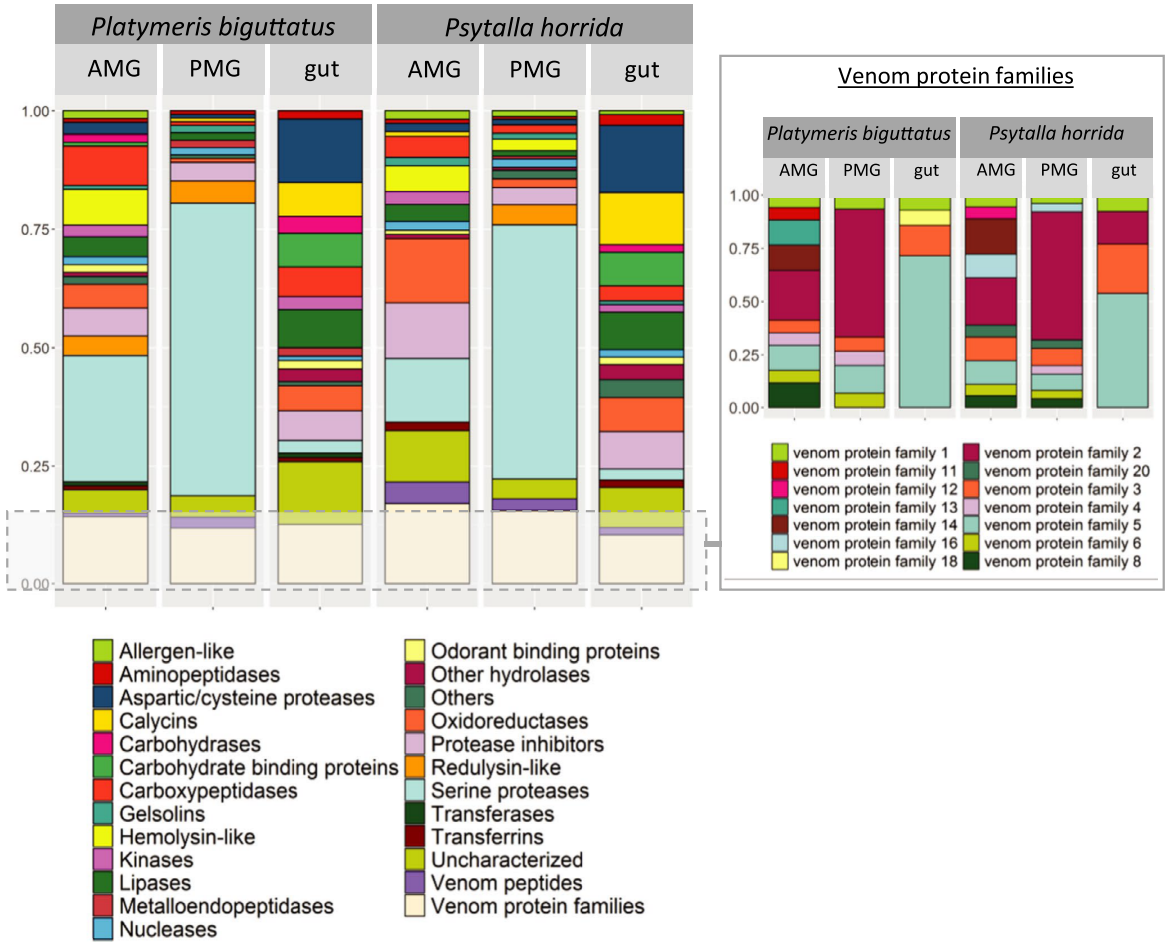
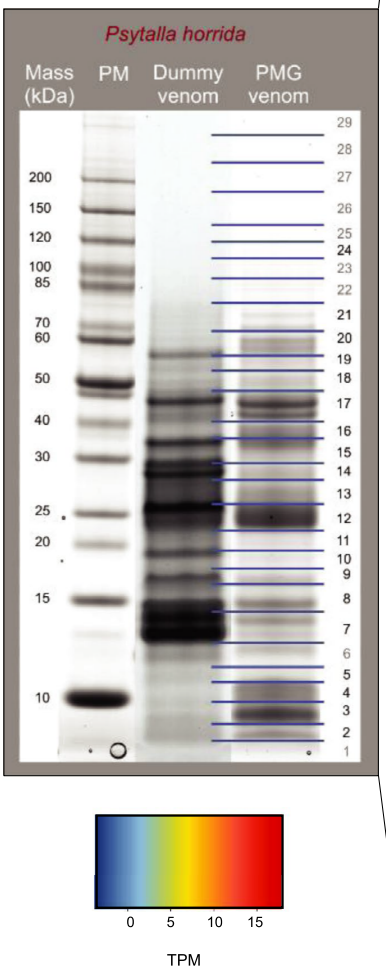


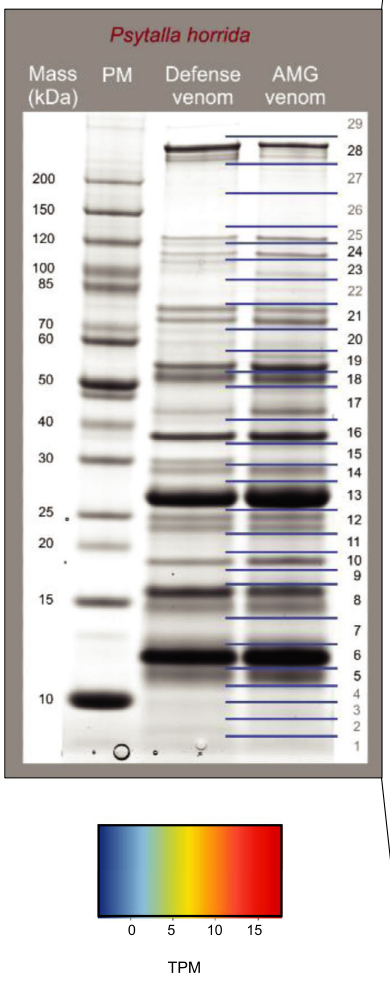
FIGURE 5 Protein composition of the AMG, PMG, and gut secretions of *P. biguttatus* and *P. horrida*. Color-coded blocks show the number of contigs identified in transcriptome datasets encoding specific classes of functional proteins. The venom protein families are shown separately in the inset box



No.	kDa	Dummy venom		PMG venom		Description	PMG	AMG	Gut	RB
		Total score	Assigned peptides	Total score	Assigned peptides					
24	101.5			369	5	Nuclease-like protein				
	13.2	191	3	103	1	Xanthine-dehydrogenase				
21	74.3	314	5			Transferrin-like protein				
	73.3	409	27	867	39	S1 protease (CUB domain)				
	65.1	467	7			Glucose dehydrogenase				
	62.7	441	15	1054	30	S1 protease				
	40.4	64	2			Acid phosphatase				
20	61.2	574	20	1266	37	S1 protease (CUB domain)				
		320	14	854	26	S1 protease				
19	61.1			149	2	Metalloproteinase				
		517	13	453	14	S1 protease (CUB domain)				
	51.8	73	3	132	2	Nuclease-like protein				
	55.1			77	1	Aminopeptidase				
		60	1			Unknown protein				
18	56.7	141	4	227	6	Uncharacterized protein				
	50.1	140	2	69	1	Lipase-like protein				
	48.5	320	19	773	23	S1 protease (CUB domain)				
				197	3	Nuclease-like protein				
	45.2	534	29	703	30	S1 protease				
17	46.6	508	30	1219	45	S1 protease				
	40.7	137	3	363	6	Protease inhibitor (serpin)				
	30.8	335	4	721	9	Carboxypeptidase				
	41.7	256	3	88	1	Hyaluronidase-like protein				
16	42.2	659	26	637	16	Gelsolin-like protein				
	35.2	139	3	156	2	Cathepsin B				
	35.2	853	29	895	22	S1 protease				
		82	2	126	3	S1 protease (CUB domain)				
15	36.8	229	5	314	8	Venom protein family 2 protein				
	33.8	278	13	925	26	S1 protease				
	33.4	290	13	493	20	S1 protease (CUB domain)				
14	29.6	205	3	376	5	Metalloproteinase				
	28.4	131	8	170	6	S1 protease				
	25.1	63	1	139	3	Venom protein family 2 protein				
13	44.7	124	2	95	2	Carbonic anhydrase				
	27.5			278	4	Venom protein family 2 protein				
	25	261	8	351	9	Redulysin-like protein				
	20.6	197	5	217	4	S1 protease (CUB domain)				
12		72	2	88	2	Redulysin-like protein				
	29.4	603	13	514	10	Venom protein family 2 protein				
	23.8	183	6	241	6	Venom protein family 5 protein				
11	22.2	73	1	221	3	Venom protein family 5 protein				
	18.2	310	4	300	5	Venom protein family 2 protein				
10	17.1	122	1	120	3	Venom protein family 4 protein				
	13.2			86	1	Uncharacterized protein				
		69	1			Unknown protein				
9	18.2	446	8	410	11	CUB domain protein				
8	16.8	146	3	322	4	Venom protein family 6 protein				
	16	65	1	65	1	Uncharacterized protein				
	12	79	1	79	1	Venom protein family 3 protein				
				76	1	Redulysin-like protein				
		67	1			Venom protein famly 16 protein				
7	16	137	2	348	4	Venom protein family 1 protein				
		73	1	94	1	Unknown protein				
5	12.9	125	3	233	4	Protease inhibitor (cystatin)				
4	10			85	2	Unknown protein				
3		280	5	524	11	Redulysin-like protein				
2	4.7	118	2	79	1	Ptu1-like peptide				

FIGURE 6 Proteins of the *P. horrida* PMG and prey dummy venom identified by LC-MS/MS. The Coomassie-stained protein gel on the left yielded the PMG venom proteins shown on the right, including the predicted protein masses (kDa), the total score, number of assigned peptides and descriptions. The excised bands are indicated with numbers and lines on the right side of the protein gel. For the proteins identified by LC-MS/MS, gene expression levels (log2 TPM) in the PMG, AMG, gut, and remaining body tissues are shown in the heat map. PM = protein marker. See Table S1 for the identity of matching predicted proteins in the *P. horrida* transcriptome

allowing the identification and quantitation of venom-associated transcripts. RNA was isolated from the AMG, PMG, gut, and remaining body tissue of both species for Illumina sequencing, which yielded 45–68 million reads per sample. The de novo reference transcriptome assembly for *P. biguttatus* contained 47,377 contigs, with an N50 contig size of 1,579 bp and a maximum contig length of 20,166 bp, whereas the equivalent assembly for *P. horrida* contained 37,424 contigs, with an N50 contig size of 1,623 bp and a maximum contig length of 20,054 bp. BUSCO analysis revealed 91.9% and 92.4% complete gene coverage in addition to 2.3% and 1.8% missing



No.	kDa	Defense venom		AMG venom		Description	AMG	PMG	Gut	RB
		Total score	Assigned peptides	Total score	Assigned peptides					
28	69	139	2	347	6	Unknown protein				
	50.4	321	7	505	11	Unknown protein				
	38.2			61	1	Uncharacterized protein				
	25.6	199	6	434	8	Unknown protein				
		312	6	252	4	Venom protein family 14 protein				
		137	4	137	2	Unknown protein				
24	101			74	1	Nuclease-like protein				
23	25.4	85	1	85	1	Unknown protein				
21	74.3	581	8	832	14	Transferrin-like protein				
	27.2	658	13	549	10	Glucose dehydrogenase				
20	66.7	173	3	82	2	S8 protease				
	27.2	579	14	233	3	Glucose dehydrogenase				
19	57.2	680	22	966	17	Hemolysin-like protein				
	56.8			618	8	Catalase				
	55.1			232	3	Aminopeptidase				
	34.4	72	1	78	1	Lipase-like protein				
	46.7	178	2	282	4	Nuclease-like protein				
18	50	543	7	784	11	Kinase-like protein				
	40.9	79	1	201	2	Cathepsin D				
	30.8	408	6	412	7	Carboxypeptidase				
17	44.2			69	1	S1 protease (CUB domain)				
	44.3	120	2	361	5	Nuclease-like protein				
	44.2			206	3	Gelsolin-like protein				
	40.7	439	6	559	9	Protease inhibitor (serpin)				
	35.2	137	2	302	4	S1 protease				
		331	5	140	2	Carboxypeptidase				
16	42.2	75	1	75	1	Gelsolin-like protein				
	40.4			84	1	Lysosomal aspartic protease				
	31.5	397	8	357	6	Unknown protein				
15	48.3			63	1	Kinase-like protein				
	33.7			147	4	S1 protease				
	37.9			365	6	Phytanoyl-coa dioxygenase				
14	33.5	505	15	341	6	Bpi-like protein				
13	24.5	192	9	392	9	Hemolysin-like protein				
	16.6	492	8	497	17	S1 protease (CUB domain)				
		80	1			Unknown protein				
	20.6	384	5	451	12	Nuclease-like protein				
12	25.5	388	4	500	6	Venom protein family 5 protein				
	22.3	324	4			Lipocalin				
	23.5	68	1			Allergen-like protein				
11	22.3	155	2	378	5	Hemolysin-like protein				
	21.7	72	1	154	2	Venom protein family 12 protein				
	111.9			60	1	Aminopeptidase				
	22			96	1	Venom protein family 2 protein				
	25.9	70	1			Venom protein family 2 protein				
				219	5	Venom protein family 5 protein				
10	20.3	518	11	594	12	Hemolysin-like protein				
		83	1	201	3	Protease inhibitor (pacifastin)				
9	17.6			319	4	Kinase-like protein				
	13.6	81	1	81	1	Lysozyme-like protein				
	14	160	2	255	4	Heme-binding protein				
	14.1	153	3	271	6	Venom protein family 3 protein				
8	17.4	169	2	290	6	Unknown protein				
	16.8	225	4	288	5	Venom protein family 6 protein				
	15.3			283	5	Fatty acid binding protein				
	5.9			70	1	Venom peptide				
7	16	79	1	137	2	Venom protein family 1 protein				
	15	230	4	230		Unknown protein				
	12.2	137	3	137	3	Venom protein family 3 protein				
	11.6	263	6	316	7	Venom protein family 8 protein				
	5.1			77	2	CUB domain protein				
6	10.1	322	7	318	9	Venom protein family 16 protein				
	10.3	116	2	420	6	Protease inhibitor (cystatin)				
5	12.9	193	5	482	10	Protease inhibitor (cystatin)				
	8.2	149	3	171	3	Uncharacterized protein				

FIGURE 7 Proteins of the *P. horrida* AMG and defense venom (mild harassment) identified by LC-MS/MS. The Coomassie-stained protein gel on the left yielded the AMG venom proteins shown on the right, including the predicted protein masses (kDa), the total score, number of assigned peptides and descriptions. The excised bands are indicated with numbers and lines on the right side of the protein gel. For the proteins identified by LC-MS/MS, gene expression levels (log2 TPM) in AMG, PMG, gut, and remaining body tissues are shown in the heat map. PM = protein marker. See Table S1 for the identity of matching predicted proteins in the *P. horrida* transcriptome

genes for the *P. biguttatus* and *P. horrida* transcriptome assemblies, respectively. The contigs were screened against the nonredundant NCBI protein database, and functional annotations were added. For digital gene expression analysis, the Illumina reads were remapped onto the assemblies to calculate expression values. Although comparisons of RPKM and TPM levels revealed no major differences, we used the log2 TPM value for between-tissue comparisons. Potential venom-associated contigs were selected based on their BLAST hits, annotations, and expression levels. Furthermore, the candidate proteins were checked for signal peptides and matched against the proteome data (see above). We ultimately selected 128 (PMG) and 120 (AMG) venom protein candidates for *P. biguttatus* as well as 166 (PMG) and 111 (AMG) for *P. horrida*. The candidates were classified according to their domains, predicted molecular functions, and family memberships.

The venom gland transcriptomes of *P. horrida* and *P. biguttatus* are compared in Figure 5. The comparative profiles of the AMG, PMG, and gut transcriptomes revealed major tissue-specific but only minor species-dependent differences. Most of the PMG venom transcripts in both species could be assigned to S1 family proteases (with many containing a CUB domain) or the different venom protein families identified in *P. plagipennis* (particularly venom protein families 1, 2, 3, 4, 5, and 6). The PMG transcriptome of *P. horrida* also contained matches to venom protein families 8 and 20. Furthermore, we found six (*P. biguttatus*) and seven (*P. horrida*) redulysin-like sequences in the PMG transcriptome among several other groups including gelsolins, protease inhibitors, and Ptu1-like peptides (Figure 5). The contig with the highest PMG-specific expression in both species encoded a redulysin-like protein, followed by S1 proteases and proteins from venom protein families 1 and 2 (Figure 6, Figure S1). Other abundant transcripts in the PMG transcriptome encoded a gelsolin-like protein, a metalloproteinase, and other venom protein family members. We identified three (*P. biguttatus*) and four (*P. horrida*) ICK family peptides in the transcriptome datasets, but only one was also detected in the PMG proteome. This Ptu1-like peptide was homologous to a peptide in *P. plagipennis* and showed strong PMG-specific expression in both *P. biguttatus* and *P. horrida*. The main components and composition of the PMG venom therefore appeared to be similar in both species, although more complex in the case of *P. horrida*.

The AMG venom showed more species-dependent differences in composition. S1 proteases and venom protein family members were predominant in both species, but the *P. biguttatus* AMG transcriptome featured more than double the number of S1 protease sequences compared to *P. horrida*. Venom protein families 1, 2, 3, 5, 6, 8, and 14 were represented in both species, whereas venom protein families 4, 11, and 13 were specific to *P. biguttatus* and venom protein families 12, 16, and 20 were specific to *P. horrida*. We also identified transcripts representing several hemolysins, protease inhibitors (including cystatins, serpins, and pacifastins), and an odorant-binding protein in both species (Figure 5). In *P. horrida*, the contig with the highest AMG-specific expression encoded a member of venom protein family 16, followed by venom protein family 8, a hemolysin, and two cystatin and pacifastin protease inhibitors (Figure 7). In *P. biguttatus*, the contig with the highest AMG-specific expression encoded a hemolysin, followed by an uncharacterized protein, two protease inhibitors (cystatin and pacifastin), and an S1 protease with a CUB domain (Figure S2). In both species, the AMG appeared to secrete a more complex protein mixture than the PMG.

Looking more closely at the venom protein families, we observed some groups that were present in the venom gland and gut transcriptomes (Figure 5). The majority of these tissue-wide venom protein families were members of venom protein families 3 and 5, with transcripts in the AMG and PMG, and several that were solely expressed in the gut (Figure 8). Members of venom protein families 1, 2, and 18 were also found in the gut transcriptomes (Figure 8). The role of these venom protein family proteins is not clear, but it is likely that not all of them possess venom-specific functions.

The venom glands of both *P. horrida* and *P. biguttatus* produced several uncharacterized proteins and unknown proteins with no hits in the protein database. Some of them were identified on the basis of specific domains or Interpro family memberships, and others via BLAST searches of the translated ORF containing the sequence predicted by LC-MS/MS. However, most of these proteins could not be assigned to any known family or associated with any specific domain or motif. The *P. horrida* AMG in particular featured many unknown transcripts with strong tissue-specific expression representing proteins that were abundant in the corresponding proteome (Figures 5 and 7). The functions of these abundant proteins should be investigated in future experiments. In both species, some transcripts were strongly expressed not only in the venom glands but also in the gut and remaining body tissues. These included transcripts encoding transferrins, serpins, kinases, carboxypeptidases, and aminopeptidases. The proteins were also present in the AMG and/or PMG proteomes, indicating they are genuine secreted proteins that might play an important role in the venom secretions. However, their expression in nonglandular tissues suggests they possess additional functions that are unrelated to the effects of AMG and PMG venom.

3.4 | Differentiation of internal and extra-oral digestion

Predatory bugs cannot take up solid food and must predigest their prey extra-orally before sucking up the liquefied tissues for further digestion in the gut. The properties of extra-oral digestion enzymes differ from those of enzymes in the gut in terms of pH preference and target substrates. Given that the PMG secretions of *P. horrida* and *P. biguttatus* are required for prey killing and digestion, we compared the PMG and gut transcriptomes of both species and found that the two tissues secrete different sets of proteins (Figure 5). Specifically, serine proteases were predominant in the PMG transcriptomes whereas cysteine and aspartic proteases were more common in the gut. Furthermore, the gut transcriptomes also contained sequences encoding several lipase-like proteins, carbohydrate-binding proteins, and calycins that were not represented in the PMG. Interestingly,

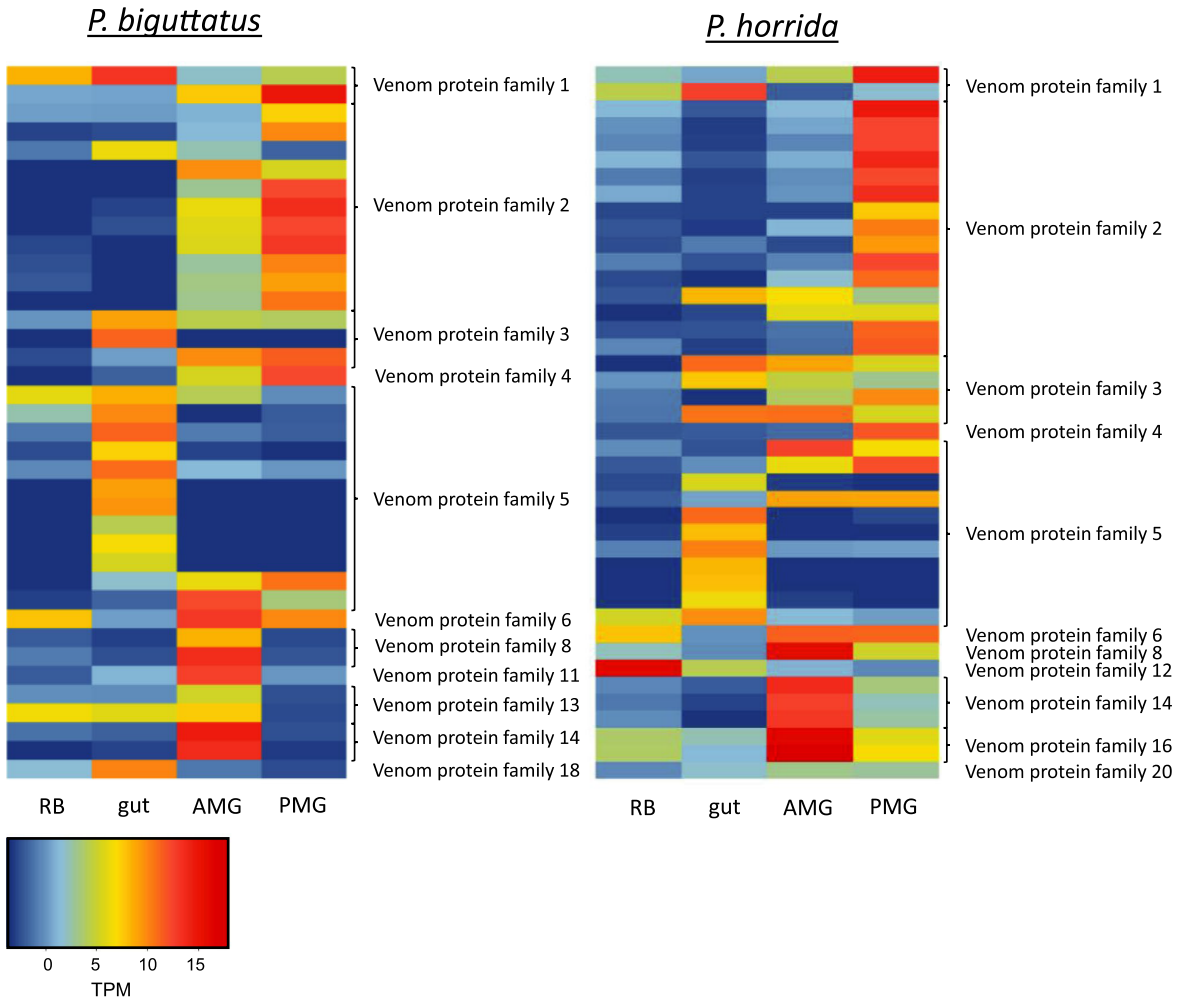


FIGURE 8 Gene expression levels (log2 TPM) of proteins from different venom protein families in the rest of body tissue (RB), gut, AMG, and PMG for *P. biguttatus* and *P. horrida*

members of venom protein family 5 were also prevalent in the gut transcriptomes, indicating a potential digestive function.

4 | DISCUSSION

To determine the glandular origins and utilization of different types of venom in the two reduviid species *P. biguttatus* and *P. horrida*, we used three nonlethal collection methods that mimic natural stimuli. Electrostimulation is often used to collect venom from arthropods, and this approach has been successful in Hymenoptera (Mueller et al., 1981), Heteroptera (Walker, Hernández-Vargas, et al., 2018; Walker, Mayhew, et al., 2018), centipedes (Jenner, von Reumont, Campbell, & Undheim, 2019; Malta et al., 2008), spiders (Barbaro, Cardoso, Eickstedt, & Mota, 1992; da Silveira et al., 2002), and scorpions (Carcamo-Noriega, Possani, & Ortiz, 2019; Rowe & Rowe, 2008). The advantage of electrostimulation is that it yields large volumes of venom (Glenn, Straight, & Snyder, 1972; Rocha-e-Silva, Sutti, & Hyslop, 2009) that is usually free from tissue contamination (Mueller et al., 1981). However, it is an unnatural stimulus, and secretions obtained in this manner may not always match the composition and effects of natural venom, as reported for the ant *Myrmecia pilosula* Smith (Wiese et al., 2008). We therefore established a more realistic method to collect prey-killing venom using a prey dummy to mimic a natural attack scenario, which in our hands allows the collection of high-quality venom from many Heteroptera. Defensive venom can be collected by harassment (Walker, Mayhew, et al., 2018; Walker, Rosenthal, et al., 2018), and similarly, we applied mild and intense harassment as well as cold stress in order to provoke the secretion of defensive venom by *P. biguttatus* and *P. horrida*. Finally, venom can be obtained directly by the extraction of dissected venom glands, although this method is lethal (Drenth, 1974; Heep et al., 2019;

Laurino et al., 2016; da Silveira et al., 2002; Walker, Hernández-Vargas, et al., 2018; Walker, Mayhew, et al., 2018). In contrast to whole gland homogenates clarified by high-speed centrifugation, we found that low-speed centrifugation (Walker, Mayhew, et al., 2018) produced clean extracts without tissue contamination by avoiding pressure-induced cytolysis. The comparison of secretions from *P. plagipennis* collected using various stimuli and direct extracts from dissected AMG and PMG tissues revealed differences that allowed the secretions obtained by mild harassment to be defined as defensive venom and suggested that the lethal neurotoxic venom obtained by electrostimulation is probably used for prey immobilization and digestion (Walker, Mayhew, et al., 2018). Furthermore, Walker et al. (2019) found that the mild harassment of *P. rhadamanthus* induced venom spitting/spraying, which was defined as defensive venom.

The venoms of zoophagous bugs are used to paralyze and liquefy prey insects (Cohen, 1993; Edwards, 1961; Walker et al., 2017, 2019; Walker, Mayhew, et al., 2018), thus facilitating the ingestion of nutrients via the proboscis (Cohen, 1998). Recently, the PMG was identified as the glandular origin of prey-killing venom in *P. plagipennis* and *P. rhadamanthus* (Walker, Mayhew, et al., 2018; Walker et al., 2019). Our integrated transcriptomics and proteomics approach confirmed that the PMG is also the source of prey-killing venom in *P. biguttatus* and *P. horrida*. Protein bands of venom samples collected using the prey dummy were similar to the PMG extracts, and most of the prey-killing venom proteins identified by LC-MS/MS were also present in the PMG extracts (Figure 6, Figure S1). The corresponding transcripts were strongly expressed in a PMG-specific manner confirming that the PMG is the source of venom used for prey immobilization and extra-oral digestion in both species.

One of the key functions of heteropteran prey-killing venom is the immobilization of prey. Heteropteran venoms were thought to lack neurotoxins (Azevedo et al., 2007; Edwards, 1961), but several putative neurotoxic peptides have been identified and isolated (Bernard et al., 2001; Corzo et al., 2001; Walker et al., 2017, 2019). These include Ptu1, an ICK family peptide isolated from the assassin bug *Peirates turpis* Walker, which can reversibly block Cav2.2 voltage-gated calcium channels in a similar manner to the homologous ω-conotoxins from cone snails (Corzo et al., 2001; Kasai, Aosaki, & Fukuda, 1987). The ICK motif features a cystine knot and an antiparallel, triple-stranded β-sheet (Lavergne, Alewood, Mobli, & King, 2015; Norton & Pallaghy, 1998; Pallaghy, Norton, Nielsen, & Craik, 1994). Such intra-chain disulfide bonds often stabilize the tertiary structure of peptides in animal venoms (Lavergne et al., 2015). In our experiments, PMG extracts from *P. horrida* and *P. biguttatus* triggered rapid paralysis when injected into *G. mellonella* larvae, with low PD_{50} values of 9.8 μg (37.0 μg/g) and 6.2 μg (23.4 μg/g) total protein, respectively. This matches the paralytic effects of *P. plagipennis* and *P. rhadamanthus* PMG venom (Walker, Mayhew, et al., 2018; Walker et al., 2019). We identified three specific peptides homologous to *P. plagipennis* Ptu1-like peptides in the PMG transcriptomes of *P. horrida* and *P. biguttatus*, and one peptide homologous to the ICK family peptide Ado1 from *Agriosphodrus dohrni* Signoret in the PMG transcriptome of *P. horrida*. Among these sequences, only one Ptu1-like peptide was found in the PMG proteome and was present in both *P. horrida* and *P. biguttatus*. Given the similarity of these sequences to ICK peptides from other venoms, their strong gland-specific expression, and the presence of signal peptides, we are confident that these other ICK peptides are secreted by *P. horrida* and/or *P. biguttatus*, but we did not detect them due to the limitations of the Bis-Tris polyacrylamide gels used in our experiment. Uncharacterized proteins such as members of venom family 1 are also thought to possess neurotoxic activity in reduviid bugs (Walker et al., 2017). We identified tissue-specific transcripts representing venom protein family 1 not only in the PMG and AMG, but also in the gut of both species, suggesting these sequences are unlikely to encode neurotoxic peptides and probably fulfill housekeeping or digestive functions. In contrast, an uncharacterized member of venom protein family 4 was specific to (and strongly expressed in) the PMG transcriptome and may therefore represent a novel neurotoxin.

Extra-oral digestion is a common trait among zoophagous invertebrates, allowing even small predators to ingest large prey species (Cohen, 1995). Predatory Heteroptera achieve nonrefluxing extra-oral digestion by injecting digestive enzymes from the salivary glands into their prey and sucking up the liquefied tissues (Cohen, 1998). Likewise, phytophagous species typically pre-digest plant material before ingesting it (Mehrabadi, Bandani, & Dastranj, 2014; Zhu, Yao, & Luttrell, 2016). We observed the rapid digestion of *G. mellonella* larvae injected with *P. biguttatus* PMG venom, indicating the presence of efficient digestive enzymes. Endopeptidases, particularly serine proteinases, appear to play a key role during extra-oral digestion by heteropteran insects and are abundant in the salivary secretions of phytophagous (Mehrabadi et al., 2014; Zhu et al., 2016), hematophagous (Amino, Tanaka, & Schenkman, 2001; Meiser et al., 2010), and zoophagous species (Bell, Down, Edwards, Gatehouse, & Gatehouse, 2005; Walker, Hernández-Vargas, et al., 2018; Walker et al., 2017, 2019). We found that S1 proteases were predominant in *P. horrida* and *P. biguttatus* PMG venom, some with very high expression levels. We also identified one *P. horrida* dipeptidase, three *P. horrida* exopeptidases, and two *P. biguttatus* exopeptidases in the PMG transcriptomes and proteomes, but all were expressed nonspecifically. In contrast, mainly cysteine and aspartic endopeptidases (and several exopeptidases) were present in the gut transcriptomes. This indicates that extra-oral digestion in the two reduviid species focuses on initial proteolysis by serine-type endopeptidases so that further protein digestion by endopeptidases and exopeptidases can take place in the gut (Bell et al., 2005; Cohen, 1993). Extra-oral digestion also breaks down lipids, especially cell membranes and storage lipids (Cohen, 1995). We identified one triacylglycerol lipase in the PMG of *P. horrida* and two in the PMG of *P. biguttatus*, but we found no phospholipases. Other strongly expressed lipase-like proteins, including triacylglycerol lipases, carboxylesterases, and phospholipases, were found in the guts of both species. This indicates that extra-oral digestion prioritizes the predigestion of storage lipids by triacylglycerol lipases, whereas most lipid catabolism, including the digestion

of phospholipids, takes place in the gut. In contrast to other true bugs (Boyd et al., 2002; Swart et al., 2006; Zeng & Cohen, 2000), no carbohydrase-like proteins were found in the PMG transcriptomes or proteomes of either species, but two glucosidases were present in the gut transcriptomes of both.

Heteropteran venoms need to fulfill several additional functions to overcome prey defenses (Ayyachamy, Sahayaraj, & Rivers, 2016; Sahayaraj & Muthukumar, 2011), improve the spread of venom (Edwards, 1961), and optimize nutrient availability (Cohen, 1995). Furthermore, the predator needs to protect itself from microbial colonization of the salivary gland complex and infections caused by the ingestion of pathogens. The saliva of *Rhynocoris* species triggers rapid hemolysis in its prey, thus suppressing initial defense mechanisms including hemocyte spreading and aggregation (Ayyachamy et al., 2016; Sahayaraj & Muthukumar, 2011). The venoms of *Rhynocoris marginatus* Fabricius and *Catamirus brevipennis* Servile suppress Gram-positive and Gram-negative bacteria, with greater efficacy against the latter (Sahayaraj, Borgio, Muthukumar, & Anandh, 2006). *P. rhadamanthus* venom increases calcium influx in mouse dorsal root ganglion cells, probably by forming pores in cell membranes (Walker et al., 2019). We tested the hemolytic, antimicrobial, and cytotoxic activity of *P. horrida* and *P. biguttatus* venoms and found that AMG venom displayed none of the above-mentioned effects whereas PMG venom was able to lyse erythrocytes (with greatest efficacy against human cells), inhibit the growth of *E. coli*, and reduce the viability of cultured insect cells. Redulysin-like proteins were abundant in the PMG venoms of both species, and have previously been identified in *P. plagipennis* and *P. rhadamanthus*, where they may act as pore-forming proteins with a cytolytic motif (Walker et al., 2017, 2019). Redulysin-like proteins are homologous to trialysin, a protein found in the saliva of the blood-feeding reduviid *Triatoma infestans* Klug (Amino et al., 2002) that can lyse bacteria, protozoans, and mammalian cells (Amino et al., 2002; Martins et al., 2006). The PMG-specific redulysins in *P. horrida* and *P. biguttatus* are probably responsible for the observed hemolytic, antimicrobial, and cytotoxic effects, but other components may also contribute. For example, we identified one strongly expressed PMG-specific gelsolin-like protein in both assassin bugs. Gelsolin facilitates membrane ruffling and cytoskeletal deconstruction by enhancing actin depolymerization (Harms et al., 2004; Sun, Yamamoto, Mejillano, & Yin, 1999), and gelsolin-derived peptides are active against Gram-negative and Gram-positive bacteria (Bucki et al., 2004).

The use of separate venomous secretions for prey killing and defense is a rare trait only reported in scorpions (Inceoglu et al., 2003) and cone snails (Dutertre et al., 2014) until recently. The first insect shown to deploy separate venoms was the harpactorine bug *P. plagipennis*, which uses AMG venom for defense and PMG venom to subdue and digest prey (Walker, Mayhew, et al., 2018). However, this is not a common trait among reduviid species. *P. rhadamanthus* mostly uses PMG venom for prey killing and defense, leaving the role of AMG venom unclear (Walker et al., 2019). *P. biguttatus* appears to follow the same strategy, primarily secreting PMG venom (and small quantities of AMG venom) in response to different stress situations. In contrast, *P. horrida* appears to use its PMG and AMG venoms defensively in a context-dependent manner. All three species belong to the subfamily Reduviinae, but the phylogenetic relationships between *P. rhadamanthus*, *P. biguttatus*, and *P. horrida* remain unclear because the subfamily is likely to be polyphyletic (Hwang & Weirauch, 2012; Weirauch & Munro, 2009). In our experiments, the PMG venom was secreted in response to mild harassment without restraint (allowing the bug to escape from serious confrontation), possibly representing the response to intraspecific conflict. The insects did not attack the forceps but secreted a droplet of venom that stuck to the proboscis tip. This may serve as warning behavior among conspecifics in order to avoid cost-intensive fighting. In contrast to mild harassment, more intense stress (conceived as a predator attack) was countered by the secretion of AMG venom, which is inactive against insects but may be effective against vertebrates (Walker, Mayhew, et al., 2018). The use of AMG venom for defense against vertebrates in *P. plagipennis* (Walker, Mayhew, et al., 2018) matches our observations in *P. horrida* and indicates that it may be used to induce pain. We detected hemolysin-like proteins that were strongly expressed specifically in the AMG of both *P. horrida* and *P. biguttatus*, but these are unlikely to possess hemolytic activity (despite their name) given the results of our functional assays. Such proteins may instead act on mast cells, thus triggering the release of pain-inducing compounds (Schmidt, Blum, & Overal, 1983), although specific bioassays would be required to confirm this hypothesis. This is the mechanism used by melittin in bee venoms (Chen, Guan, Sun, & Fu, 2016; Tosteson & Tosteson, 1981) and α-hemolysin produced by *Staphylococcus aureus* Rosenbach, which induces pain by binding to the receptor ADAM10 in nociceptor neurons and triggering calcium influx by pore formation (Chiu, 2018; Wilke & Wardenburg, 2010). It is unclear why *P. horrida* alone uses AMG venom defensively, whereas *P. biguttatus* relies mainly on PMG venom. One major difference between the AMG venoms of these species is the absence in *P. biguttatus* of venom protein family 16. A detailed functional characterization of this venom protein is necessary to determine its role in predator deterrence. Other AMG venom proteins that may fulfill important defensive functions of include cystatins, pacifastins, cysteine-rich secreted proteins, and additional uncharacterized venom protein families. The specific function of the AMG in *P. biguttatus* remains unclear because our experiments did not find any evidence that AMG venom is used for defense. Future research should focus on the characterization of strongly expressed proteins and peptides that are restricted to the AMG or PMG, and should also look at the many uncharacterized venom protein families.

Our comprehensive analysis of venom composition, effects, and deployment by the two reduviid species *P. horrida* and *P. biguttatus* revealed intriguing species-dependent differences in composition and context-dependent use. Given that venom regeneration can take several days and uses energy reserves, venom deployment (and reservoir depletion) probably results in considerable disadvantages for the insects until the reserves are replenished (Morgenstern & King, 2013). The AMG is much smaller than the

PMG and yields less venom, probably because the insects need less of it. *P. horrida* may deploy AMG secretions only as a last line of defense, such as when caught by a (vertebrate) predator or disturbed by larger animals, including humans. Such strategic use of defensive venom has also been observed in scorpions, which can adjust the amount of venom injected, the venom composition, and the frequency of stings according to threat levels (Nisani & Hayes, 2011). Although both reduviid species share the same habitat and prey range, and their AMG and PMG secrete similar cocktails of proteins, only *P. horrida* appears to distinguish between different threats and respond accordingly. It is unclear how the injection of specific venom types is regulated and whether the release of AMG venom by *P. biguttatus* can be triggered by stimuli other than those tested in our experiments. Our results highlight the complexity of assassin bug behavior and its context dependence. Furthermore, although the use of AMG secretions for defense is not restricted to the subfamily Harpactorinae, it is not a consistent trait among the Reduviidae and can clearly differ even between closely related species.

ACKNOWLEDGMENTS

MLF, HV, NW, and DGH acknowledge funding from the Max Planck Society, Germany. We thank Henriette Ringys-Beckstein for technical assistance in laboratory work and Richard M Twyman for editing the manuscript.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Maike L. Fischer: Data curation (lead); Formal analysis (equal); Investigation (equal); Methodology (equal); Visualization (lead); Writing-original draft (lead). **Natalie Wielsch:** Data curation (supporting); Methodology (supporting); Writing-review & editing (supporting). **David G. Heckel:** Funding acquisition (supporting); Supervision (supporting); Writing-review & editing (supporting). **Andreas Vilcinskas:** Conceptualization (supporting); Funding acquisition (equal); Supervision (supporting); Writing-review & editing (equal). **Heiko Vogel:** Conceptualization (lead); Data curation (supporting); Formal analysis (equal); Funding acquisition (equal); Supervision (lead); Writing-review & editing (lead).

DATA AVAILABILITY STATEMENT

The short-read data described herein have been deposited in the EBI Sequence Read Archive with accession numbers ERS4259175–ERS4259178 for *P. biguttatus* and ERS4259179–ERS4259182 for *P. horrida*. The complete study can also be accessed directly using the following URLs: http://www.ebi.ac.uk/ena/data/view/PRJEB36335 and http://www.ebi.ac.uk/ena/data/view/PRJEB36336. Supplemental Methods, Figures, and Table S1 with detailed proteomic data analysis information are deposited in the Open Access Data Repository EDMOND and can be directly accessed at the following https://dx.doi.org/10.17617/3.4b

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Fischer ML, Wielsch N, Heckel DG, Vilcinskas A, Vogel H. Context-dependent venom deployment and protein composition in two assassin bugs. *Ecol Evol*. 2020;00:1–16. https://doi.org/10.1002/ece3.6652

3.3. Manuscript III

An assassin's secret: multifunctional cytotoxic compounds in the predation venom of the assassin bug *Psytalla horrida* (Reduviidae, Hemiptera)

Maike L. Fischer, Benjamin Fabian, Yannick Pauchet, Natalie Wielsch, Silke Sachse, Andreas Vilcinskas, Heiko Vogel

in preparation to be submitted to *Toxins*

An assassin's secret: multifunctional cytotoxic compounds in the predation venom of the assassin bug *Psytalla horrida* (Reduviidae, Hemiptera)

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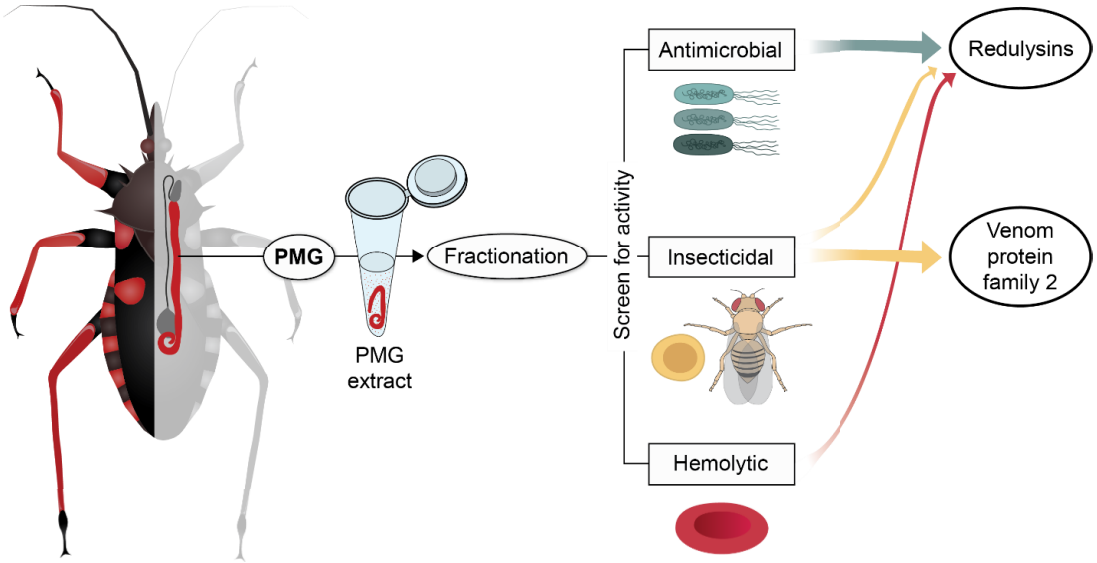
Abstract

Predatory assassin bugs produce venomous saliva that enables them to overwhelm, kill and pre-digest large prey animals. Venom from the posterior main gland (PMG) of the African assassin bug *Psytalla horrida* has strong cytotoxic effects, but the responsible compounds are yet unknown. Using cation-exchange chromatography, we fractionated PMG extracts from *P. horrida* and screened the fractions for toxicity. Two venom fractions strongly affected insect cell viability, bacterial growth, erythrocyte integrity and intracellular calcium levels in *Drosophila melanogaster* olfactory sensory neurons. LC-MS/MS analysis revealed that both fractions contained gelsolin, redulysins, S1 family peptidases and proteins from the uncharacterized venom protein family 2. Synthetic peptides representing the putative lytic domain of redulysins had strong antimicrobial activity against *Escherichia coli* and/or *Bacillus subtilis* but only weak toxicity towards insect or mammalian cells, indicating a primary role in preventing the intake of microbial pathogens. In contrast, a recombinant venom protein family 2 protein significantly reduced insect cell viability but exhibited no antibacterial or hemolytic activity, suggesting that it plays a role in prey overwhelming and killing. The results of our study show that *P. horrida* secretes multiple cytotoxic compounds targeting different organisms to facilitate predation and antimicrobial defense.

Keywords: Venomics, Reduviidae, cytotoxicity, redulysin, venom protein family 2

Key contribution: The assassin bug *Psytalla horrida* secretes multiple toxins from different gene families that exhibit antimicrobial, hemolytic and insecticidal activity.



Graphical abstract

Introduction

Assassin bugs (Reduviidae) comprise a diverse group of hematophagous and zoophagous heteropterans found in terrestrial habitats around the world. Predacious assassin bugs inject toxic saliva to capture their prey [1,2], but they are also known for their painful defensive bites [3,4]. Their venom gland complex is usually subdivided into an anterior and posterior main gland (AMG and PMG, respectively) and an accessory gland (AG) [5,6]. Recent studies have shown that some reduviid species are capable of using venoms from different glands in a context-dependent manner. While only PMG venom is involved in prey envenomations, AMG venom is injected in response to harassment in two species, the harpactorine bug *Pristhesancus plagipennis* Walker and the reduviine bug *Psytalla horrida* (Stål) [5,7]. Defensive venom is directed primarily against vertebrates and usually causes pain and tissue damage to deter predators [3,4,8]. In contrast, venom used for prey capture must rapidly immobilize and kill prey and facilitate the extra oral digestion (EOD) of invertebrate tissue [1,2,7,9,10]. In addition, venom likely contributes to the suppression of microbial growth to prevent the ingestion of potential pathogens or microbial infestation of the venom glands. [11,12]. To fulfill these distinct functions, assassin bugs need complex venoms that are active against a variety of targets.

The AMG and PMG of reduviid bugs secrete distinct and complex protein mixtures, but PMG venom in particular confers potent toxic activity towards different targets. PMG venom from *P. horrida* and *Platymeris biguttatus* (L.) was found to be highly active against *Escherichia coli*, erythrocytes and insect cells, and is able to rapidly paralyze and digest *Galleria mellonella* (L.) larvae [7]. A strong paralyzing and liquefying action of PMG venom has also been demonstrated for *P. plagipennis* [1,5]. In addition, venom collected from *Rhynocoris iracundus* (Poda) was shown to have toxic effects towards mammalian cells, bacterial cells, and helminths [12,13]. Defensively propelled PMG venom from *Platymeris rhadamanthus* Gerstaecker has major lytic effects on mammalian neuronal cells, leading to rapid calcium influxes [14].

Although the venom composition of several assassin bug species has been analyzed recently, little is known about the compounds responsible for the observed effects. The most abundant peptides in assassin bug venoms include digestive enzymes, protease inhibitors, putative pore-forming proteins and neurotoxins, and a large number of uncharacterized proteins [1,5,7,12,14,15]. A recent study attempted to identify the insecticidal venom components of the red tiger assassin bug *Havinthus rufovarius* Bergroth by determining the toxic activity of venom fractions. The fractions with the strongest paralytic and lethal effects on sheep blowflies contained primarily a CUB domain protein and a cystatin, suggesting that one or both proteins contribute to the venom's insecticidal activity [16]. In the hematophagous bug *Triatoma infestans* Klug, trialysin has been identified as a major toxic component that exerts cytotoxic effects against bacteria, protozoa and mammalian cells by disrupting cell membranes. It was shown that upon proteolytic activation, trialysin forms voltage-dependent channels in lipid bilayers, thus permeabilizing cells [11,17]. In predacious reduviids, homologous proteins called redulysins are among the highest expressed proteins in the PMG and likely contribute to the venom's toxic effects [1,7,12,14]. Moreover, trialysin/redulysin homologs are not only found in predacious true bugs but also in phytophagous species such as *Riptortus pedestris* (Fabricius), where they can act as antimicrobial agents [18]. A protein family that is also very abundant and highly expressed in the venom glands of many predatory bugs analyzed to date, is the heteropteran venom protein family 2 [5,7,14,15,19]. Similar to redulysins, family 2 proteins contain several conserved cysteine residues [19], however their function, activity and role in the predacious lifestyle are still unknown.

Venoms are an important source of novel active molecules that may be used for drug or pesticide development. Despite recent advances in the analysis of heteropteran venoms, the activity, function and mode of action of most components remain uncharacterized. This study aims at identifying the venom compounds that are responsible for the antimicrobial, hemolytic, and insecticidal effects of PMG venom from the African assassin bug *P. horrida*. We fractionated crude venom extracts and screened the fractions for toxic effects. Using LC-MS/MS, we analyzed the composition of the active protein fractions and selected candidates for peptide synthesis or heterologous expression. We found several proteins that exert toxic activity towards different targets, thus providing novel insights into venom function and activity in assassin bugs.

Results

Screening of venom fractions

Fractionation of PMG extracts using cation-exchange chromatography resulted in 43 protein fractions with partially overlapping banding patterns of different intensities (Figure 1). The fractions were screened for cytotoxic, antimicrobial and hemolytic activity. The observed cytotoxic effects were mainly caused by two fractions (fraction A = 26; fraction B = 41; Figure 1). Fraction A had strong effects on insect cell viability (22% viability; Figure 1A) and bacterial growth (33% growth after 12.5 h; Figure 2B), but no significant hemolytic activity (Figure 2C). In contrast, fraction B had no significant effects on cell viability (Figure 2A), but comparable effects on bacterial growth (27% growth after 13 h; Figure 2B) and stronger hemolytic activity (85% erythrocyte integrity; Figure 2C). In addition to cell-culture based assays, we screened for insecticidal effects on olfactory sensory neurons in the brain of *Drosophila melanogaster* Meigen using calcium imaging (Figure 3A, B). Application of unfractionated PMG venom on the exposed brains led to an alternating decrease-increase response of intracellular calcium levels ($[Ca^{2+}]_i$) in the antennal lobe.

Although both the timing and amplitude of the effects varied between replicates, the general pattern remained the same for all flies tested (Figure 3C). In contrast, application of fraction A did not lead to the above pattern, but resulted in a quick drop of fluorescence intensity (Figure 3D). The fluorescence intensity after 10 min of scanning was significantly lower in flies treated with fraction A than in those treated with the negative controls (Figure 3E, F). Fraction B triggered similar calcium responses as fraction A, but the results cannot be interpreted with confidence due to strong buffer effects (data not shown).

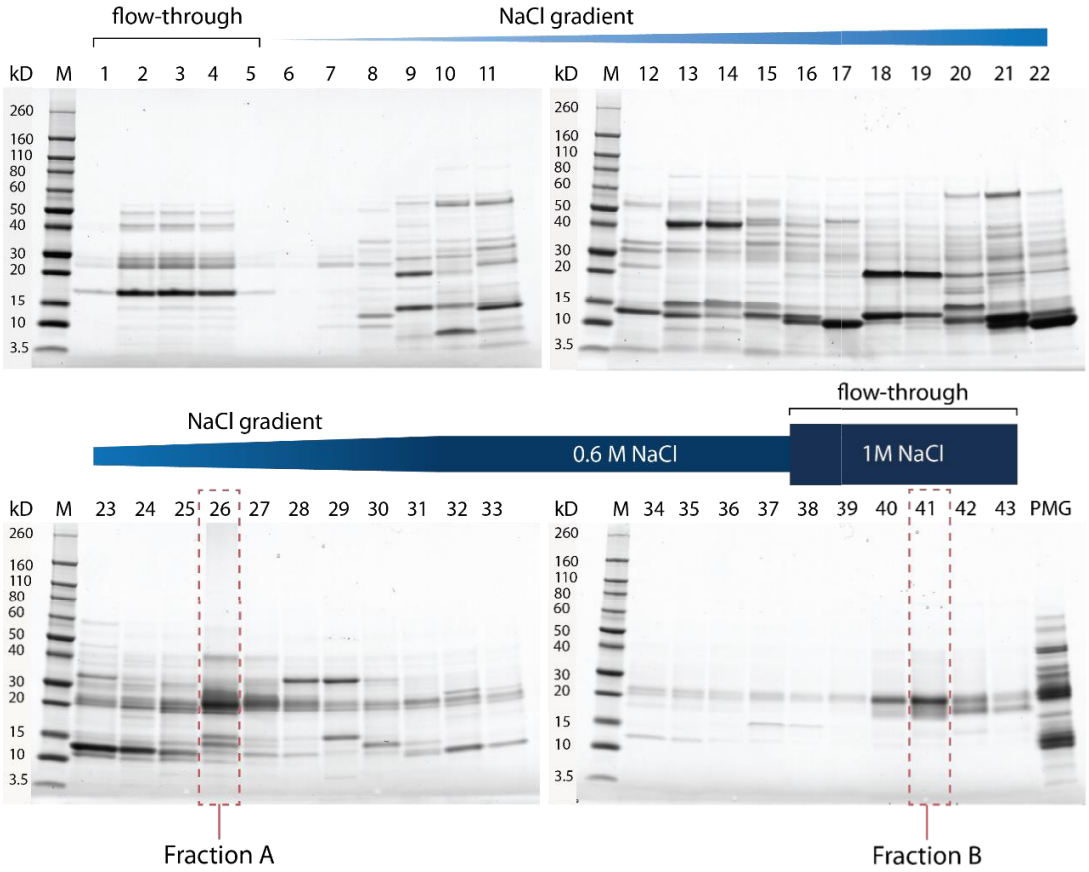


Figure 1: SDS-PAGE analysis of fractions of *P. horrida* PMG venom. 1 – 43 = venom fractions obtained through cation exchange chromatography; PMG = unfractionated PMG venom; M = protein marker. Fraction A and fraction B are highlighted in red. The first five fractions correspond to the flow-through with buffer A, whereas the last six fractions correspond to the flow-through with buffer B.

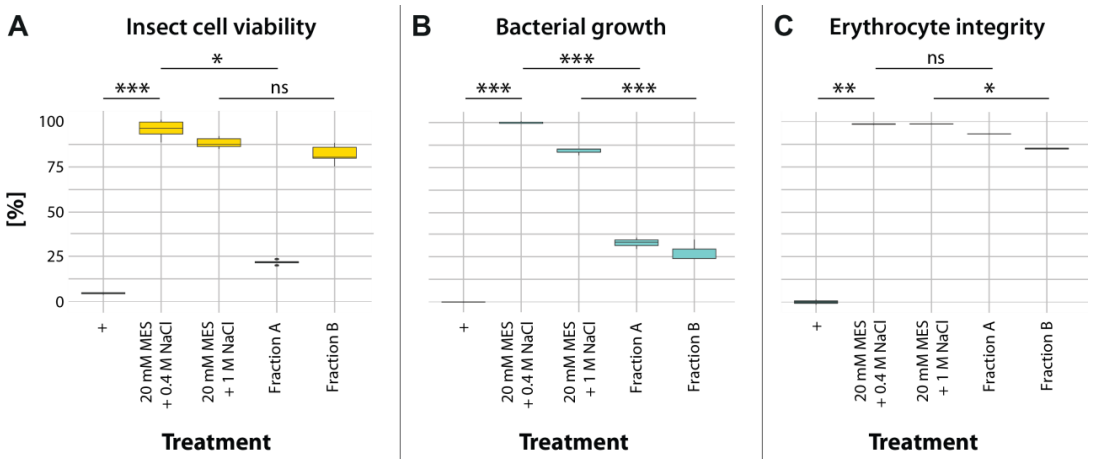


Figure 2: Bioactivity of fraction A and fraction B in comparison to a negative control (20 mM MES + 0.4 M NaCl or 20 mM MES + 1 M NaCl, respectively), and (+) a positive control treatment. Significant differences compared to the respective negative control are highlighted with asterisks (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). **A** Fraction A led to reduced viability of treated sf9 cells; (+) = 0.1% Triton x-100. Statistical test: Dunn's tests, n = 6. **B** Both fractions significantly delayed growth of *E. coli*; (+) = 0.05 mg/mL gentamycin. Statistical test: pairwise t-tests, n = 3. **C** Fraction B caused mild hemolysis of horse erythrocytes; (+) = 0.1% Triton x-100. Statistical test: Dunn's tests, n = 3.

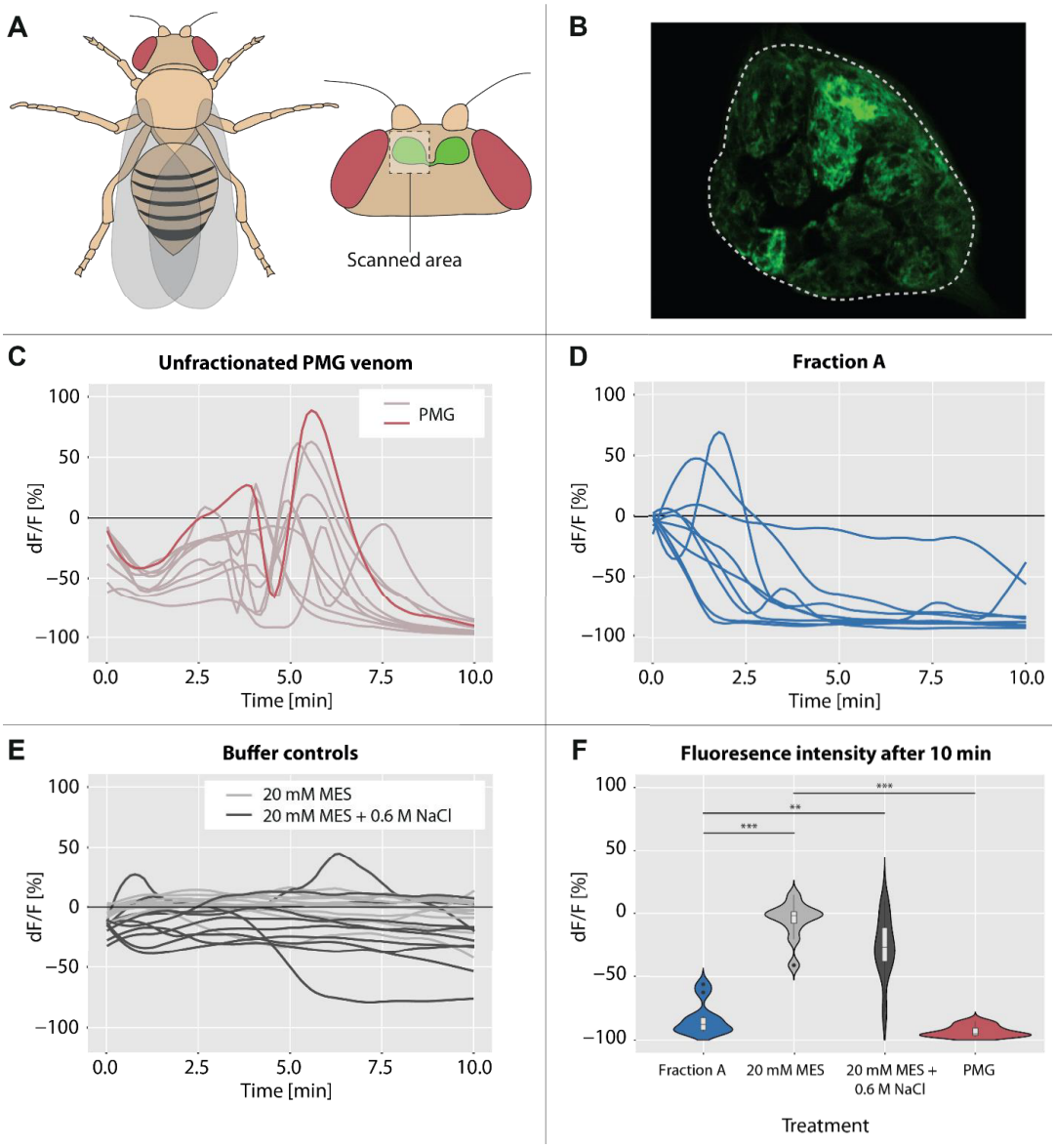


Figure 3: Calcium imaging of *D. melanogaster* antennal lobes using genetic expression of the calcium-sensitive protein GCaMP6s. **A** Schematic of *D. melanogaster* highlighting the left antennal lobe that was imaged. **B** Representative image of an antennal lobe with GCaMP6s expression in olfactory sensory neurons without venom treatment. The dashed line represents the region selected with the ROI manager to extract the brightness values. **C** Changes of dF/F (representing fluorescence changes as an indicator for intracellular calcium concentration) after treatment with unfractionated PMG venom; for highlighting the observed representative course of the fluorescence change, one replicate is marked in dark red. **D** Changes of dF/F after treatment with fraction A. **E** Changes of dF/F after treatment with 20 mM MES and 20 mM MES + 0.6 M NaCl (negative controls). **F** Changes of fluorescence intensity after 10 min. Boxplots

within the violin plots represent median (line), interquartile range (box) and data range (whiskers). Significant differences between treatments are highlighted with asterisks (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001, Dunn's tests, n = 10 (PMG, fraction A, 20 mM MES + 0.6 M NaCl), n = 11 (20 mM MES)).

Protein composition of active fractions

Proteomic analysis revealed that fractions A and B were highly similar in their composition. Most identified proteins could be assigned to the S1 peptidase family (12 in fraction A, seven in fraction B). Furthermore, we detected redulysins (one in fraction A, one in fraction B), venom protein family 2 proteins (three in fraction A, two in fraction B)) and gelsolins (1 in both fractions). Most identified proteins were strongly expressed in the PMG. In both fractions, gelsolin had the highest total score in the LC-MS/MS analysis (Figure 4). Due to their high PMG-specific expression levels and abundancy in predacious true bugs, redulysin and venom protein family 2 were selected as candidate families for further analysis of cytotoxic activity.

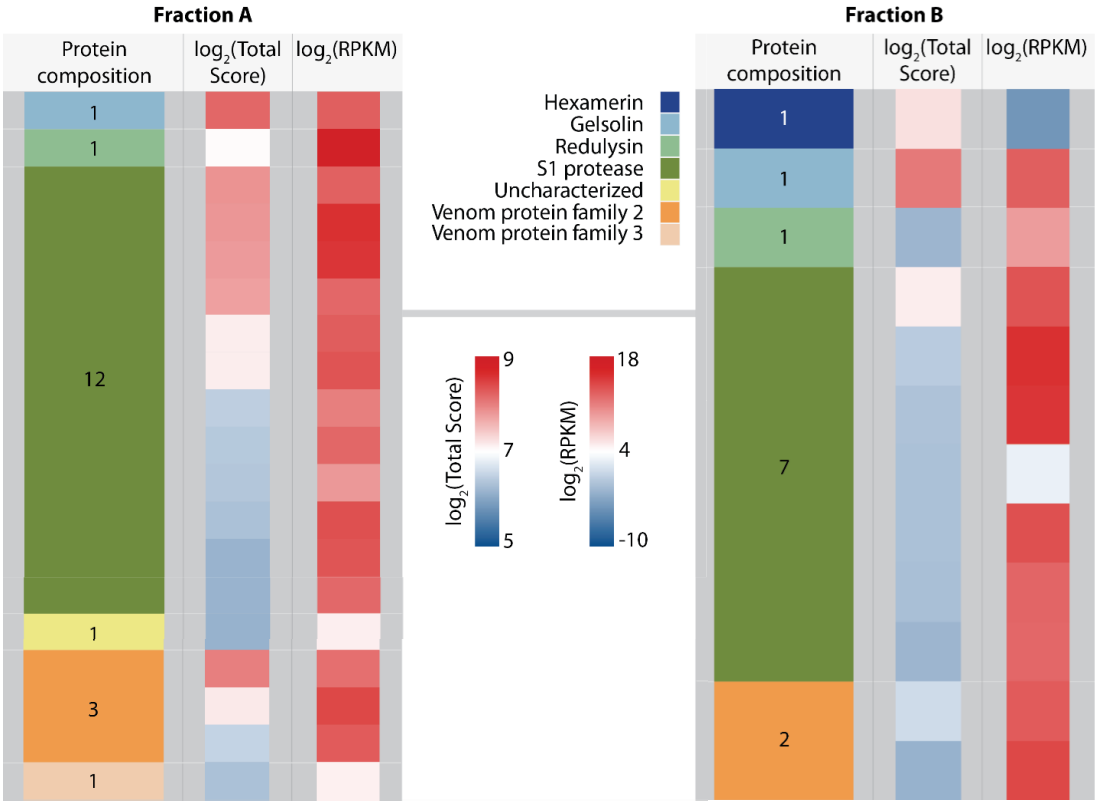


Figure 4: Protein composition of fraction A and fraction B. Color-coded blocks represent the number of contigs identified in transcriptome datasets and verified by proteomic analysis, which encode specific classes of functional proteins. Log_2(Total Score) depicts the logarithmic total score of all matched peptides identified by LC-MS/MS. Log_2(RPKM) shows the expression level of the respective contig in the PMG.

Activity of synthetic redulysin peptides

We identified 12 redulysins in the genome of *P. horrida*, seven of which were also present in the PMG transcriptome and six in the proteome (Table S3). Eleven out of 12 redulysins that were identified in the genome of *P. horrida* contained a conserved motif homologous to the DEER cleavage site in *T. infestans* (8 x DEER, 2 x NEER, 1 x DEQE) [11], which was followed by a lysine-rich region and a region with usually up to eight cysteine residues. The main lytic regions of these redulysins were predicted based on their homology to the lytic region of the characterized *T. infestans* trialysin (Genbank accession: AAL82381.1) [17] and synthesized by solid-phase synthesis (Table S3). The two redulysins g1037.t1 and g1038.t1 differed from other isoforms by their exceptionally high molecular weight (Table S3). In both proteins, we found several acidic regions with potential cleavage sites (4 x GILK, 3 x DEEK, 2 x DEEQ, 1 x DILK) followed by stretches of predominantly basic amino acids, which matches the typical pattern of the lytic region of *T. infestans* trialysin (Figure S1). Moreover, peptides from different regions of g.1038.t1 (Phor_Comb_C9529 in the transcriptome) were detected only in protein bands below 15 kDa (Figure S2). We thus hypothesized that these proteins evolved multiple lytic regions through domain duplictions, resulting in several post-translationally liberated toxins. Therefore, one and six additional putative lytic peptides (peptides 1-6 and 8) were synthesized from g1037.t1 and g1038.t1, respectively. To test whether redulysins from predatory and herbivorous true bugs have different activities, one peptide was synthesized based on a redulysin homolog, which we detected in the salivary gland transcriptome of the phytophagous species *Lygus rugulipennis* Poppius (Table S4). Only two (peptides 9 and 18) and six (peptides 10 – 15 and 18) synthetic peptides had weak effects on insect cell viability and erythrocyte integrity, respectively. While none of the synthetic peptides inhibited *B. thuringiensis* growth, five and nine peptides had strong inhibitory effects on *E. coli* and *B. subtilis* growth, respectively, at concentrations of 10 µM. Peptide 17 was only active on *E. coli* and peptides 9, 11 and 16 only on *B. subtilis* (Figure 5B). Dose-response curves for *E. coli* were generated with four peptides that showed strong toxicity below 10 µM. Peptide 12, which corresponds to the lytic region of redulysin g2022.t1, exhibited the most potent effects with an ED_{50} of < 1 µM after 14 h (Figure 5C). Calcium imaging of *D. melanogaster* olfactory neurons treated with 100 µM of peptide 18, which had the strongest effects on insect and red blood cells, caused a slight increase in fluorescence within 10 min. After 10 min, the fluorescence intensity was significantly higher than in the control flies (23.8 % compared to -5.2 % on average, respectively; Figure 5D). In contrast, peptides 7, 10 and 12, which had strong antimicrobial activity, had no effects on *D. melanogaster* neurons (Figure S3). None of the peptides corresponding to the hypothetically duplicated lytic regions (peptides 1 – 6, 8) had (negative) effects on insect cell viability, erythrocyte integrity or microbial growth (Figure 5B).

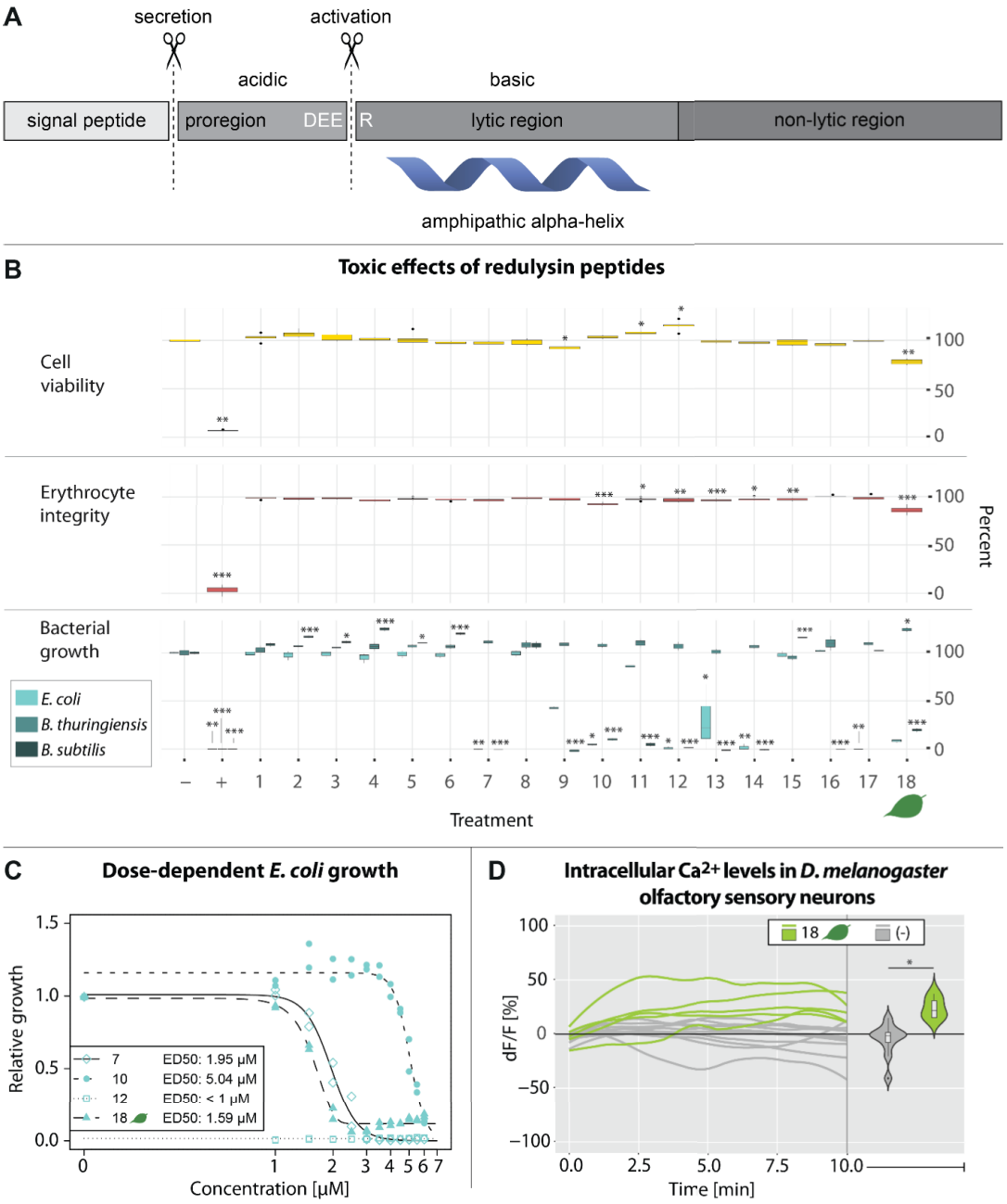


Figure 5: Bioactivity of synthetic redulysin peptides. **A** Structure of redulysins consisting of a signal peptide, a proregion, a lytic region and a non-lytic region. After cleavage of the proregion at the DEER motif, the amphipathic pore-forming alpha-helix is exposed. **B** *B. subtilis*, *B. thuringiensis* and erythrocyte integrity, insect cell viability and *E. coli* growth in presence of 10 µM redulysin peptide; (-) = 20 mM MES pH 5.5 (= 100%), (+) = 0.5 mg/mL gentamycin or 0.1% triton-x 100. Significant differences compared to the negative control are highlighted with asterisks (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001, pairwise t-tests or

Dunn's tests, n = 3). **C** Dose-dependent growth of *E. coli* 14 h after treatment with selected redulysin peptides at varying concentrations. The data were fitted to a logistic model and plotted as dose-response curves (n = 2). **D** Change of intracellular Ca^{2+} levels (represented by dF/F) in *D. melanogaster* olfactory sensory neurons after treatment with 100 µM redulysin peptide 18. Violin plots represent the change in fluorescence intensity after 10 min (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001, Kruskal-Wallis test). Boxplots within the violin plots represent median (line), interquartile range (box) and data range (whiskers). (-) = 20 mM MES pH 5.5. Peptide 18, which corresponds to the redulysin from the phytozoophagous bug *L. rugulipennis* is marked with a leaf symbol in **B, C** and **D**.

Activity of recombinant venom protein family 2 protein

Recombinant Vpf2 significantly reduced insect cell viability to approximately 70 % at concentrations of 0.7 mg/mL, but the toxic effects did not occur when denatured Vpf2 was used (Figure 6A). Vpf2 had no effects on *E. coli* growth or erythrocyte integrity (data not shown). In addition, the recombinant protein did not affect $[Ca^{2+}]_I$ of *D. melanogaster* olfactory sensory neurons at concentrations of 0.15 mg/mL (Figure 6B).

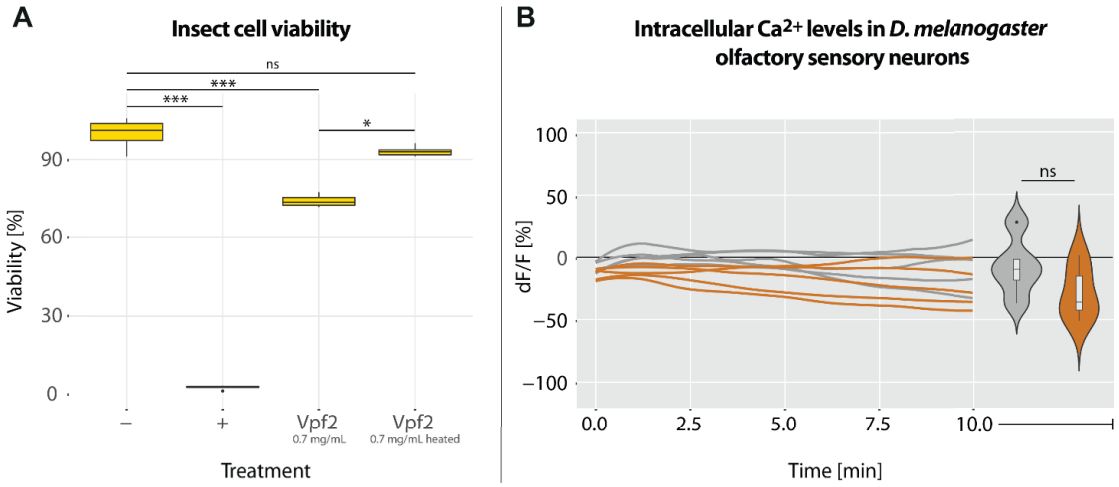


Figure 6: Bioactivity of a recombinant venom protein family 2 (Vpf2) protein. **A** Insect cell viability in presence of 0.7 mg/mL Vpf2 and heated Vpf2; (-) = PBS (= 100%), (+) = 0.1% triton-x 100. Significant differences compared to the negative control are highlighted with asterisks (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001, Dunn's tests, n = 5). **B** Course of intracellular Ca^{2+} levels in *D. melanogaster* olfactory neurons after treatment with 0.2 mg/mL Vpf2 or PBS. Violin plots represent the change in fluorescence intensity after 10 min (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001, Kurskal-Wallis test). Boxplots within the violin plots represent median (line), interquartile range (box) and data range (whiskers).

Discussion

Assassin bugs secrete complex venoms that have toxic effects towards a variety of different cell types [12-14]. PMG venom from the African assassin bug *P. horrida* facilitates predation and defense and has strong insecticidal and cytotoxic activity [7]. However, the compounds responsible for these effects are yet unknown. We fractionated PMG venom using cation-exchange chromatography and conducted different bioassays to identify the cytotoxic compounds. The toxicity towards bacterial, insect and red blood cells was limited to two fractions (Figure 2, 3), which both contained gelsolin, redulysins, S1 family peptidases and uncharacterized proteins from the heteropteran venom protein family 2 (Figure 4). The toxic effects could be partly reproduced by synthetic redulysin peptides (Figure 5) and recombinant Vpf2 (Figure 6A), thus suggesting that both protein families contribute to the cytotoxic effects of *P. horrida* PMG venom.

The redulysins are a family of highly abundant and strongly expressed proteins in the PMG of predatory true bugs [1,7,12,15]. Their homology to the pore-forming protein trialysin from *T. infestans* suggests a broad cytolytic activity, as observed in various assassin bug species including *P. rhadhamanthus*, *P. plagipennis*, *R. iracundus*, *P. biguttatus* and *P. horrida* [5,7,11,13-15]. Trialysin forms voltage-dependent α-helical membrane pores and is cytotoxic towards bacterial, mammalian and protozoan cells as it rapidly destroys the membrane potential [11,20]. It is activated by cleavage of the acidic proregion at the Asp-Glu-Glu-Arg (DEER) site, resulting in the mature peptide that consists of a basic, lysine-rich lytic region and a non-lytic region [17] (Figure 5A). We identified redulysins in the two cytotoxic fractions and expected that they contribute strongly to the effects on bacterial, insect and mammalian cells. Indeed, several synthetic redulysin peptides had antimicrobial activity against *E. coli* and/or *B. subtilis*, including the peptide from *L. rugulipennis* (Figure 5B, C). The specificity of some peptides to only *E. coli* or *B. subtilis* indicates that *P. horrida* secretes several toxic polypeptides directed against different organisms. Such differential toxicity of antimicrobial venom peptides has been described for example in scorpions of the genus *Mesobuthus*, which secrete multiple peptides targeting different bacterial and fungal species [21]. Since trialysins/redulysins are probably stored in the salivary glands as an inactive propeptide and are activated only upon injection [11,17], it is unlikely that they play a role in keeping the glands sterile as previously suggested [11,12]. More likely, they act as antimicrobial agents to suppress the growth of microorganisms from prey or plant tissue and thus prevent the ingestion of potential pathogens. Although several *P. horrida* redulysin peptides showed weak toxicity towards insect or mammalian cells (Figure 5B), this may not reflect the actual activity range of the native redulysins. In nature, the active peptides consist not only of the basic lysine-rich region but also of the non-lytic C-terminal region, which was omitted in our synthetic peptides. For example, the cytolytic activity of synthetic trialysin peptides was reduced up to 100-fold compared to the native protein [11]. Martins, et al. [20] found considerable differences in toxicity and target-specificity between synthetic peptides representing different portions of the lytic region of trialysin. Similarly, the synthetic fractions of the antimicrobial checacin from the pseudoscorpion *Chelifer cancroides* (L.) exhibited significantly reduced antimicrobial activity compared to the full-length peptide, probably due to a reduced positive net charge [22]. It is therefore possible that the mature full-length *P. horrida* redulysins also act on insect and mammalian cells and may contribute to liquefaction, paralysis and pain induction, although the synthetic peptides were not or only weakly active. Interestingly, the synthetic peptide corresponding to the redulysin from the phytophagous *L. rugulipennis* exhibited the strongest cytotoxic effects towards insect and red blood cells (Figure 5B). Although *L. rugulipennis* can occasionally feed on other soft-bodied arthropods [23-25], we doubt that the insecticidal activity of its redulysin has an ecological function in this opportunistic predatory behavior. It is more likely a non-target effect of a general cytolytic activity, whose main function is to sterilize food items. However, further

studies on the ecology and feeding behavior of *L. rugulipennis* as well as on the mode of action of the native redulysin are necessary to confirm this assumption. Due to their unusually high molecular weight (Table S3) and redundancies in the amino acid sequences, we hypothesized that the *P. horrida* redulysins g1037.t1 and g1038.t1 might be cleaved multiple times upon activation to produce several active peptides (Figure S1). Such a multidomain multiproduct protein has been found in the venom of the banded Gila monster *Heloderma suspectum cinctum* Bogert & Martín Del Campo, which expresses several post-translationally liberated toxins encoded for by a single mRNA [26]. However, none of the redulysin peptides representing these putative additional domains (peptides 1-6 and 8) conferred cytotoxic activity against any of the tested cell types (Figure 5B). Further research is needed to clarify the function, post-translational activation and activity spectra of these large redulysins. If they in fact are multidomain multiproduct proteins, they might be active against organisms not covered in this study, such as protozoans, fungi or other bacterial species.

The strong and broad cytotoxic effects of *P. horrida* PMG venom indicate that more than one protein family is involved its insecticidal activity. The uncharacterized heteropteran venom protein family 2 is a very diverse and abundant protein family in predatory true bugs that has been detected in venoms of nepomorphan and cimicomorphan species [1,7,14]. Due to their high similarity to sequences from various bilaterian and cnidarian animals, Walker, et al. [19] suggested that this protein family has been recruited from a widespread non-venom protein, probably prior to the divergence of the Nepomorpha. Moreover, they found weak homology to trialysins/redulysins and hypothesized that both protein families originated from the same ancestral gene [19]. Venom family 2 proteins from *P. horrida* PMG venom contain 6 – 10 cysteine residues in the mature peptide after signal peptide cleavage, similar to the non-lytic region of redulysins (Figure S4). Due to their high PMG-specific expression and their presence in the cytotoxic fractions (Figure 4), we hypothesized that family 2 proteins contribute to the venom's toxicity. Although many different expression systems were tested, we were unable to produce sufficient amounts of active recombinant family 2 proteins, neither in insect or bacterial cells nor in cell-free systems. The main problems included low cell viability upon induction of expression as well as low protein yields and solubility, indicating a cytotoxic nature of the candidate proteins. Finally, the heterologous expression of one selected family 2 protein, Vpf2, in CHO cells yielded sufficient amounts of soluble protein. Vpf2 was toxic towards insect cells (Figure 6A) but showed no major activity against *E. coli* or red blood cells, thus suggesting that its main ecological role involves prey overwhelming, killing and/or liquefaction. As the venom protein family 2 is a highly diverse protein family with at least 13 genes in *P. horrida* [7], we assume that other family 2 proteins may have stronger effects or act on other cell types. Future research should therefore focus on analyzing the activity of different venom protein family 2 proteins from different species to determine their ecological function and mode of action.

In addition to cell culture based assays, the effects of *P. horrida* venom on $[Ca^{2+}]_i$ of a subset of neurons in the brain of *D. melanogaster* were tested. Calcium ions play an important role in neuronal signaling, muscle contraction and apoptosis [27]. Moreover, calcium influx is commonly used as an indicator for pain, as it is a crucial step in the activation of nociceptive neurons. Several studies demonstrated the rapid increase of $[Ca^{2+}]_i$ in mammalian sensory neurons upon treatment with defensive venom secretions [14,28,29]. Most likely, these effects are due to the disruption and depolarization of membranes by pain-inducing lytic proteins, as shown for the assassin bug *P. rhadhamanthus* [14]. We expected similar effects of *P. horrida* PMG venom on *D. melanogaster* neurons, due to its strong cytolytic activity. Interestingly, we observed a highly complex pattern of fluctuations in $[Ca^{2+}]_i$ upon treatment with unfractionated venom (Figure 3C), indicating that several compounds with different kinetics and modes of action act on insect

neurons. As a first rapid reaction, the relative change in fluorescence intensity dropped down to approx. -50% in less than a minute, likely representing the effects of fast-acting compounds such as neurotoxins. Venom peptides from assassin bugs that have been suggested to act as neurotoxins and cause prey paralysis include ptu1-like peptides, cystatins and CUB domain proteins [16,30]. The fast decrease in $[Ca^{2+}]_i$ was followed by a gradual increase over several minutes, in some cases exceeding the baseline. The subsequent rapid $[Ca^{2+}]_i$ drop was followed by a sharp increase that can likely be explained by a complete breakdown of membrane potential due to pore-forming proteins, leading to the death of the fly. Similar to what has been described for other cytolytic insect venoms, the fluorescence intensity then gradually decreased, probably due to leakage of the fluorescent dye through membrane pores [14,31]. The complex effects could not be fully recreated by applying the cytolytic fractions A or B, thus indicating that other non-lytic compounds not present in these fractions contribute to the venom's toxicity towards insect neurons. Interestingly, application of fraction A did not lead to an increase of $[Ca^{2+}]_i$ as would be typical for pore-forming proteins [14,31], but instead resulted in a rapid decrease in most replicates (Figure 3D). LC-MS/MS analysis revealed the presence of gelsolin in both fractions (Figure 4), a calcium binding protein that is associated with actin depolymerization in prey animals [32,33]. We assume that large amounts of extracellular calcium were bound by gelsolin after application of the fractions, resulting in a change of the concentration gradient between intracellular and extracellular media. Under these conditions, the action of pore-forming proteins such as redulysins would lead to a decrease of $[Ca^{2+}]_i$ to balance the gradient. Application of the synthetic *L. rugulipennis* redulysin peptide, which had the strongest effects on insect cell viability, resulted in a slight increase in $[Ca^{2+}]_I$ (Figure 5D), thus confirming its presumed pore-forming activity. Although other tested redulysin peptides from *P. horrida* showed no effects on $[Ca^{2+}]_I$ (Figure S3), we assume that they generally share a mode of action with the *L. rugulipennis* redulysin. As stated above, the activity range and potency of the synthetic peptides likely differ from the native redulysins. Moreover, it is possible that redulysins act synergistically with other molecules, thus enhancing and/or expanding their potency and target range. The effects of the synthetic *L. ruglulipennis* redulysin on $[Ca^{2+}]_I$ in *D. melanogaster* brains were observed at concentrations 10-fold higher than the toxic effects on cultured insect cells, thus indicating that redulysins may rely on other compounds, supporting translocation to their target sites in more complex (i.e. *in vivo*) environments. The central nervous system of *D. melanogaster* consists of 10% glial cells, which form a 2 – 3 µm thick layer around the brain and fulfil a variety of functions, including the generation of a blood-brain barrier to provide chemoprotection [34,35]. It is likely that these protective glial cells impede the action of redulysins on *D. melanogaster* neuronal cells. An important so-called "venom spreading factor" found in many venoms is hyaluronidase, a carbohydrase that degrades polysaccharides in the extracellular matrix of animals and therefore facilitates the rapid spread of venom molecules to reach their site of action [10,36,37]. *Psytalla horrida* venom also contains hyaluronidase [7] and we hypothesize that it enhances the potency of cytotoxic peptides such as redulysins. Future experiments should include combinations of redulysin peptides with potential spreading factors such as hyaluronidase or proteases to verify whether they act synergistically. Similarly, Vpf2 should be tested in combination with putative spreading factors, to further investigate its role as an insecticidal peptide.

Materials and methods

Insects

P. horrida specimens were obtained from an insectarium breeding source (Jörg Bernhardt, personal communication). They were kept at room temperature in terraria containing sand, coconut fibers and bark and fed once per week with *Gryllus assimilis* (Fabricius), which were obtained from Tropic Shop (Nordhorn, Germany).

L. rugulipennis specimens were obtained from Katz Biotech AG (Baruth/Mark, Germany) and used directly for RNAseq.

For calcium imaging, 5 -7 days old *D. melanogaster* flies expressing GCaMP6s in olfactory sensory neurons were used (genotype: +/+;UAS-GCaMP6s/UAS-GCaMP6s;Orco-Gal4/Orco-Gal4 (from Ilona Grunwald Kadow, Silke Trautheim, MPI-CE Jena)). The flies were reared in plastic vials at 12 h/12 h light/dark cycle at 25 °C and 70% humidity. Every two weeks, they were transferred into clean vials containing fresh diet (standard cornmeal medium: sugar beet molasses, beer yeast, powdered agar, polenta, propionic acid and Nipagin and water).

Venom extraction

PMG venom was extracted from the venom glands of fifth-instar or adult assassin bugs. Individuals were anaesthetized at –20 °C for 5 min prior to dissection in phosphate-buffered saline (PBS). The posterior lobe was separated from the AMG and AG and transferred to a pre-cooled tube containing 100 µL of 20 mM MES (pH 5.5) on ice. After briefly vortexing, the samples were centrifuged (4,000 g, 3.5 min at 4 °C) and the supernatant further clarified by an additional centrifugation (13,000 g, 3 min, 4 °C). The venom extracts of several individuals were pooled and stored at –20 °C for analysis. The total protein concentration in the venom samples was determined using an N60 nanophotometer (Implen).

Venom fractionation

PMG venom extracts were fractionated by fast protein liquid chromatography (FPLC). 2 mL of venom (7.6 mg/mL) in 20 mM MES at pH 5.5 (buffer A) were filtered through a 0.22 µm syringe filter and loaded onto a 1 mL RESOURCE S cation-exchange column (GE Healthcare), which was pre-equilibrated with buffer A. Chromatography was carried out at a flow rate of 0.5 mL/min. After initial elution with buffer A, the salt concentration was gradually increased by adding 20 mM MES containing 1 M NaCl at pH 5.5 (buffer B) up to a maximum salt concentration of 600 mM NaCl (= 60% buffer B). In total, 43 protein-containing fractions were collected and stored at -20 °C for analysis.

Gel electrophoresis and LC-MS/MS

The proteins in the venom fractions were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–12% Criterion XT gradient gels (BioRad) with XT MES running buffer (BioRad). The gels were run at 125 V for 1 h, alongside a pre-stained protein standard (Thermo Fisher Scientific) and subsequently stained with a 1:1 mixture of Coomassie Brilliant Blue R-250 and colloidal Coomassie Brilliant Blue G-250 (Thermo Fisher Scientific) for 1.5 h. After washing in Millipore water overnight to remove excess dye, the stained gels were scanned and analyzed. From selected gel lanes, the protein bands were excised for tryptic digestion [38] and reconstituted in 50 µL of 1 % formic acid in water. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed as described previously

[7]. Briefly, the peptide mixture were injected into an ultra-high performance liquid chromatography (UPLC) M-class system (Waters) coupled online to a Synapt G2-si mass spectrometer (Waters). Pre-concentrated and desalted samples were eluted onto an ACQUITY UPLC HSS T3 analytical column (100 Å, 75 µm X 200, 1.8 µm particle size) at a flow rate of 350 nL/min. The gradient of mobile phase A (0.1% aqueous formic acid) and B (acetonitrile plus 0.1% formic acid) increased from 2-10% B over 5 min, 10–40% B over 40 min, 40–70% B over 7 min, 70-95% B over 3 min, isocratic at 95% B for 2 min, and a return to 1%. The eluted peptides were injected into the mass spectrometer operating in V-mode and positive ESI mode. The resolving power was at least 20,000 full width at half height (FWHM).

Data were collected by data-dependent acquisition (DDA). The acquisition cycle consisted of a survey scan covering the m/z range 400–1,800 Da, which was followed by MS/MS fragmentation of the 10 most intense precursor ions collected at 0.5-s intervals in the m/z range 50–2,000. We used dynamic exclusion to minimize multiple fragmentations for the same precursor ions. The MassLynx v4.1 software (Waters) was used to collect MS data.

Proteomic data processing and protein identification

The ProteinLynx Global Server (PLGS) v2.5.2 (Waters) was used to process DDA raw data. The spectra were screened against a sub-database of common contaminants including human keratins and trypsin. Unmatched spectra were interpreted *de novo* to yield peptide sequences for homology-based searching using MS BLAST [39] installed on a local server. We carried out MS BLAST searches against the Arthropoda database (download from NCBI on 10 12 January February 2019) and the *P. horrida* sub-database obtained from *in silico* translation of the transcriptome generated by Fischer, et al. [7]. In addition, the pkl files generated from raw data were searched against the NCBInr database (downloaded on 12 February 2019) combined with the *P. horrida* sub-database using MASCOT v2.6.0. The following parameters were used: fixed precursor ion mass tolerance of 15 ppm for survey peptide, fragment ion mass tolerance of 0.1 Da, 1 missed cleavage, fixed carbamidomethylation of cysteines and possible oxidation of methionine.

RNAseq of Lygus rugulipennis

The salivary gland complex (SG), gut and remaining body tissue of adult *L. rugulipennis* specimens were dissected in 1x PBS and transferred into separate ceramic bead tubes containing 500 µL of TRI Reagent (Sigma-Aldrich). The tissues of six individuals were pooled and homogenized using a TissueLyser LT (Qiagen). The Direct-zol RNA Miniprep Kit was used to extract total RNA according to the manufacturer's instructions (Zymo Research). RNA quantity and integrity were determined using a N60 nanophotometer and an Agilent 2100 Bioanalyzer and RNA Nanochip (Agilent Technologies), respectively.

The SG, gut and remaining body tissue transcriptomes were sequenced by the Max-Planck Genome Center Cologne (http://mpgc.mpipz.mpg.de/home/) using an Illumina HiSeq3000 Genome Analyzer platform. Poly-A mRNA was isolated from 1 µg total RNA using oligo-dT attached to magnetic beads. The RNA was then fragmented to an average of 250 bp and sequencing libraries were generated using the TruSeq RNA library preparation kit. Paired-end (2 × 150 bp) read technology was used for sequencing. Detailed information on sequencing and assembly are summarized in Table S2. An in-house assembly and annotation pipeline was used to process the generated reads. Quality control measures (filtering of high-quality reads, removal of reads containing primer/adaptor sequences and trimming of read lengths) were applied using CLC Genomics Workbench v11.1. The transcriptome assembly for all tissue samples combined was prepared using CLC Genomics Workbench v11.1 standard settings and two additional CLC-

based assemblies with different parameters. The presumed optimal consensus transcriptome was then selected, as previously described [40]. Annotations were added using BLAST, Gene Ontology and InterProScan in OmicsBox (https://www.biobam.com/omicsbox) as described by [41]. For BLASTx searches against the non-redundant NCBI protein database (NR database), up to 20 best NR hits per transcript were retained (E-value cutoff: $\leq 10^{-3}$; minimum match length: 15 amino acids). Transcriptome completeness was assessed using a Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis (http://busco.ezlab.org) by comparing the assembled transcriptome against a set of highly-conserved single-copy orthologs. Using the BUSCO v3 pipeline [42], the predicted proteins of the *L. rugulipennis* transcriptome were compared to the predefined set of 1658 Insecta single-copy orthologs from the OrthoDB v9.1 database. Digital gene expression analysis was performed in CLC Genomics Workbench v11.1. For that, BAM files were generated and the sequences were counted to estimate expression levels, using previously described parameters for read mapping and normalization [43]. To estimate gene expression levels mapped read values were normalized as implemented in CLC Genomics Workbench v11.1 and ArrayStar, and the reads per kilobase per million mapped reads (RPKM) values were calculated.

Synthesis of redulysin peptides

Potential lytic regions of *P. horrida* redulysins were synthesized by Genscript Biotech Corporation using solid-phase synthesis. Lyophilized peptides were reconstituted in 20 mM MES pH 5.5 to obtain stock solutions of 1 mM, which were stored at -20 °C.

Heterologous expression of venom protein family 2 proteins

One venom protein family 2 protein (Phor_Comb_C31143; hereafter Vpf2) homologous to venom protein family 2 protein 4 from *P. plagipennis* (Genbank accession: AQM58361.1) was heterologously expressed in CHO cells by Genscript Biotech Corporation. The recombinant proteins were purified from cell culture supernatants using HisTrap™ FF Crude+ HiLoad™ 26/600 Superdex. Proteins were eluted in PBS buffer (pH 7.2), purity was confirmed with SDS-PAGE under non-reducing condition (≥95%) and SEC-HPLC (>99%) and purified proteins were stored at -80 °C.

Cell viability assay

Sf9 cells were cultured in sterile 96-well plates (Greiner) using Sf-900 II SFM medium (Gibco) containing 0.05 mg/mL gentamycin. After 24 hours, the culture medium was replaced by 100 µL of a 1:10 dilution of venom fraction or dissolved redulysin peptide (100 µM) in *Sf9* culture medium or a 1:1 dilution of recombinant Vpf2 in *Sf9* culture medium (n = 6) and incubated at 27 °C for 4 h. The positive control was a 0.1% Triton x-100 solution in *Sf9* (n = 6). Negative controls were 20 mM MES (pH 5.5) for synthetic redulysin peptides, 20 mM MES + 0.4 M NaCl (pH 5.5) as well as 20 mM MES + 1 M NaCl (pH 5.5), for fraction A and B, respectively (n = 6). For the cell viability assay with recombinant Vpf2, we used PBS and denatured protein (heated at 95 °C for 10 min) as negative controls. After incubation, the culture medium was replaced with medium containing 0.5 mg/mL thiazolyl blue tetrazolium bromide (MTT, Sigma Aldrich). After incubation at 27 °C for 2 h, the MTT mixture was removed and 50 µL of dimethyl sulfoxide (DMSO, Sigma Aldrich) were added. The plates were incubated at 27 °C for 10 min, briefly vortexed and the absorbance at 540 nm was read in an Infinite m200 plate reader (Tecan). The average absorbance value of pure DMSO was subtracted from all values and the relative cell viability was calculated in relation to the negative control (defined as 100%).

Bacterial growth inhibition assay

50 µL of an *E. coli* overnight culture in lysogeny broth (LB) medium were added to 5 mL LB medium and incubated at 37 °C for 2-3 h. The culture was then diluted with LB medium to an OD_{600} of 0.003. In a 96-well plate (Greiner), 10 µL of venom fraction or dissolved redulysin peptide (100 µM) were mixed with 90 µL of bacteria dilution (n = 3). Similar dilutions were prepared with gentamycin (0.5 mg/mL) as a positive control (n = 3). Negative controls were 20 mM MES (pH 5.5) for synthetic redulysin peptides, and 20 mM MES + 0.4 M NaCl (pH 5.5) as well as 20 mM MES + 1 M NaCl (pH 5.5), for fraction A and B, respectively (n = 3). The mixtures were incubated at 30 °C in an Infinite m200 plate reader (Tecan) and the absorbance at 595 nm was read in 5 min intervals over a period of 24 h. The average absorbance value of PBS in sterile LB medium (1:10) was subtracted from all values and the relative growth after 12.5 h (OD_{595} of a PBS control ~ 0.36, log-phase) was calculated in relation to the respective negative control (defined as 100%). For the dose-response analysis, varying concentrations (0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 µM) of selected redulysin peptides were tested (n = 2) and the OD_{595} values after 14 h compared.

Hemolysis assay

Erythrocytes were harvested by centrifuging defibrinated horse blood (Thermo Fisher Scientific) at 1,500 g for 3 min. The cells were washed three times with PBS and a 1:10 erythrocyte suspension in PBS was prepared. 20 µL of venom fraction or dissolved redulysin peptide (100 µM) were mixed with 180 µL cell suspension (n = 3) in a 96-well plate and incubated at 37 °C for 1 h. The positive control was 1% Triton x-100 (n = 3). Negative controls were 20 mM MES (pH 5.5) for synthetic redulysin peptides, and 20 mM MES + 0.4 M NaCl (pH 5.5) as well as 20 mM MES + 1 M NaCl (pH 5.5), for fraction A and B, respectively (n = 3). After incubation, the cell suspensions were centrifuged at 2,000 g for 10 min and the supernatants were transferred into a new clear 96-well plate. The absorbance at 440 nm was read in an Infinite m200 plate reader (Tecan). The average absorbance value of the negative control was subtracted from all values and the relative cell integrity was calculated in relation to the negative control (defined as 100%).

Dissection of flies for calcium imaging

D. melanogaster females were anesthetized on ice prior to dissection. The flies were immobilized by pushing the cervical region into a slit of a copper plate (Athene slot diaphragm, 125-µm slot, Plano) that was glued to a mounting stage made of Plexiglas. A fine needle (minutiens 0.10 mm, Austerlitz Insect Pins) was pressed on top of the proboscis and fixed with beeswax on both sides of the mounting stage in order to immobilize the head. Furthermore, the backside of the head was glued to the copper plate by using 3-component dentist glue (Protemp™ II, 3M). Both ends of a fine tetrode wire (Redi Ohm 800, H.P. Reid Inc.) were fixed to a plastic plate with beeswax. The wire was inserted into the ptilinal suture. Using screws in the mounting stage, the plastic plate with the attached wire were slowly pushed forward to displace the antennae slightly to the front. A plastic plate with a hole was glued to a polyethylene foil and an additional hole with a smaller diameter was punched through the foil. The plate was placed on top of the fly's head, so that the hole in the foil exposed the head. 2-component silicon (Kwik-Sil™, World Precision Instruments) was used to seal the space between the edges of the foil and the fly's head capsule. Then, a droplet of Ringer's solution (NaCl: 130 mM, KCl: 5 mM, MgCl2: 2 mM, CaCl2: 2 mM, Sucrose: 36 mM, HEPES-NaOH (pH 7.3): 5 mM) was added onto the head. The head capsule was opened dorsally with a fine scalpel (Micro Knive, Fine Science Tools) and fat body tissue, glands and tracheae were removed with fine forceps to ensure optical access to the brain.

Calcium imaging

In order to monitor calcium signals in the fly brain, we functionally imaged calcium changes in the antennal lobe, the first olfactory neuropil of insects, with and without venom application at a 2-photon microscope. Prior to the experiment, the droplet of Ringer's solution added during the dissection was removed with tissue paper and replaced with 40 µL Ringer's solution. The dissected fly was placed on the stage of a ZEISS LSM 710 NLO confocal microscope (Zeiss) equipped with an infrared Chameleon Ultra diode-pumped laser (Coherent). The ZEN software (Zeiss) was used to control the microscope. The fluorophore of the expressed GCaMP6s was excited using a laser wavelength of 925 nm. A 63x water immersion objective (W Plan-Apochromat 63x/1.0 VIS-IR, Zeiss) was used to visualize a plane of the right antennal lobe containing the DM1 and DM2 glomeruli. The laser power was set between 10% and 30%, depending on the expression level of GCaMP6s in individual flies and the master gain was set to 700. The frame size of acquired time series was 248 x 250 pixels and the frame rate 4 Hz.

In order to confirm that the dissected flies were alive and the brains undamaged, their response to an odor (3-hexanone at a concentration of 10^{-2}) was tested prior to the experiments. An electronically controlled odor delivery system consisting of flexible Teflon tubes guiding two converging airstreams (0.5 L/min each) to the antennae was used. A solenoid valve controlled by the LabVIEW software (National Instruments) was installed in one of these airstreams and switched between a tube transporting pure air and a tube that entered a 50 mL glass bottle (Schott) containing 1 mL of a diluted odorant. A time series of 10 s was acquired, in which the odor pulse began after 2 s and lasted for 2 s. After confirming that flies were alive, a time series of 15 s was acquired in order to determine the GCaMP6s base fluorescence. Subsequently, 5 µL of unfractionated PMG venom (18 mg/mL in MES buffer), venom fractions, redulysin peptides (900 µM) or recombinant Vpf2 (1.4. mg/mL) were added to the ringer solution and a time series of 10 min was started immediately afterwards. PBS as well as 20 mM MES (pH 5.5) and 20 mM MES containing 0.6 M NaCl (pH 5.5) and were used as negative controls, representing the lowest highest possible NaCl concentrations in the fractions (excluding flow-through).

Data analysis

Data analysis was performed using R v4.0.3 and the integrated development environment RStudio v1.2.1335 [44]. For the hemolysis, cell viability and bacterial growth inhibition assays, we performed one-way analysis of variance (ANOVA) with pairwise t-tests or Kruskal-Wallis rank sum tests with pairwise Dunn's tests (Table S1) using the FSA package [45]. Dose-response curves for *E. coli* treated with redulysin peptides were plotted after fitting the data for each treatment to a logistic model using the drc package [46]. All plots were created using the ggplot2 package [47] or the R built-in plot() function. To analyze calcium imaging scans, the ImageJ software [48,49] was used. The ROI manager was applied to select the antennal lobe and extract the average brightness value of pixels within the selected region (including movements) across the entire time series. The extracted values were used to calculate and plot the change in fluorescence intensity (ΔF/F) in percent. For each individual, the mean value of the 15 s time series representing the base fluorescence was subtracted from the values of the 10 min time series to calculate ΔF. Then, ΔF was divided by the base fluorescence to calculate ΔF/F and multiplied by 100 to obtain ΔF/F [%].

Acknowledgements

We thank Yu Okamura for annotating the genome of *P. horrida* and Bianca Wurlitzer, Domenica Schnabelrauch and Henriette Ringys-Beckstein for technical assistance in the laboratory.

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4. DISCUSSION

Venom use is an adaptive trait that has evolved independently at least 14 times in insects and plays a major role in diversification and the exploitation of new ecological niches (Arbuckle & Harris, 2021; Walker et al., 2018c). Predatory Heteroptera have been largely neglected in venom research, although they were one of the first insect groups to evolve venom use (Walker, 2020). Most research thus far has focused on individual species from the families Reduviidae and Belostomatidae, but comparative ecological studies are rare and most true bugs venom compounds remain uncharacterized regarding in terms of their function and ecological role. This thesis sets out work aiming to decipher and compare the venom composition and deployment in different heteropteran species to define species-dependent adaptations to ecological niches and the corresponding active substances.

4.1. Ecological niche influences venom composition and activity

The Nepomorpha and Cimicomorpha are the largest heteropteran infraorders that have retained the ancestral predatory feeding style (Weirauch et al., 2019). Even so, they differ greatly in their ecology. The Cimicomorpha, like the most recent common ancestor (MRCA) of Heteroptera, inhabit terrestrial environments, whereas the Nepomorpha transitioned to aquatic habitats approximately 240 million years ago (Walker et al., 2018a; Wang et al., 2016). This habitat change required substantial adaptations, especially in terms of thermoregulation, osmoregulation, respiration, locomotion and feeding (Dijkstra et al., 2014). The effects of such niche shifts on salivary/venom protein composition and activity in Heteroptera have not yet been studied in detail. Using an integrated proteomics and transcriptomics approach combined with bioactivity assays, we compared the salivary/venom protein composition and activity of two cimicomorphan and four nepomorphan species. The reduviines (Cimicomorpha: Reduviidae) *P. horrida* and *P. biguttatus* are generalist predators of arthropods and are found only in open tropical and subtropical areas of Africa, with overlapping habitats. In contrast, the nepomorphans *Ilyocoris cimicoides* (L.) (Naucoridae), *Notonecta glauca* L. (Notonectidae), *Nepa cinerea* L. (Nepidae) and *Corixa punctata* (Illiger) (Corixidae) are common European water bug species, which co-occur in the same aquatic habitats but occupy different ecological

niches. We found evidence that the ecological niche, including habitat, microhabitat, feeding style and foraging strategy, strongly influence saliva/venom composition and activity, particularly with respect to EOD, cytotoxicity and paralysis (**manuscript I**).

Digestive enzymes are needed by true bugs for EOD and are directly related to their feeding habits (Cantón & Bonning, 2020; Yoon et al., 2022; Zeng & Cohen, 2000). Plants are rich in carbohydrates whereas animals are generally richer in proteins and lipids, so different sets of enzymes are required for efficient tissue digestion and nutrient utilization. In **manuscript II**, we showed that *Galleria mellonella* (L.) larvae envenomated by *P. biguttatus* were liquefied almost completely within 30 min, indicating the presence of potent digestive enzymes in the venom. Similar effects have been demonstrated for *P. rhadamanthus* (Edwards, 1961). Proteotranscriptomic analysis revealed that the strictly predatory species *P. horrida*, *P. biguttatus*, *I. cimicoides*, *N. glauca* and *N. cinerea* secrete a similar set of digestive enzymes dominated by family S1 endopeptidases but containing few exopeptidases, lipases and carbohydrases, which were instead more abundant in the gut (**manuscripts I and II**). This reflects their adaptation to a diet of protein-rich and lipid-rich animals, such as EOD ensures tissue dissociation and the initial degradation of polypeptides and storage lipids, whereas further protein digestion by exopeptidases and most lipid and carbohydrate catabolism occur in the gut. Similar enzyme profiles have also been described for other predatory species from different infraorders (Oliveira et al., 2006; Rügen et al., 2021; Walker et al., 2018a; Walker et al., 2017; Walker et al., 2019). In contrast, venom/saliva from *C. punctata* was rich in cysteine proteases (cathepsins) from peptidase family C1 and carbohydrases from glycoside hydrolase families GH 1, GH 13, GH 27 and GH 38 (**manuscript I**). The feeding style of Corixidae is disputed, with different studies claiming zoophagy (Esenbekova et al., 2015; Jansson & Scudder, 1972), saprophagy (Warren, 1989) and omnivory (Sutton, 1951). The abundance of salivary carbohydrases, including putative amylases and mannosidases, as well as the presence of salivary cathepsins, which are strongly associated with herbivorous hemipterans (Guo et al., 2020; Huang et al., 2021; Lomate & Bonning, 2018), indicate that *C. punctata* has adapted to a predominantly plant-based diet (**manuscript I**). Carbohydrase (especially amylase) and protease activity are often used to predict feeding habits in heteropterans because high carbohydrase activity is usually associated with phytophagy and high protease activity with zoophagy (Agustí & Cohen, 2000; Zeng & Cohen, 2000). However,

glycogen and starch, the main storage carbohydrate in animals and plants, respectively, are both substrates for the midgut amylases of *Andralus spinidens* (Fabricius) (Sorkhabi-Abdolmaleki et al., 2014). We detected salivary amylases in the predatory nepomorphans *I. cimicoides* and *N. cinerea* and the putatively herbivorous *C. punctata* and found that their venom extracts can degrade both starch and glycogen **(manuscript I**). This is clear evidence for the dual function of salivary amylases in nepomorphan species, allowing them to optimize nutrient extraction from both animal and plant tissues. Therefore, the presence of salivary amylases should not automatically be linked to phytophagy because they may be important enzymes in both predatory and herbivorous species, facilitating opportunistic and permanent dietary switches. Hyaluronidases are carbohydrases from GH 56 that break down the extracellular matrix in animals and act as venom spreading factors and allergens in many animal venoms (Girish et al., 2002; Sobotka et al., 1976; Tu & Hendon, 1983). We identified GH 56 hyaluronidases in *P. horrida*, *P. biguttatus*, *N. glauca* and *I. cimicoides*. Whereas *I. cimicoides* also secreted other carbohydrases from families GH 37 and GH 13, and *N. glauca* likewise secreted GH 18, GH 37 and GH 38 proteins, the two assassin bug species secreted no additional carbohydrases (**manuscripts I and II**). Carbohydrases are usually abundant in herbivores, but GH 56 (putative hyaluronidase), GH 13 (putative amylase/glycogenase) and GH 18 (putative chitinase) seem to play important roles in the degradation of abundant polysaccharides in animals and are therefore beneficial for predatory Heteroptera. Our results show that feeding styles should not be predicted based exclusively on the abundance of carbohydrases and proteases but must take into account the nature and activity of individual enzymes and their putative ecological roles.

Cytotoxic compounds are common in many animal venoms and may paralyze and kill prey, liquefy issued, induce pain and/or provide antimicrobial defense (Amino et al., 2002; Walker et al., 2017). *Psytalla horrida* and *P. biguttatus* PMG venom was highly toxic towards insect, bacterial and mammalian cells (**manuscripts II and III**), as previously described for other predatory reduviids (Rügen et al., 2021; Walker et al., 2017; Walker et al., 2019). Moreover, *P. horrida* PMG extracts elicited complex calcium-mediated responses in *Drosophila melanogaster* Meigen brains, indicating the presence of several insecticidal compounds (**manuscript III**). Among the Nepomorpha, only *N. cinerea* venom showed strong toxic effects against insect and mammalian cells and was able to delay the

growth of *E. coli*. In contrast, *I. cimicoides* and *N. glauca* venom showed weak cytotoxicity and *C. punctata* venom was non-toxic (**manuscript I**). The cytotoxicity of animal venoms often reflects the presence of membrane pore-forming compounds (Amino et al., 2002; Matsuzaki et al., 1997). We identified several redulysins – homologs of the pore-forming trialysin – in *P. horrida* and *P. biguttatus* PMG venom, which were strongly expressed in a tissue-specific manner. Using assay-guided venom fractionation and peptide synthesis, we found that redulysins are antimicrobial agents in *P. horrida* venom (**manuscripts II and III**) suggesting a role in prey sterilization to prevent the ingestion of microbial pathogens. Redulysins/trialysins are very common in Reduviidae and other predatory and herbivorous true bugs (Fu et al., 2021; Walker et al., 2017; Walker et al., 2019; Yoon et al., 2022), but are not present in nepomorphan venoms (Walker et al., 2018a; Yoon et al., 2022) (**manuscript I**). This suggests that they rely on other compounds and/or mechanisms to quickly kill prey, liquefy animal tissue and defend against predators or pathogens. Whereas *N. cinerea* seems to secrete lytic proteins other than redulysins, *N. glauca* and *I. cimicoides* venoms featured no major lytic activity (**manuscript I**), indicating that they may rely on other modes of action or less potent lytic peptides. Moreover, *N. glauca* and *I. cimicoides* are active predators that can probably escape from their own predators quickly (Brooks et al., 2009; Peták et al., 2014), whereas *N. cinerea* is a slow-moving ambush predator that is probably more susceptible to predation (Esenbekova et al., 2015; Peták et al., 2014; Pineda et al., 2014). We therefore hypothesize that the strong cytotoxic activity of *N. cinerea* PMG venom is a species-dependent adaptation that confers predator deterrence in a microhabitat with a high risk of predator exposure. Furthermore, the complete absence of cytotoxic activity in *C. punctata* venom (**manuscript I**) is further evidence that this species is not predatory but rather feeds on plants and/or detritus.

Many predatory true bugs can overwhelm prey animals that are many times larger than themselves (Edwards, 1961; Ohba, 2019; Ohba et al., 2008). Mechanisms that may facilitate this remarkable ability include barbed raptorial forelegs (Gorb, 1995; Ohba, 2019; Ohba et al., 2008) and neurotoxic venom components that quickly paralyze prey (Corzo et al., 2001; Wait et al., 2020; Walker et al., 2017). We found that *G. mellonella* larvae injected with PMG extracts of the reduviids *P. horrida* and *P. biguttatus* were rapidly paralyzed (PD_{50} 37.0 µg/g and 23.4 µg/g, respectively), indicating the presence of

potent neurotoxins (**manuscript II**). We identified several putative paralytic compounds in *P. horrida* and *P. biguttatus* venoms, including CUB domain proteins, cystatins and Ptu1-like peptides (**manuscript II**). Although the paralytic activity of Ptu1 isolated from *P. turpis* has not been confirmed *in vivo*, it can reversibly block calcium channels in cell culture, confirming that it acts as a neurotoxin (Corzo et al., 2001). Water bugs can also paralyze their prey, in some cases even large vertebrates such as frogs, snakes and turtles (Ohba & Nakasuji, 2006; Ohba, 2011, 2019). We identified Ptu1-like peptides only in *I. cimicoides* venom and predict that it contributes to prey overwhelming in this species (**manuscript I**). Whereas *N. cinerea* probably relies on its specialized raptorial forelegs to subdue large prey, *N. glauca* does not have this morphological adaptation but can nevertheless quickly paralyze prey animals (Giller, 1980; Gittelman, 1974). We assume that peptides unrelated to Ptu1 are responsible for prey paralysis in this species (**manuscript I**). However, further research is needed to determine whether uncharacterized protein families or small non-peptide molecules help nepomorphans like *N. glauca* to overwhelm their prey.

Most predatory Heteroptera feed on arthropods, but some nepomorphan species, particularly those in the family Belostomatidae, also prey on vertebrates (Ohba & Nakasuji, 2006; Ohba, 2011, 2019). For example, *I. cimicoides* and *N. glauca* can feed on tadpoles and small fish (Gamboa et al., 2012; González & Leal, 1995; Le Louarn & Cloarec, 1997; Ramos & Van Buskirk, 2012). The overwhelming and EOD of vertebrate prey requires specific adaptations due to differences in the nervous system, circulatory system and tissue composition compared to invertebrates (Arenas Gomez et al., 2020; Cobb & Pentreath, 1978; Monahan-Earley et al., 2013; Smarandache-Wellmann, 2016). We identified a protein homologous to 5' nucleotidase 1 from the venom of the giant waterbug *L. distinctifemur* in *I. cimicoides* and *N. glauca* venom, but not in the other nepomorphan or cimicomorphan species we analyzed (**manuscript I**). This enzyme is an apyrase commonly found in the venoms of animals that feed on vertebrates and it probably inhibits ATP/ADP-induced platelet aggregation (Dhananjaya et al., 2006; Hart et al., 2008; Walker et al., 2018a). The presence of 5'-nucleotidase 1 only in *I. cimicoides* and *N. glauca* venom suggests that they have recruited this enzyme into their venom to expand their diet to include opportunistic feeding on vertebrates, similar to the giant water bugs.

Our results show that the ecological niche, especially habitat, microhabitat and

feeding style, strongly determines the saliva/venom composition in terrestrial and aquatic Heteroptera. The two assassin bug species *P. horrida* and *P. biguttatus* do not differ much in terms of venom composition and activity, reflecting their close phylogenetic relationship and similar ecological niche (**manuscript II**). In contrast, we identified more specific adaptations among the nepomorphan species. The strictly predatory species *I. cimicoides*, *N. glauca* and *N. cinerea* produced mixtures of digestive enzymes similar to those of the cimicomorphan predators, but they also secreted several carbohydrases representing different GH families that were absent in *P. horrida* and *P. biguttatus* (**manuscripts I and II**). Our results also suggest that they have evolved different strategies to overwhelm and kill their prey, exemplified by the differences in venom cytotoxicity. *Corixa punctata* secreted mostly herbivory-associated enzymes and lacked any cytotoxic components, suggesting a mostly plant-based diet (**manuscript I**). Future research should include a broader range of heteropteran species from different ecological niches and families in order to define the general ecological characteristics of species based on their salivary/venom protein composition and activity.

4.2. Context-dependent venom deployment in Heteroptera

The complex venom systems of true bugs usually consist of three distinct venom chambers (AMG, PMG and AG) suggesting the functional compartmentalization of different venom mixtures (Figure 3B). The assassin bug *P. plagipennis* was the first insect shown to use distinct venom cocktails originating from different gland parts: PMG for predation and AMG for defense. Whereas defensive venom is released following mild harassment with tweezers, predation-associated venom was initially harvested following the unnatural process of electrostimulation (Walker et al., 2018b). We developed a method that allows the collection of predation-associated venom using a more natural stimulus, involving a prey dummy (**manuscript II**). This mimics a natural attack scenario and allowed the collection of clean predation venom from all species except *C. punctata* (**manuscripts I and II**). All the cimicomorphan and nepomorphan species we analyzed secreted distinct protein mixtures from the AMG and PMG. Although some proteins were produced in both glands, others were gland-specific, such as hemolysins and redulysins in the AMG and PMG, respectively (**manuscripts I and II**). This is consistent with other

studies of heteropteran venoms (Walker et al., 2018a; Walker et al., 2018b; Walker et al., 2019). Despite differences between the glands, context-dependent venom deployment was only observed for the assassin bug *P. horrida*. A prey-dummy stimulus or mild harassment without fixation triggered the secretion of PMG venom, whereas strong harassment with fixation and squeezing led to the secretion of AMG venom (**manuscript II**). We hypothesize that the secretion of AMG venom by *P. horrida* is associated with a predator attack and may have strong deterrent effects on vertebrates. In contrast, *P. biguttatus*, *I. cimicoides*, and *N. glauca* used exclusively PMG venom for predation and defense (**manuscripts I and II**). Although the gland morphology of all species would allow for stimulus-dependent venom use, this trait seems to be restricted to certain species. This is particularly intriguing given the close phylogenetic relationship between the assassin bugs *P. horrida* and *P. biguttatus*. They share a very similar ecological niche, anatomy, and venom activity, with only the PMG venom having strong cytolytic effects, similar to other reduviid species (**manuscript II**). It remains unclear why context-dependent venom deployment is not ubiquitous in predatory true bugs, despite the presence of morphological prerequisites. Moreover, it calls into question the function of the AMG in heteropterans without an obvious context-dependent venom deployment, given the substantial differences in protein composition between the AMG and PMG extracts (**manuscripts I and II**). Perhaps AMG venom is released in response to stimuli that were not represented by our tests, such as a last reserve when encountering large (vertebrate) prey to minimize the use of metabolically intensive – and therefore costly – venom. A comparable strategy has been described for scorpions, which inject a simplistic pre-venom first, but switch to the peptide-rich main venom in high-threat situations (Inceoglu et al., 2003). Furthermore, AMG venom may be injected at a later stage of predation, which was not included in our analysis, to maximize the extraction of nutrients from difficult-to-access tissues. More extensive studies focusing on the biological activity of AMG venom, the regulation of context-dependent venom use and its distribution within true bugs are needed to clarify the function and ecology of venom use in Heteroptera. In particular, AMG-specific proteins such as hemolysins should be functionally characterized to determine their activity and role in envenomation.

4.3. Evolution of a versatile cytotoxin repertoire in predatory Reduviidae

Predatory assassin bugs are known for their painful stings that can cause severe, long-lasting symptoms (Haddad Jr et al., 2010; Hartwig, 1977). The strong cytotoxicity of the venom and its multiple ecological functions (prey paralysis and killing, EOD and defense) suggest that reduviid venoms contain diverse toxins that act on multiple targets. Peptide toxins are usually recruited from non-venom proteins, either through the co-option of single-copy genes or via gene duplication and neofunctionalization (Casewell, 2012; Drukewitz et al., 2019; Fry, 2005; Martinson et al., 2017; Walker, 2020). The increases gene diversification by releasing pleiotropic constraints (Walker, 2020). Therefore, many venom protein families are highly diversified, with individual members possessing different functions (Kordiš & Gubenšek, 2000).

The most diverse protein families in the *P. horrida* and *P. biguttatus* PMG transcriptomes are the peptidase S1A chymotrypsin family, redulysins, and the uncharacterized heteropteran venom protein family 2 (**manuscript II**). The pore-forming redulysins may be the main cytotoxic agents in reduviid venom (Rügen et al., 2021; Walker et al., 2017), and we confirmed the strong antimicrobial activity of synthetic peptides based on the lytic region of *P. horrida* redulysins (**manuscript III**). We screened synthetic peptides representing 10 putative redulysin genes identified in the *P. horrida* genome containing a conserved motif homologous to the cleavage site (Asp-Glu-Glu-Arg [DEER]) in *T. infestans* trialysin (Amino et al., 2002; Martins et al., 2008) (**manuscript III**). Six of these peptides inhibited the growth of both *E. coli* and *Bacillus subtilis*, two inhibited *B. subtilis* alone and one inhibited *E. coli* alone (**manuscript III**). This suggests that the diversification of redulysins in *P. horrida* led to a shift in target specificity, enabling them to combat different microbial species, including both Gram-positive and Gram-negative bacteria. A similar functional divergence was shown for *D. melanogaster* peptides from the multigene drosomycin family that are act on different fungal strains (Yang et al., 2006). One limitation of our study is that the synthetic peptides only represent the lytic region of redulysins, whose activity spectra may differ from the mature full-length peptides. Several studies have reported lower activity and target-shifts in synthetic toxins compared to native peptides (Amino et al., 2002; Krämer et al., 2022). Therefore, it is possible that the mature full-length redulysins may not only possess antimicrobial activity but also act

on other cell types, although we found no or only weak activity towards insect and red blood cells (**manuscript III**).

Domain duplications, resulting in domain repeats within one gene, can also play an important role in venom evolution. For example, the banded Gila monster *Heloderma suspectum cinctum* Bogert & Martín Del Campo encodes several toxins – one natriuretic peptide and four helokinestatins – from a single mRNA transcript, which evolved from a monodomain gene that underwent several domain duplications. The individual toxins are released by post-translational cleavage (Fry et al., 2010). We found two *P. horrida* redulysin genes (g1037.t1 and g1038.t1) containing four and six putative duplicated lytic domains, respectively, each characterized by a potential cleavage site homologous to the DEER motif followed by a lysine-rich region (**manuscript III**). The putative duplicated regions from g1038.t1 (Contig Phor_Comb_C9529 in the transcriptome) were detected by LC-MS/MS only in protein bands with a molecular mass less than 15 kDa, suggesting the native peptide is post-translationally cleaved into multiple peptides (**manuscripts II and III**). Therefore, we assessed the cytotoxic activity of seven additional synthetic peptides representing the putative duplicated lytic regions from g1037.t1 and g1038.t1 but none of them showed toxicity against bacterial, insect or mammalian cells (**manuscript III**). Further studies are needed to determine whether redulysins g1037.t1 and g1038.t1 are multidomain, multiproduct proteins that are cleaved into several active peptides and to characterize their activity spectra. They may be toxic towards cell types not tested in this study such as fungal pathogens or protozoan parasites. Activity against the latter would be ecologically and medically important because many heteropteran species carry parasites such as trypanosomes that cause diseases in plants and animals, including Chagas disease in humans (Dollet, 1984; Mitchell, 2004).

Venom protein family 2 is an uncharacterized protein family that is highly abundant, diverse and strongly expressed in predatory Heteroptera (Walker et al., 2018a; Walker et al., 2018b; Walker et al., 2019; Yoon et al., 2022) (**manuscripts I and II**). We identified 10, 15, 9, 18 and 12 contigs representing venom protein family 2 in the PMG transcriptomes and proteomes of *P. biguttatus*, *P. horrida*, *I. cimiciodes*, *N. glauca* and *N. cinerea*, respectively. This made it the most diverse uncharacterized venom protein family among the predatory species, but was completely absent from *C. punctata* venom (**manuscripts I and II**). Based on the PMG-specific expression pattern in predacious bugs

and the presence of venom protein family 2 in the cytotoxic fractions of *P. horrida* venom, we hypothesized that these proteins may contribute to the cytotoxic venom activity of true bugs (**manuscripts I, II and III**). To test this hypothesis, we attempted to express several candidates of venom protein family 2 from *P. horrida*. The production of recombinant toxins can pose major challenges, such as their inability to fold properly and maintain native activity and their toxicity towards host cells in cell-based systems (Rivera-de-Torre et al., 2022). We tried several cell-free as well as bacterial and insect cell-based expression systems, but none resulted in significant amounts of soluble, active protein. We encountered problems such as low protein expression, cell death after the induction of expression, low protein solubility after purification, and the inactivity of purified proteins (**manuscript III**). The low expression levels and cell death indicate that the proteins are indeed toxic. We therefore commissioned an external provider to screen insect, yeast and mammalian cell-based expression systems, which resulted in the successful expression of one *P. horrida* venom protein family 2 protein (Phor_Comb_C31143, hereafter Vpf2) in Chinese hamster ovary (CHO) cells. The purified protein significantly reduced insect cell viability but had no significant effects on *E. coli* growth or erythrocyte integrity (**manuscript III**). This indicates that Vpf2 contributes to the strong insecticidal effects of *P. horrida* venom and its role in overwhelming, killing and liquefying animal prey. Although no additional recombinant proteins were tested in this thesis, we assume that other members from venom protein family 2 also contribute to the toxicity of *P. horrida* venom. In contrast to redulysins, venom protein family 2 is found not only in reduviid venoms but also in nepomorphan venoms (Walker et al., 2018a; Walker et al., 2017; Walker et al., 2019) (**manuscript I**), suggesting that it was recruited prior to the divergence of the Panheteroptera approximately 258 million years ago (Wang et al., 2016). Given this evolutionary age and the remarkable diversity, we hypothesize that different family 2 proteins may have evolved distinct functions and activity spectra to attack a range of targets. Therefore, future studies should characterize the different venom protein family 2 proteins to determine their activity, mode of action and distribution among Heteroptera.

4.4. Conclusion and future perspectives

This work provides new insights into the ecological function, composition and deployment of venom in predatory Heteroptera. We found strong evidence that the ecological niche, particularly habitat, microhabitat, feeding style and foraging strategy, influence venom/saliva composition and activity in the Reduviidae and Nepomorpha (**manuscripts I and II**). We cannot conclude there is a direct causality between venom profiles and ecological niches, but we propose that the compositional analysis of saliva/venom in true bugs can be used as a basis for the ecological assessment of a given species, although assumptions should be supported by ecological studies and activity assays. Detailed analysis of the main gland compartments revealed that all studied species secrete distinct protein mixtures in the AMG and PMG, although only *P. horrida* uses both venom types in a context-dependent manner (**manuscripts I and II**). We analyzed the PMG/prey-killing venom from *P. horrida* in more detail and identified cytotoxic redulysins and members of the uncharacterized venom protein family 2 (**manuscript III**). This clearly shows that the complex cytotoxic effects of the venom are not due to a single protein (family), but that *P. horrida* secretes multiple toxic proteins to attack different targets. Altogether, our results indicate that adaptive evolution plays a fundamental role in the composition of true bug venoms, especially in multigene families that have evolved diverse functions and activities.

The conclusions drawn from this thesis raise new questions and perspectives for future studies because many aspects of venom evolution in Heteroptera remain unclear. Expanding the range of organisms to include true bug species from different infraorders, families, feeding styles and habitats would help to confirm patterns in venom/saliva protein composition associated with specific ecological niches. Neglected infraorders such as Dipsocoromorpha, Enicocephalomorpha and Leptopodomorpha as well as species with mixed feeding styles or high levels of specialization should be prioritized to fully understand the adaptive evolution of venoms. Moreover, research should focus on proteins from diverse multigene families that are present in the salivary secretions of both predatory and phytophagous species to elucidate their activity, biological role and contribution to niche shifts.

We identified an insecticidal protein from the uncharacterized venom protein

family 2 in *P. horrida*, but the activity and function of other family 2 proteins remains unclear. Therefore, activity assays with different family 2 proteins from *P. horrida* and other predatory species are needed to determine the function of this gene family in true bug envenomations. Similarly, the ecological function and mode of action of different redulysins in their mature full-length form and the role of putative multidomain multiproduct proteins within this gene family warrants further investigation.

True bugs have been largely neglected in drug discovery, but our results show that their venoms have complex toxic effects on a variety of targets that may be of interest for use as insecticides or therapeutics. Most importantly, candidates for drug development must be potent, stable, target-specific and suitable for mass production (King, 2013; Lewis & Garcia, 2003). The synthetic redulysin peptides tested in this thesis are interesting candidates for antimicrobial therapeutics because they were highly toxic to bacteria but had little effect against the other cell types tested (**manuscript III**). Although some peptides were specifically active against either *E. coli* or *B. subtilis*, their target specificity requires more research covering a broad spectrum of different microbes and cell types. For *T. infestans* trialysin, the potency and specificity of synthetic peptides representing different portions of the lytic region can vary strongly depending on the structural features of the N- and C-termini (Martins et al., 2006). Therefore, future research should cover different structural variants of synthetic *P. horrida* redulysin peptides obtained by targeted amino acid deletions or substitutions to improve potency and target specificity. Similarly, further research is needed on the insecticidal activity of venom protein family 2 proteins and their potential for bioinsecticide development. Altogether, our study makes an important contribution to advances in insect venomics and sheds new light on venom evolution and ecology in Heteroptera.

5. SUMMARY

The true bugs (Heteroptera) are a hyperdiverse suborder of phytophagous, mycopahous, hematopagous and zoophagous insects that inhabit a wide range of habitats around the world. With their piercing-sucking mouthparts, they inject protein-rich saliva into their food, pre-digest it, and take up the liquefied components. Predatory Heteroptera have evolved venomous saliva, which enables them to overwhelm, kill and pre-digest animal prey, in some cases many times larger than themselves. In addition, most predacious bugs can inflict painful bites to deter predators. However, the protein composition and activity of most true bug venoms is unknown and their role in the adaptation of true bugs to new ecological niches has not been studied in detail.

In this thesis, I analyzed and compared the venom/salivary protein composition in two closely related African reduviid bugs (*Psytalla horrida* and *Platymeris biguttatus*) and four nepomorphan bugs (*Ilyocoris cimicoides*, *Notonecta glauca*, *Nepa cinerea* and *Corixa punctata*) occupying different ecological niches. The main objective was to identify species-dependent differences in venom composition, activity and deployment that can be linked to ecology. Moreover, I analyzed the venom of *P. horrida* in detail to identify and characterize the major cytotoxic venom components.

By combining proteotranscriptomic analysis and bioassays, we found interspecific differences in venom composition and activity that probably reflect adaptions to the microhabitat, feeding style and foraging strategy of different species (Figure 5). Whereas the strictly predatory species are well adapted to the extra-oral digestion of animal tissues, secreting mainly S1 family peptidases and lipases, the presumably omnivorous *C. punctata* secretes a wide range of carbohydrases and C1 family peptidases, suggesting a mainly plant-based diet. We identified amylases in predatory and omnivorous species and found evidence of dual activity on starch and glycogen, suggesting that amylases play a role in the digestion of both plant and animal carbohydrates. In addition, we found multiple species-dependent proteins probably associated with opportunistic vertebrate feeding, predator deterrence and prey overwhelming.

All species secreted distinct protein mixtures in the two main gland compartments, the anterior and posterior main gland (AMG and PMG, respectively). However, only *P. horrida* deployed AMG and PMG venom in a context-dependent manner, using PMG

venom for prey killing and AMG venom for defense. Accordingly, only PMG venom had significant digestive, antimicrobial, hemolytic and insecticidal activity and was analyzed further to identify the compounds responsible for the strong cytotoxic effects. The activity was restricted to two fractions obtained by cation-exchange chromatography. These were analyzed by liquid chromatography tandem mass spectrometry, revealing that they mainly contained gelsolin, S1 family peptidases, redulysins and proteins from the uncharacterized heteropteran venom protein family 2. Synthetic redulysin peptides significantly reduced microbial growth and a recombinant venom protein family 2 protein showed insecticidal activity, indicating that these protein families play a key role in antimicrobial defense, prey overwhelming, prey killing and/or liquefaction.

This thesis is an important contribution to our knowledge concerning the evolution and ecology of venom in predatory Heteroptera. It provides evidence that the ecological niche strongly influences venom composition, activity and deployment (Figure 5), suggesting that venomous Heteroptera are valuable models to study adaptive evolution. In addition, the identification and characterization of cytotoxic components in *P. horrida* venom paves the way to study heteropteran venom as a potential source of novel therapeutics, bioinsecticides and biotechnological tools.

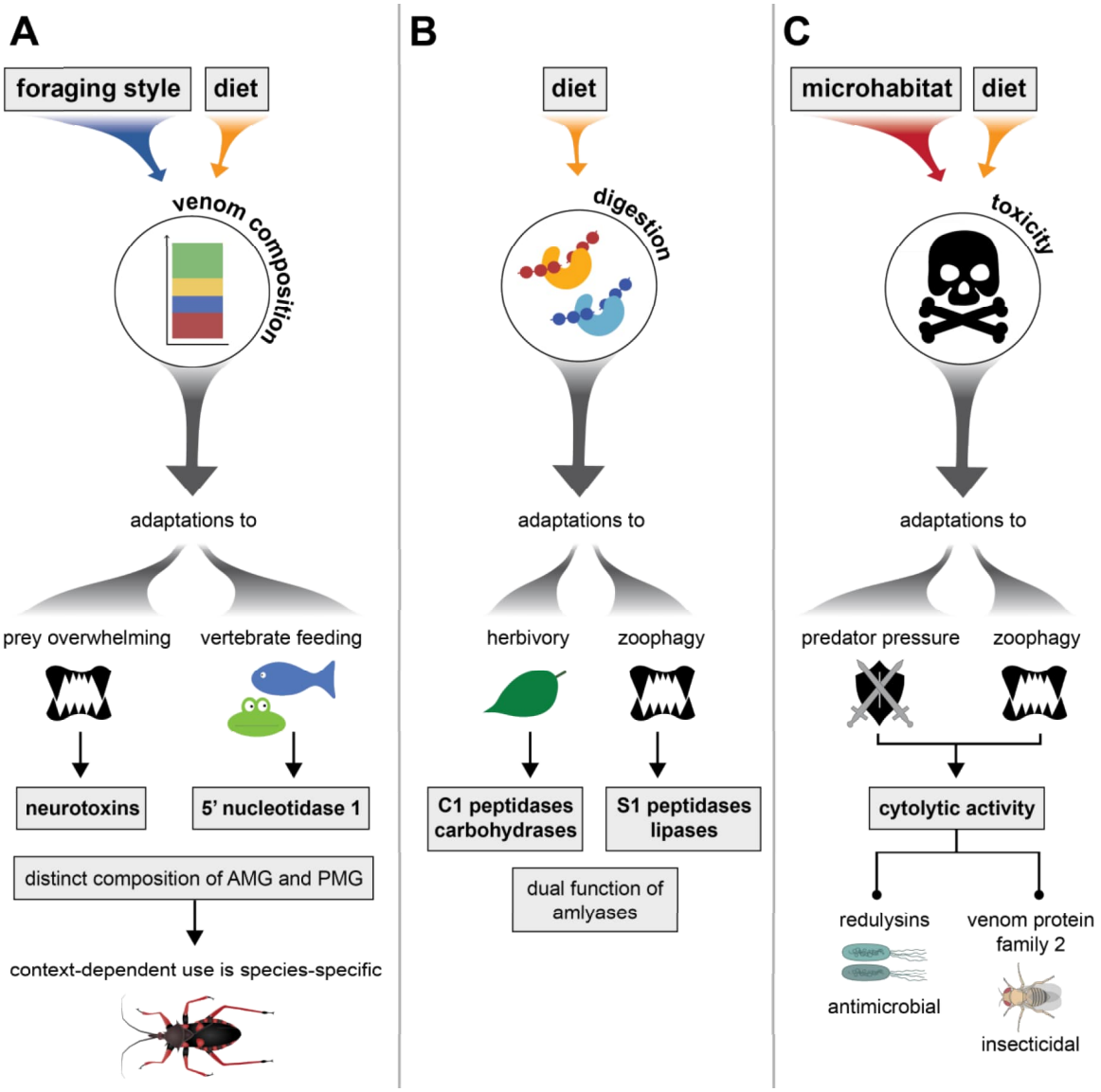

Figure 5: Graphical summary of the results described in this thesis. The ecological niche (including microhabitat, foraging strategy and diet) influences venom composition and activity in heteropterans. (A) We found considerable interspecific differences in venom composition that probably improve prey overwhelming and/or facilitate feeding on vertebrates. Although all the species we studied secrete different protein mixtures in the AMG and PMG, only *P. horrida* deploys both venom types in a context-dependent manner. **(B)** The repertoire of digestive salivary enzymes differs considerably between omnivorous/herbivorous and predatory species and is well adapted to each diet. Amylases probably play a role in herbivory and zoophagy due to their dual ability to break down both starch and glycogen. **(C)** The venom of predatory species has strong cytotoxic effects and contributes to predator deterrence as well as prey overwhelming, killing and liquefaction. The major cytotoxic protein families in the venom of *P. horrida* are redulysins and venom protein family 2.

6. ZUSAMMENFASSUNG

Wanzen (Heteroptera) sind eine sehr diverse Unterordnung phytophager, mykophager, hämatophager und zoophager Insekten, die eine Vielzahl unterschiedlicher Lebensräume auf der ganzen Welt nutzen. Mit ihren stechend-saugenden Mundwerkzeugen injizieren sie proteinreichen Speichel in ihre Nahrung, verdauen sie vor und nehmen die verflüssigten Bestandteile auf. Räuberische Wanzen haben giftigen Speichel entwickelt, mit dem sie teils vielfach größere Beutetiere überwältigen, töten und vorverdauen können. Darüber hinaus können die meisten räuberischen Wanzen schmerzhaft stechen um Fressfeinde abzuschrecken. Die Proteinzusammensetzung und Aktivität der meisten Wanzengifte sowie ihre Rolle bei der Anpassung an neue ökologische Nischen sind jedoch noch nicht bekannt.

In dieser Arbeit analysierte und verglich ich die Zusammensetzung von Gift- bzw. Speichelproteinen zweier eng verwandter afrikanischer Wanzenarten (*Psytalla horrida* und *Platymeris biguttatus*) der Familie Reduviidae und vier nepomorpher Wanzenarten (*Ilyocoris cimicoides*, *Notonecta glauca*, *Nepa cinerea* und *Corixa punctata*), die unterschiedliche ökologische Nischen besetzen. Das Hauptziel bestand darin, artspezifische Unterschiede in der Zusammensetzung, Aktivität und Nutzung der Gifte zu ermitteln, die mit der jeweiligen Ökologie der Arten in Verbindung gebracht werden können. Darüber hinaus habe ich das Gift von *P. horrida* weiter analysiert, um die wichtigsten zytotoxischen Giftkomponenten zu identifizieren und zu charakterisieren.

Mithilfe eines integrierten proteotranskriptomischen und Bioassay-basierten Ansatzes fanden wir interspezifische Unterschiede in der Zusammensetzung und Aktivität der Gifte, die wahrscheinlich Anpassungen an das Mikrohabitat, den Ernährungsstil und die Futtersuchstrategie der Arten widerspiegeln (Figure 5). Während die rein räuberisch lebenden Arten offenbar gut an die extraorale Verdauung von tierischem Gewebe angepasst sind und hauptsächlich Peptidasen der S1-Familie und Lipasen absondern, produziert die vermutlich allesfressende Art *C. punctata* eine breite Palette verschiedener Kohlenhydrasen und Peptidasen der C1-Familie, was auf eine hauptsächlich pflanzliche Ernährung schließen lässt. Wir identifizierten Amylasen sowohl bei räuberischen als auch bei allesfressenden Wasserwanzenarten und fanden Hinweise auf eine duale Aktivität gegenüber Stärke und Glykogen, was darauf hindeutet, dass Amylasen eine Rolle bei der

Verdauung sowohl pflanzlicher als auch tierischer Kohlenhydrate spielen. Zudem fanden wir mehrere artspezifische Proteine, die wahrscheinlich mit der opportunistischen Ernährung von Wirbeltieren, der Abwehr von Räubern und der Überwältigung von Beutetieren zusammenhängen.

Alle Arten sonderten in den beiden Hauptdrüsenkompartimenten, der vorderen und hinteren Hauptdrüse (AMG bzw. PMG), unterschiedliche Proteinmischungen ab. Allerdings setzte nur *P. horrida* AMG- und PMG-Gift kontextabhängig ein, wobei PMG zur Tötung der Beute und AMG zur Verteidigung genutzt wurde. Dementsprechend hatte nur das PMG-Gift eine signifikante Verdauungs-, antimikrobielle, hämolytische und insektizide Wirkung und wurde weiter analysiert, um die Verbindungen zu identifizieren, die für die starke zytotoxische Aktivität verantwortlich sind. Die toxischen Wirkungen beschränkten sich auf zwei Fraktionen, die durch Kationenaustauschchromatographie gewonnen wurden. Diese wurden mithilfe von Flüssigchromatographie-Tandem-Massen-spektrometrie analysiert, wobei sich herausstellte, dass sie hauptsächlich Gelsolin, Peptidasen der S1-Familie, Redulysine und Proteine der nicht charakterisierten Heteropteren-Giftproteinfamilie 2 enthielten. Synthetische Redulysin-Peptide und ein rekombinantes Protein der Giftproteinfamilie 2 verringerten das mikrobielle Wachstum bzw. die Lebensfähigkeit von Insektenzellen signifikant, was darauf hindeutet, dass diese Proteinfamilien eine entscheidende Rolle bei der antimikrobiellen Abwehr und der Überwältigung, Tötung und/oder Verflüssigung der Beute spielen.

Diese Arbeit trägt wesentlich zum Wissen über die Evolution und Ökologie von Giften in räuberischen Wanzen bei. Sie liefert Beweise, dass die ökologische Nische die Zusammensetzung, Aktivität und den Einsatz der Gifte stark beeinflusst (Figure 5), was darauf hindeutet, dass giftige Wanzen optimale Modelle zur Untersuchung der adaptiven Evolution darstellen. Darüber hinaus ebnet die Identifizierung und Charakterisierung zytotoxischer Verbindungen in *P. horrida* Gift den Weg zur Untersuchung des Potenzials von Wanzengiften als Therapeutika, Bioinsektizide oder biotechnologische Werkzeuge.

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8. ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor **Dr. Heiko Vogel** for giving me the opportunity to work on this topic and for his excellent supervision and guidance. Thank you for introducing me to the world of venomics and teaching me a lot about transcriptomics, proteomics and much more. Your door was always open for me and you supported all my ideas with patience, motivation and good advice. I would also like to thank **Prof. David Heckel** for his mentoring, support and feedback and for creating a great research environment in the former Department of Entomology. I would also like to thank **Prof. Andreas Vilcinskas** for making this project possible and for his constant support, valuable advice, and expertise in animal venomics. In addition, I would like to thank my FSU supervisor **Prof. Nicole van Dam** for her support and feedback in the PhD committee meeting.

I would also like to thank **Prof. Martin Kaltenpoth** for accepting me into the Department of Insect Symbiosis. Thank you for creating such a warm and welcoming atmosphere and for your support.

I would like to thank all current and former members of the Department of Entomology and the Department of Insect Symbiosis for the great working environment and the incredible help I have received from you. I have always felt welcome here, from the very beginning.

Thank you, **Jette**, for supporting me in the lab, for always helping me when I had difficulties and for answering many (stupid) questions. I really appreciate your helpfulness and expertise in the lab.

Thank you, **Domenica**, for sequencing many samples for me, for your valuable help and advice in the lab, and for providing me with tea in the office.

Thank you, **Bianca**, for always providing me with fresh cells and for your advice and support in cloning, expression and FPLC. Also, I would like to thank **Yannick** and **Roy** for the many things they taught me about proteins and for helping me a lot when I was struggling with the fractionation and protein expression.

I would like to thank **Natalie** and **Steffi** for their help with the proteomic analysis and interpretation of my data.

Thank you, **Benjamin**, for dissecting countless flies for me (which I could never have done

myself) and for doing the calcium imaging experiments with me.

I would like to thank my master's student and Hiwi **Sol** for her help in the lab, her creativity and her enthusiasm for insects.

I would also like to thank **Ana C.**, **Bine**, **Camila**, **Domenica**, **Jette**, and **Nomthi** for being the best office mates I can imagine.

Thank you, **Pauline**, **Theresa**, **Wiebke**, **Ana B.** and **Ana C.** for always having an open ear for me, cheering me up, telling bad jokes and for all the great bouldering sessions that helped me a lot after a stressful day.

I would like to thank my **family** for their constant support, for fostering my curiosity and encouraging me to explore. Without your help and support, I would not be here.

Last but not least, I would like to thank my partner **Jakob** for moving to Jena with me, believing in me and supporting me in every possible way. Thank you for staying up with me during long experiments and cheering me up after a bad day.

10. EHRENWÖRTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass mir die geltende Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt ist. Ich bestätige entsprechend § 5 Abs. 4 der Promotionsordnung, dass ich die vorliegende Dissertation mit dem Titel „Drugs from bugs: Venomics of predatory Heterotpera“ selbst angefertigt habe und keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe. Zudem habe ich alle benutzten Hilfsmittel und Quellen in der Arbeit angegeben. Personen, die mich bei der Auswahl und Auswertung des Materials und bei der Herstellung der Manuskripte unterstützt haben, sind in der Auflistung der Manuskripte (Kapitel 2, *Manuscript Overview*) genannt oder werden, im Falle von Beiträgen geringeren Ausmaßes, in der Danksagung genannt. Ich habe keine Hilfe einer kommerziellen Promotionsvermittlung oder –beratung in Anspruch genommen. Außerdem wurden im Zusammenhang mit dem Inhalt der vorliegenden Dissertation keine unmittelbar oder mittelbar geldwerte Leistungen an Dritte weitergegeben. Ich habe die Dissertation nicht bereits als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Zudem bestätige ich, dass keine gleiche, in wesentlichen Teilen ähnliche oder andere Abhandlung bei einer anderen Hochschule oder anderen Fakultät eingereicht wurde.

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Ort, Datum Unterschrift

11. AUTHOR CONTRIBUTIONS

11.1. Manuscript I

Kurzreferenz Fischer et al. (accepted), *Proceedings of the Royal Society B: Biological Sciences*

Beitrag des Doktoranden / der Doktorandin

Abbildungen 2, 3	☐	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	X	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: 90% Kurzbeschreibung des Beitrages: *Conduction and analysis of* in-vitro *assays and electrophoretic separation of proteins; pre-experiments were performed by SYV*

Abbildungen 4, 5, S2	☐	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
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	X	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: 70% Kurzbeschreibung des Beitrages: *Analysis of transcriptomes (assembled and annotated by HV); analysis of LC-MS/MS outputs (LC-MS/MS measurements done by NW)*

_____ _____

Unterschrift Kandidat/-in

Unterschrift Betreuer/-in
(Mitglied der Fakultät)

11.2. Manuscript II

Kurzreferenz Fischer et al. (2020), *Ecology and Evolution*

Beitrag des Doktoranden / der Doktorandin

Abbildungen 2, 3, 4	**X**	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	☐	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: _____% Kurzbeschreibung des Beitrages: *(z. B. „Abbildungsteile a, d und f“ oder „Auswertung der Daten“ etc)*

Abbildungen 5, 6, 7, 8, S1, S2	☐	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	X	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: 70% Kurzbeschreibung des Beitrages: *Analysis of transcriptomes (assembled and annotated by HV); analysis of LC-MS/MS outputs (LC-MS/MS measurements done by NW)*

_____ _____

Unterschrift Kandidat/-in

Unterschrift Betreuer/-in
(Mitglied der Fakultät)

11.3. Manuscript III

Kurzreferenz Fischer et al. (in preparation), in Vorbereitung zur Einreichung bei *Toxins*

Beitrag des Doktoranden / der Doktorandin

Abbildungen 1,2	**X**	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	☐	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: _____% Kurzbeschreibung des Beitrages: *(z. B. „Abbildungsteile a, d und f" oder „Auswertung der Daten" etc)*

Abbildung 3, S3	☐	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	X	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: 90% Kurzbeschreibung des Beitrages: *Calcium imaging with flies (dissected by BF); analysis of fluorescence intensity*

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(Mitglied der Fakultät)

Abbildungen 4, S2	☐	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	X	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: 70% Kurzbeschreibung des Beitrages: *Analysis of transcriptomes (assembled and annotated by HV); analysis of LC-MS/MS outputs (LC-MS/MS measurements done by NW)*

Abbildung 5, 6	☐	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	X	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: 90% Kurzbeschreibung des Beitrages: *Conduction and analysis of* in-vitro *assays; conduction and analysis of calcium imaging assays (flies dissected by BF)*

Abbildungen S1, S4	☐	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	☐	0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	X	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: 50% Kurzbeschreibung des Beitrages: *Identification of genes in the transcriptome and genome (assembled and annotated by HV)*

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Unterschrift Kandidat/-in

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Unterschrift Betreuer/-in
(Mitglied der Fakultät)

12. SUPPLEMENTARY DATA

12.1. Manuscript I

Supplemental Information for:

You are what you eat – ecological niche and microhabitat influence venom activity and composition in aquatic bugs

Maike L. Fischer, Sol A. Yepes Vivas, Natalie Wielsch, Roy Kirsch, Andreas Vilcinskas, Heiko Vogel

Proceedings of the Royal Society B: Biological Sciences

Supplementary Methods

1. LC-MS/MS, proteomic data processing and protein identification

Dried tryptic peptides were dissolved in 1% formic acid in water. Liquid chromatography-tandem mass spectrometry was performed using an UPLC M-class system online coupled to a Synapt G2-si mass spectrometer (Waters). The experimental and data acquisition methods were previously described in detail (Fischer et al. 2020). Briefly, peptides were separated on an ACQUITY UPLC HSS T3 analytical column (100Å, 75 µm X 200, 1.8 µm particle size) at a flow rate of 350 nL/min. The gradient of mobile phase A (0.1% aqueous formic acid) and B (acetonitrile plus 0.1% formic acid) increased from 2-10% B over 5 min, 10-40% B over 40 min, 40-70% B over 7 min, 70-95% B over 3 min, isocratic at 95% B for 2 min and back to 1% B. The MS survey scans were acquired at a resolution of at least 20,000 over a mass range of m/z 400-1,800 Da. Data were recorded by data-dependent acquisition (DDA) and in each cycle the 10 most intense precursor ions were subjected to fragmentations in the range of m/z 50-2,000. Multiple fragmentations for the same precursor ions were minimised using dynamic exclusion. MS data were collected using MassLynx v4.1 software (Waters).The MS raw data were processed using the ProteinLynx Global Server (PLGS) version 2.5.2 (Waters, Manchester, UK). In order to exclude background proteins, the spectra were first searched against a subdatabase containing common contaminants (human keratins and trypsin). Spectra remained unmatched were interpreted *de novo* to yield peptide sequences and subjected for homology-based searching using MS BLAST program (Shevchenko et al. 2001) installed on a local server. MS BLAST searches were performed against the Arthropoda database (downloaded from NCBI on August 29, 2020). Additionally, we used individual subdatabases derived from in silico translation of the transcriptomes of *I. cimicoides*, *N. cinerea*, *N. glauca*, and *C. punctata*. The pkl files generated from the raw data were searched additionally with the NCBI nr database (downloaded August 29, 2020) in combination with the respective subdatabase using MASCOT v2.6.0. The following searching parameters were applied: fixed

precursor ion mass tolerance of 15 ppm for survey peptide, fragment ion mass tolerance of 0.1 Da, 1 missed cleavage, fixed carbamidomethylation of cysteines and possible oxidation of methionine.

PD and D samples from *N. glauca* were analysed in-solution. Reduction and alkylation of disulphide bridges in cysteine containing proteins was performed with dithiothreitol (56°C, 30 min, 10 mM in 50 mM HEPES, pH 8.5) and 2-chloroacetamide (room temperature, in the dark, 30 min, 20 mM in 50 mM HEPES, pH 8.5), respectively. Sequencing grade Trypsin (Promega) was added in a 1: 50 ratio in 50 mM ammonium bicarbonate for overnight digestion at 37°C. The digest was stopped by the addition of formic acid to 0.1% final concentration and desalted with an OASIS® HLB µElution Plate (Waters). Samples were dried under vacuum centrifugation and reconstituted in 10 µL 1% formic acid, 4% acetonitrile and then stored at -80 °C until LC-MS analysis. An UltiMate 3000 RSLC nano LC system (Dionex) fitted with a trapping cartridge (µ-Precolumn C18 PepMap 100, 5µm, 300 µm i.d. x 5 mm, 100 Å) and an analytical column (nanoEase™ M/Z HSS T3 column 75 µm x 250 mm C18, 1.8 µm, 100 Å, Waters) was coupled directly to a QExactive Plus (Thermo) mass spectrometer using the Nanospray Flex™ ion source in positive ion mode. Trapping was carried out with a constant flow of 0.05% trifluoroacetic acid at 30 µL/min onto the trapping column for 4 minutes. Subsequently, peptides were eluted via the analytical column with a constant flow of 0.3 µL/min with increasing percentage of solvent B (0.1% formic acid in acetonitrile) from 2% to 4% in 4 min, from 4% to 8% in 2 min, then 8% to 25% for a further 41 min, and finally from 25% to 40% in another 5 min and from 40% to 80% in 4 min. The peptides were introduced into the QExactive plus via a Pico-Tip Emitter 360 µm OD x 20 µm ID; 10 µm tip (MSWIL) and an applied spray voltage of 2.2 kV. The capillary temperature was set at 275°C. Full mass scans were acquired with mass range 350-1500 m/z in profile mode with resolution of 70000. The filling time was set at maximum of 100 ms with a limitation of 3x106 ions. Data dependent acquisition (DDA) was performed with the resolution of the Orbitrap set to 17500, with a fill time of 50 ms and a limitation of 1x105 ions. A normalised collision energy of 26 was applied. Loop count 20. Isolation window 1.7 m/z. Dynamic exclusion time of 20 s was used. The peptide match algorithm was set to ‘preferred’ and charge exclusion ‘unassigned’, charge states 1, 5 - 8 and above 8 were excluded. MS2 data was acquired in centroid mode. The raw mass spectrometry data was processed with MaxQuant (v1.6.17.0) (PMID: 19029910) and searched against the subdatabase derived from in silico translation of the *N. glauca* transcriptome containing the longest open reading frames. Common contaminants were included in each search. Decoy mode was set to revert. Carbamidomethyl (C) was set as fixed modification, acetylation of N-termini and oxidation of methionine were set as variable modifications. The mass error tolerance for the full scan MS spectra was set to 20 ppm and for the MS/MS spectra to 0.5 Da. A maximum of 2 missed cleavages was allowed. For protein identification, a minimum of 1 unique peptide with a peptide length of at least seven amino acids and a false discovery rate below 0.01 were required on the peptide and protein level.

2. RNA-Seq and *de novo* transcriptome assembly

Data obtained from RNA-Seq were further processed using CLC Genomics Workbench v20.1. Quality control included filtering of high-quality reads, removal of reads containing primer/adaptor sequences, and trimming of read lengths. For transcriptome assembly, RNA-Seq data from all tissue samples (AMG, PMG, gut and remaining body tissue) were combined for each species and *de novo* transcriptome assemblies were prepared using default settings and two additional CLC-based assemblies with different parameters. For each species, the presumed optimal consensus transcriptome was selected as described previously (Vogel et al., 2014) and annotated using BLAST, Gene Ontology and InterProScan in OmicsBox (https://www.biobam.com/omicsbox) as described by Götz et al. (2008). For BLASTx searches against the non-redundant NCBI protein database (nr database), up to 20 best NR hits per transcript were considered, using an E-value cutoff of ≤10-3 and a minimum match length of 15 amino acids. A Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis (http://busco.ezlab.org) was used to assess transcriptome completeness by comparing the assembled transcriptomes against a set of highly-conserved single-copy orthologs. For this, the BUSCO v5 pipeline (Waterhouse et al., 2017) was used to compare the predicted proteins of the transcriptomes to the predefined set of 1658 Insecta single-copy orthologs from the OrthoDB v9.1 database. For digital gene expression analysis, BAM files were generated and sequences were counted using CLC Genomics Workbench v20.1, with parameters for read mapping and normalization as previously described (Pöppel et al., 2015). To estimate gene expression levels, mapped read values were normalised as implemented in CLC Genomics Workbench v20.1 and ArrayStar, and the reads per kilobase per million mapped reads (RPKM) were calculated. Potential venom-associated contigs were selected based on their annotations, expression levels, presence in the respective proteome and the presence of a signal peptide for secretion (i.e., extracellular localization) using SignalP4.1 (D-cutoff value 0.34).

References

Fischer, M. L., Wielsch, N., Heckel, D. G., Vilcinskas, A., & Vogel, H. (2020). Context-dependent venom deployment and protein composition in two assassin bugs. *Ecology and evolution*, 10(18), 9932-9947. (doi:10.1002/ece3.6652)

Götz S, Garcia-Gomez JM, Terol J., Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talon M, Dopazo J, Conesa A. 2008. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic acids research*, 36(10), 3420-35. (doi:10.1093/nar/gkn176).

Pöppel A-K, Vogel H, Wiesner J, Vilcinskas A. 2015. Antimicrobial peptides expressed in medicinal maggots of the blow fly *Lucilia sericata* show combinatorial activity against bacteria. *Antimicrobial agents and chemotherapy* 59(5), 2508-2514. (doi:10.1128/AAC.05180-14).

Shevchenko A, Sunyaev S, Loboda A, Bork P, Ens W, Standing KG. 2001. Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. *Anal Chem*. 73(9):1917-26. (doi:10.1021/ac0013709).

Vogel H, Badapanda C, Knorr E, Vilcinskas A. 2014. RNA-sequencing analysis reveals abundant developmental stage-specific and immunityrelated genes in the pollen beetle *Meligethes aeneus*. *Insect Mol Biol* 23(1), 98-112. (doi:10.1111/imb.12067).

Waterhouse RM, Seppey M, Simão FA, Manni M, Ioannidis P, Klioutchnikov G, Kriventseva EV, Zdobnov EM. 2017. BUSCO applications from quality assessments to gene prediction and phylogenomics. *Mol Biol Evol* 35(3), 543-548. (doi:10.1093/molbev/msx319).

Supplementary Tables

Table S1: Summary of *de novo* reference transcriptome assemblies from *I. cimicoides*, *N. glauca*, *N. cinerea* and *C. punctata* and candidate venom protein selection.

Species	*Number of contigs*	*N50 contig size*	*BUSCO coverage (Insecta_odb9 database)*	*Selected venom protein candidates*
I. cimicoides	*63.868*	*1.624*	*C:86.2%[S:85.5%,D:0.7%],F:5.8%,M:6.0%*	*AMG: 41; PMG: 71*
N. glauca	*56.171*	*2.171*	*C:90.4%[S:89.5%,D:0.9%],F:4.4%,M:5.2%*	*AMG: 81; PMG: 116*
N. cinerea	*55.742*	*1.824*	*C:86.5%[S:85.9%,D:0.6%],F:6.2%,M:7.3%*	*AMG: 18; PMG: 89*
C. punctata	*51.117*	*1.734*	*C:85.5%[S:84.7%,D:0.8%],F:8.0%,M:6.5%*	*AMG: 62; PMG: 78*

Supplementary Figures

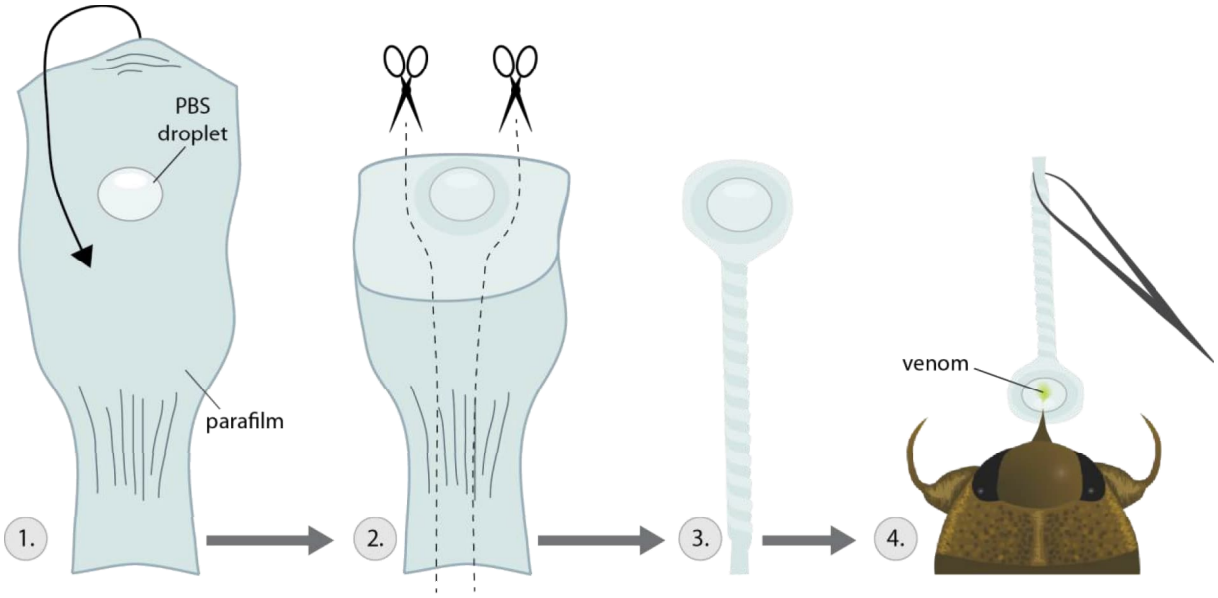


Figure S1: Schematic overview of the preparation of an artificial prey dummy for venom collection. A droplet of PBS is pipetted onto a piece of stretched parafilm and then enclosed by folding the parafilm over the droplet (1.). The material is cut off as shown (2.) and the long end is twisted into a stem for better handling (3.). The dummy is then moved on the water surface to imitate a potential prey animal (4.).

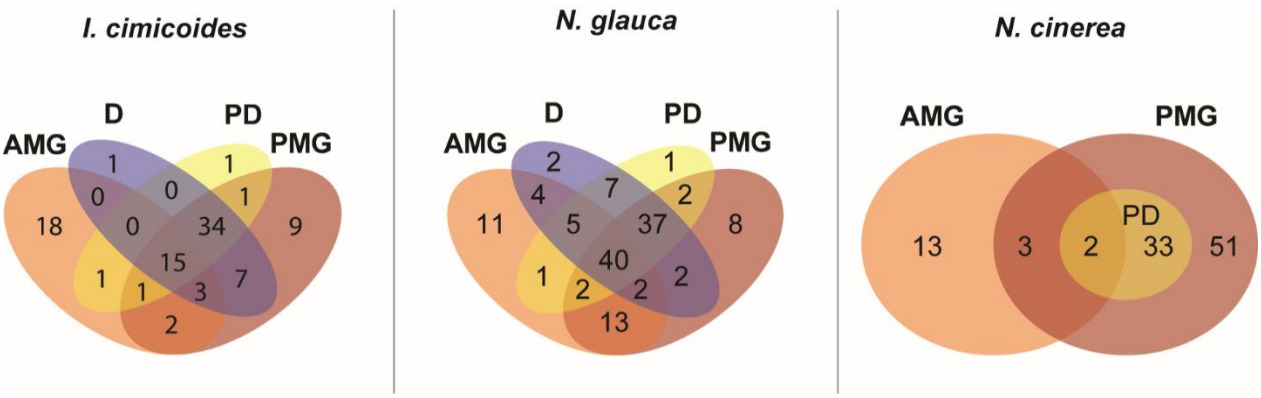


Figure S2: Protein occurrences in AMG and PMG extracts and defensive (D) and prey dummy (PD) venom of *I. cimicoides*, *N. glauca*, and *N. cinerea*.

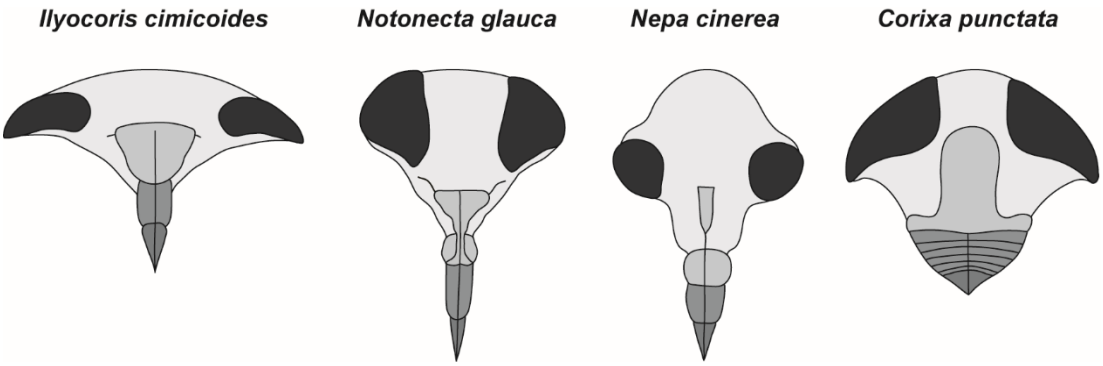


Figure S3: Mouthpart morphology of *I. cimicoides*, *N. glauca*, *N. cinerea* and *C. punctata*.

12.2. Manuscript II

Supplemental Information for:

Context-dependent venom deployment and protein composition in two assassin bugs

Maike L. Fischer, Natalie Wielsch, David G. Heckel, Andreas Vilcinskas, Heiko Vogel

Supplementary Methods

1. LC-MS/MS, proteomic data processing and protein identification

The tryptic peptides were dissolved in 50 µL aqueous 1% formic acid and 1 to 5 µL of the peptide mixture was injected into a Waters M-class ultra-high performance liquid chromatography (UPLC) system coupled online to a Synapt G2-si mass spectrometer (Waters). Samples were pre-concentrated and desalted online using a UPLC M-Class Symmetry C18 trap column (100 Å, 180 µm x 20 mm, 5 µm particle size) and 0.1% aqueous formic acid at a flow rate of 15 µL min^{-1}. The peptides were eluted onto an Acquity UPLC HSS T3 analytical column (100 Å, 75 µm X 200, 1.8 µm particle size) at a flow rate of 350 nL min^{-1} using a gradient of mobile phase A (0.1% aqueous formic acid) and B (acetonitrile plus 0.1% formic acid) increasing from 2-10% B over 5 min, 10–40% B over 40 min, 40–70% B over 7 min, 70-95% B over 3 min, isocratic at 95% B for 2 min, and a return to 1% B. The eluted peptides were injected into the mass spectrometer operating in V-mode and positive ESI mode, with a resolving power of at least 20,000 full width at half maximum height (FWHM). We injected 100 fmol $µL^{-1}$ human Glu-fibrinopeptide B in 0.1% formic acid/acetonitrile (1:1 v/v) at a flow rate of 1 µL min^{-1} via the reference sprayer every 45 s to compensate for mass shifts in MS and MS/MS fragmentation mode.

Data were collected by data-dependent acquisition (DDA). The acquisition cycle for DDA analysis consisted of a survey scan covering the *m/z* range 400–1800 Da followed by MS/MS fragmentation of the 10 most intense precursor ions collected at 0.5-s intervals in the *m/z* range 50–2000. Dynamic exclusion was applied to minimize multiple fragmentations for the same precursor ions. MS data were collected using MassLynx v4.1 software (Waters).

DDA raw data were processed and screened against a sub-database containing common contaminants (human keratins and trypsin) using ProteinLynx Global Server (PLGS) v2.5.2 (Waters). The following parameters were applied: fixed precursor ion mass tolerance = 15 ppm for survey peptide, fragment ion mass tolerance = 0.02 Da, estimated calibration error = 0.002 Da, one missed cleavage, fixed carbamidomethylation of cysteine residues, and variable oxidation of methionine. Spectra that remained unmatched following the database search were interpreted *de novo* to yield peptide sequences for homology-based searching using MS BLAST (Shevchenko et al. 2001) installed on a local server. MS BLAST searches were performed against the Arthropoda database (download from NCBI on 12 February 2019) and *P. horrida* and *P. biguttatus* sub-databases obtained from *in silico* translation of the corresponding transcriptomes. The pkl files generated from raw data were searched in parallel against the NCBInr database (downloaded on 10 January 2019) combined

with *P. horrida* and *P. biguttatus* sub-databases using MASCOT v2.6.0 and the parameters described above.

2. RNA-Seq and *de novo* transcriptome assembly

Quality control measures, including the filtering of high-quality reads, the removal of reads containing primer/adaptor sequences, and the trimming of read lengths, were applied using CLC Genomics Workbench v11.1. For transcriptome assembly, RNA-Seq data from all four tissue samples were combined for each species and *de novo* transcriptome assemblies were prepared using CLC Genomics Workbench v11.1 with standard settings and two additional CLC-based assemblies with different parameters. The presumed optimal consensus transcriptome for each species was then selected, as previously described (Vogel et al., 2014). The transcriptomes were annotated using BLAST, Gene Ontology and InterProScan in OmicsBox (https://www.biobam.com/omicsbox) as described by Götz et al. (2008). For BLASTx searches against the non-redundant NCBI protein database (NR database), up to 20 best NR hits per transcript were retained, with an E-value cutoff of $\leq 10^{-3}$ and a minimum match length of 15 amino acids. To assess transcriptome completeness, we performed a Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis (http://busco.ezlab.org) by comparing our assembled transcriptomes against a set of highly-conserved single-copy orthologs. This was accomplished using the BUSCO v3 pipeline (Waterhouse et al., 2017), comparing the predicted proteins of the *P. biguttatus* and *P. horrida* transcriptomes to the predefined set of 1658 Insecta single-copy orthologs from the OrthoDB v9.1 database. Digital gene expression analysis was carried out using CLC Genomics Workbench v11.1 to generate BAM files, and then counting the sequences to estimate expression levels, using previously described parameters for read mapping and normalization (Pöppel et al., 2015). Gene expression levels were estimated by normalizing mapped read values as implemented in CLC Genomics Workbench v11.1 and ArrayStar, calculating the reads per kilobase per million mapped reads (RPKM) and transcripts per million (TPM) values. For comparisons of expression levels between tissues, we used the log2 transformed TPM value.

Potential venom or gut transcripts were screened for the presence of a signal peptide using SignalP 4.1 and classified based on the annotations. The extracted tissue-specific gene sets were then compared and complemented with the proteins that were identified by LC-MS/MS analysis.

Supplementary References:

Götz S., Garcia-Gomez JM., Terol J., Williams TD., Nagaraj SH., Nueda MJ., Robles M., Talon M., Dopazo J. and Conesa A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic acids research, 36(10), 3420-35.

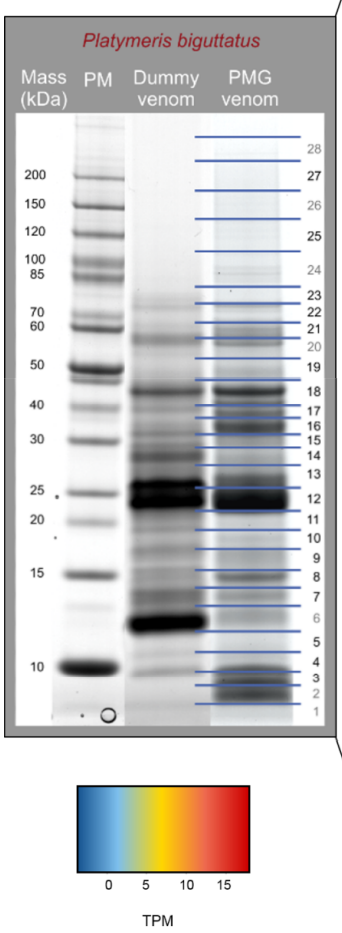
Pöppel A-K, Vogel H, Wiesner J, Vilcinskas A. 2015. Antimicrobial peptides expressed in medicinal maggots of the blow fly Lucilia sericata show combinatorial activity against bacteria. Antimicrob Agents Chemother 59:2508 –2514. doi:10.1128/AAC.05180-14.

Shevchenko A, Sunyaev S, Loboda A, Bork P, Ens W, Standing KG. 2001. Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. Anal Chem. 73(9):1917-26.

Vogel H, Badapanda C, Knorr E, Vilcinskas A. 2014. RNA-sequencing analysis reveals abundant developmental stage-specific and immunityrelated genes in the pollen beetle Meligethes aeneus. Insect Mol Biol 23:98–112. http://dx.doi.org/10.1111/imb.12067.

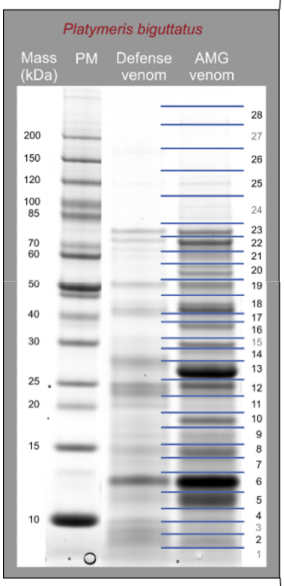
Waterhouse RM, Seppey M, Simão FA, Manni M, Ioannidis P, Klioutchnikov G, Kriventseva EV, Zdobnov EM. 2017. BUSCO applications from quality assessments to gene prediction and phylogenomics. Mol Biol Evol 35, 543-548. doi: 10.1093/molbev/msx319

Supplementary Figures



No.	kDa	Dummy venom		PMG venom		Description	PMG	AMG	AG	Gut	RB
		Total score	Assigned peptides	Total score	Assigned peptides						
27	30.1	396	16	63	1	S1 protease					
25		300	4			Xanthine dehydrogenase					
	52.8	164	3			Uncharacterized protein					
23	73.3	245	6	338	13	S1 protease (CUB domain)					
22	37.9	364	10	167	2	S1 protease					
	75.8	1088	20			Transferrin-like protein					
	83.3	426	6			Laccase					
	70.7	327	4			Hexosaminidase-like protein					
21	58.8	1357	43	359	8	S1 protease (CUB domain)					
		237	11	194	3	S1 protease					
	55			65	1	Aminopeptidase					
19	45.3	410	32	374	12	S1 protease (CUB domain)					
	46.5	867	52	646	24	S1 protease					
18	45.6	450	9	432	6	Protease inhibitor (serpin)					
	45.2	1227	113	672	23	S1 protease					
	28.6	510	24	316	4	Gelsolin-like protein					
		122	2			Nuclease-like protein					
17	43	178	3	77	1	Cathepsin B					
		288	9	90	2	S1 protease (CUB domain)					
	22.8			98	1	Uncharacterized protein					
	33.1	466	19	153	6	S1 protease					
16	59.8	125	5	123	5	S1 protease (CUB domain)					
	33.7	475	31	478	17	S1 protease					
	31	172	2	179	2	Lipase-like protein					
15	58.5	514	18	67	3	S1 protease (CUB domain)					
	30.7	658	40	492	13	S1 protease					
14	29.6	68	1	192	3	Metalloproteinase					
13	35.8	126	12	71	3	S1 protease					
	26.3	340	5	198	3	Venom protein family 2 protein					
	27.8	447	18	360	7	Redulysin-like protein					
		77	7	77	3	Chitin-binding like protein					
12	24.5	825	28	371	12	Redulysin-like protein					
	30.2	615	19	154	11	Venom protein family 2 protein					
11	24.3	195	5	158	6	Venom protein family 2 protein					
		218	4	144	2	Venom protein family 5 protein					
10	22.1	60	1	60	1	Venom protein family 2 protein					
	17	144	2	139	2	Venom protein family 4 protein					
9		77	2	77	1	Unknown protein					
8	22.4	68	1	71	1	Protease inhibitor (pacifastin)					
	18.3	568	14	288	6	Venom protein family 1 protein					
	16.8	162	2	302	5	Venom protein family 6 protein					
	15.3			78	1	Fatty-acid-binding protein					
		158	4	130	7	S1 protease					
	32.3	76	2			Nuclease-like protein					
7	14.4	159	6	153	2	S1 protease (CUB domain)					
5	12.2	73	1	71	1	Protease inhibitor (cystatin)					
4		74	1	67	1	Redulysin-like protein					
3	4.7	79	1	79	1	Ptu1-like peptide					
	2.7	75	1			Unknown protein					

Figure S1. Proteins of the *P. biguttatus* PMG and prey dummy venom identified by LC-MS/MS. The Coomassie-stained protein gel on the left yielded the PMG venom proteins shown on the right, including the predicted protein masses (kDa), the total score, number of assigned peptides and descriptions. The excised bands are indicated with numbers and lines on the right side of the protein gel. For the proteins identified by LC-MS/MS, gene expression levels (log2 TPM) in the PMG, AMG, gut, and remaining body tissues are shown in the heat map. PM = protein marker. See Table S1 for the identity of matching predicted proteins in the *P. biguttatus* transcriptome.



No.	kDa	Defense venom Total score	Defense venom Assigned peptides	AMG venom Total score	AMG venom Assigned peptides	Description	AMG	PMG	AG	Gut	RB
28	149	300	11	121	3	Unknown protein					
	71.7	80	2	71	1	Unknown protein					
26	167.8			78	1	Glycosyltransferase-like protein					
25	117.7	283	4	228	3	Mannosidase					
	101.4			221	3	Nuclease-like protein					
		65	1			Hyaluronidase-like protein					
23	75.8	449	13	820	21	Transferrin					
	6.3			60	1	Unknown protein					
22		399	11	68	2	S1 protease					
	70.1	277	4			Hexosaminidase-like protein					
	83.3	98	1			Laccase-like protein					
	57.3	622	12	1323	26	Glucose dehydrogenase					
21	69.7	264	4	375	9	S1 protease					
	62.5			212	3	Carboxylesterase					
	57.6			266	4	Catalase					
	55.5			331	4	Aminopeptidase					
	35.6	96	1	296	10	Hemolysin-like protein					
20		77	1	426	10	Carboxypeptidase					
	57.3	130	3	728	27	Hemolysin-like protein					
19	50.2	294	4	132	2	Lipase-like protein					
	49.3	412	81	870	17	Carboxypeptidase					
	48.9	721	9	789	11	Kinase-like protein					
	45.4			144	2	Aspartic protease					
				62	1	Uncharacterized protein					
		67	1			Lipase-like protein					
	45.5	409	6			Protein yellow-like					
18	45.6	669	17	1098	22	Protease inhibitor (serpin)					
		236	3	148	2	Gelsolin-like protein					
	40.1	338	5			Gelsolin-like protein					
	39.8			81	1	Unknown protein					
17	41			63	1	Venom protein family 13 protein					
	33.8	280	7	319	5	S1 protease					
16	36.7	211	4	133	2	S1 protease					
	37.9	233	4			S1 protease					
	39.4			381	6	Phytanoyl-CoA dioxygenase					
14	42	419	10	68	1	S1 protease					
	32.3			75	1	Nuclease-like protein					
	24.3			73	2	Hemolysin-like protein					
				122	2	Chitin-binding protein					
	13.7	123	3	190	4	S1 protease (CUB domain)					
	32.4	277	7			S1 protease					
	73.3	273	8			S1 protease (CUB domain)					
		73	1			Lipase-like protein					
13	36.4	354	7	128	2	S1 protease					
	24.5	151	7	623	15	Hemolysin-like protein					
	24.6	66	1	82	2	Redulysin-like protein					
		79	1	148	3	Unknown protein					
	46.5	551	11			S1 protease					
	58.8	287	7			S1 protease (CUB domain)					
12	33.5	187	4	528	17	Bpi-like protein					
	24.5	275	9	219	6	Redulysin-like protein					
	24.4	240	3	513	8	Venom protein family 5 protein					
	21	80	1	97	1	Venom protein family 2 protein					
	47.5	92	1			S1 protease (CUB domain)					
11	25.9	131	3	70	1	Venom protein family 2 protein					
10	20.3			475	17	Hemolysin-like protein					
	22.1	178	5	66	1	Venom protein family 2 protein					
	17	122	2	67	1	Venom protein family 4 protein					
9	42	465	18	133	4	S1 protease					
	27	89	2	78	1	Redulysin-like protein					
	24.3	144	2	422	8	Venom protein family 11 protein					
	17.6			79	1	Kinase-like protein					
				67	1	S1 protease (CUB domain)					
	26.2	71	1			S1 protease (CUB domain)					
	22.7	258	8			S1 protease					
8	18.3	231	4	223	3	Venom protein family 1 protein					
	16.8	381	7	324	6	Venom protein family 6 protein					
		204	5	72	1	S1 protease					
	4.3	77	1	77	1	Unknown protein					
	14			178	2	Odorant binding protein					
				68	1	Venom protein family 8 protein					
7		71	1			S1 protease					
	57.7	85	2			S1 protease (CUB domain)					
6		119	2	197	6	Protease inhibitor (cystatin)					
	8.2			120	4	Uncharacterized protein					
5	78.3			120	2	Cysteine protease					
	13	193	4	491	9	Protease inhibitor (cystatin)					
	11.6	83	1	91	1	Venom protein family 8 protein					
	8.2			209	4	Protease inhibitor (pacifastin)					
4		92	1	92	2	Carboxypeptidase					
		67	1			Redulysin-like oprotein					
	12.2	65	1			Protease inhibitor (cystatin)					
2	5.5			75	1	Protease inhibitor (kazal)					

Figure S2. Proteins of the *P. biguttatus* AMG and defense venom (mild harassment) identified by LC-MS/MS. The Coomassie-stained protein gel on the left yielded the AMG venom proteins shown on the right, including the predicted protein masses (kDa), the total score, number of assigned peptides and descriptions. The excised bands are indicated with numbers and lines on the right side of the protein gel. For the proteins identified by LC-MS/MS, gene expression levels (log2 TPM) in AMG, PMG, gut, and remaining body tissues are shown in the heat map. PM = protein marker. See Table S1 for the identity of matching predicted proteins in the *P. biguttatus* transcriptome.

12.3. Manuscript III

Supplemental information for:

An assassin's secret: multifunctional cytotoxic compounds in the predation venom of the assassin bug *Psytalla horrida* (Reduviidae, Hemiptera)

Maike L. Fischer, Benjamin Fabian, Yannick Pauchet, Natalie Wielsch, Silke Sachse, Andreas Vilcinskas, Heiko Vogel

Supplementary tables

Table S2: Summary of the tests used to statistically analyze the results from the bioassays.

	Assay		**Test**	**P**	**Test**
Fractions	Bacterial growth inhibition		ANOVA	5.7e-10	Pairwise t-tests
	Cell viability		Kruskal-Wallis	1.9e-08	Pairwise Dunn's tests
	Hemolysis		Kruskal-Wallis	0.007735	Pairwise Dunn's tests
	Calcium imaging		Kruskal-Wallis	9.9e-08	Pairwise Dunn's tests
Redulysins	Bacterial growth inhibition	*E. coli*	Kruskal-Wallis	7.1e-05	Pairwise Dunn's tests
		B. subtilis	ANOVA	1.9e-11	Pairwise t-tests
		B. thuringiensis	ANOVA	4.4e-10	Pairwise t-tests
	Cell viability		Kruskal-Wallis	6.4e-13	Pairwise Dunn's tests
	Hemolysis		Kruskal-Wallis	7.6e-10	Pairwise Dunn's tests
	Calcium imaging		Kruskal-Wallis	0.00184	
Vpf2	Cell viability		Kruskal-Wallis	2.2e-07	Pairwise Dunn's tests
	Calcium imaging		ANOVA	0.1866	

Table S2: Summary of *de novo* reference transcriptome assembly from *L. rugulipennis*.

Species	**Number of contigs**	**N50 contig size**	**BUSCO coverage (Insecta_odb9 database)**
L. rugulipennis	66.754	1.576	C:91.4%[S:90.3%,D:1.1%],F:3.9%,M:4.7 %

Table S3: *P. horrida* redulysins identified in the genome, PMG transcriptome and proteomes of PMG venom, fraction A and fraction B. The molecular weight of the unprocessed proteins is given in kDa.

Genome (name)	Molecular weight (kDa)	Transcriptome PMG (name)	Proteome PMG	Proteome Fraction A	Proteome Fraction B
g1038.t1	69.9	Phor_Comb_C9529	**X**	**X**	
g1037.t1	55.2				
g1039.t1	30.7				
g2020.t1	27.1	Phor_Comb_C34871	**X**		
g2021.t1	27.2	Phor_Comb_C46120	**X**		
g2022.t1	26.7	Phor_Comb_C52128	**X**		**X**
g2023.t1	27.1				
g2565.t1a	18.7	Phor_Comb_C25695	**X**		
g2565.t1b	25.1	Phor_Comb_C44851			
g2565.t1c	19.6				
g17426.t1a	25.3	Phor_Comb_C27614	**X**		
g17426.t1b	40.7				

Table S4: Sequences of synthesized redulysin peptides from *P. horrida* and *L. rugulipennis*.

No	Species	Gene name		Sequence
1	*P. horrida*	g1038.t1	1	ETWGKMKDHYLNLGKETLAKLKELKEKGQG
2		domain	2	DLPYTTKVLLILKETLDKLKAATGEEKEKLIQKIKG
3			3	EAWGKMKNHYLNLGKATLAKLNELKENYKT
4			4	DPALTAKILLKLKEYLEKLKAAAGKEKEKLILKIKE
5			5	KETFGKLKDRWSQLTKAQLQKVIQTLKN
6			6	QIIQKYVQKLKDFYEKIKAAVGAKKEELKKRLEE
7			7	GWLGKLGKGMKKVGKKFVKKMSSAMKAGCKKGMK MLKD
8		g1037.t1	1	EAWGKMKDHYLNLGKATLAKLNELKENYKT
9		domain	5	GWLGKLGKGMKKVGKKFMKKMSSTMKAGCKKGMK MLKD
10		g2020.t1		GKVGDWFKKYWKNFKNAMKKLSKEIKEACNKGREFL KK
11		g2021.t1		GFGDWAQGVWNDAKNVFKKLKKAVKQKCKEGREYL KK
12		g2022.t1		GKGLDWFKKQWGKMKNSWKKVGAKVKAVFNKGRD FLKK
13		g2023.t1		GKVGDWFKNQWGKMKNSFKKVGAKMKAVFNKGRE FLKK
14		g2565.t1a		KTWTTLKMAVNKLLSRYRKLSKEDFKKILK
15		g2565.t1b		GRWDTFKEKVKIFAKDKKELAKQWAGKFKEWLITTKE NAKIKLKE
16		g17426.t1a		GRKNDKYIGDVIKERLRKLKKSMVEGLRRLKN
17		g17426.t1b		GNKVTSKDPSKPKKKKKFSLKGIGESMKKWAKKGLEIL KS
18	*L. rugulipennis*	Lrug_Comb_C538		SKIGSMGKHVIKQIGKVGLNMLKKGLKVLKSMYYKFG GKRLLKRAKEYLKK

Supplementary figures

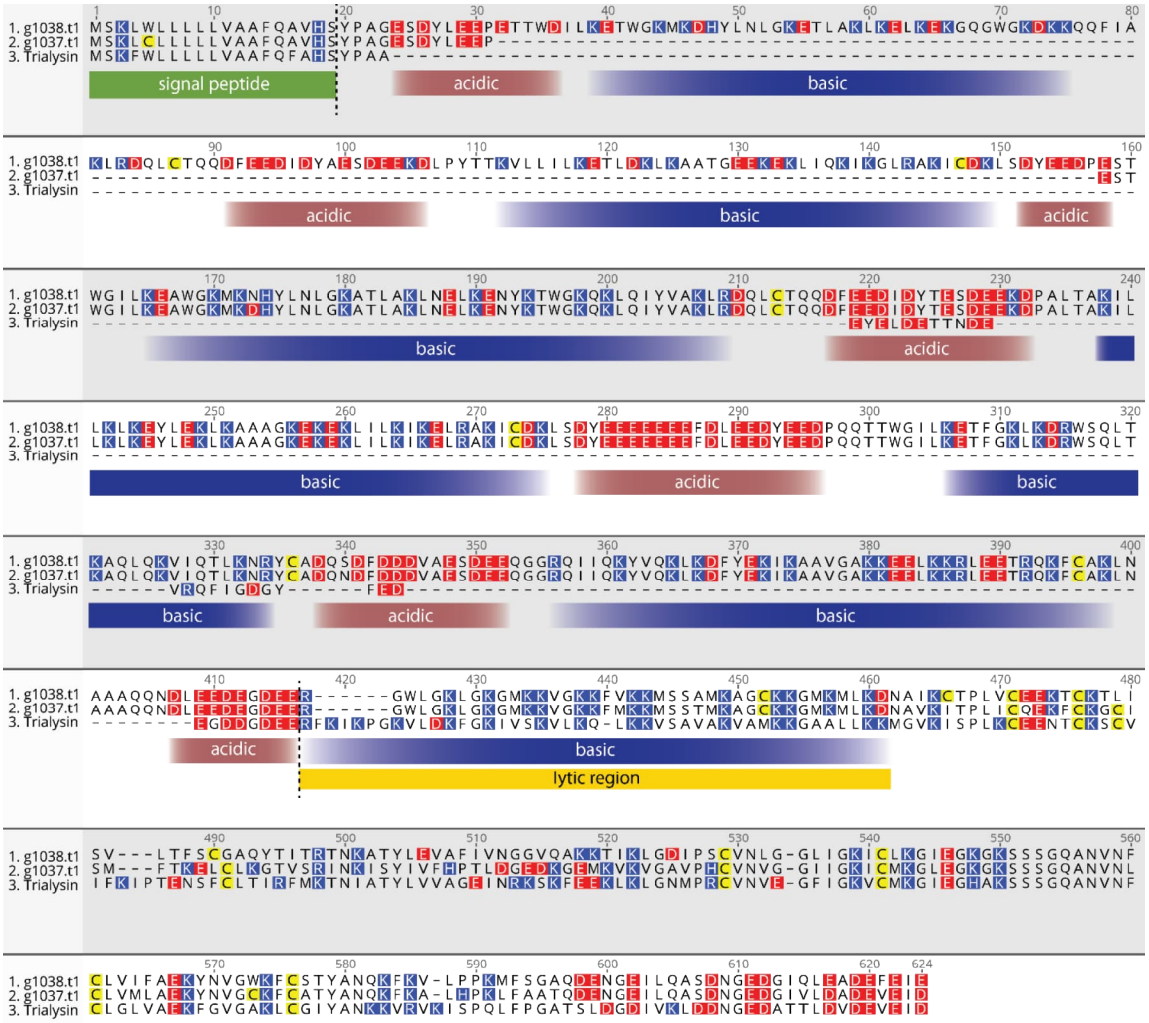


Figure S1: Alignment of *P. horrida* redulysins g1038.t1, g1037.t1 and trialysin from *T. infestans* (Genbank accession: AAL82381.1). Acidic and basic amino acids are highlighted in red and blue, respectively. Cysteine residues are highlighted in yellow. The signal peptide sequence is marked in green and the lytic region according to Amino, et al. [1] in yellow. Dotted vertical lines represent the putative restriction sites after the signal peptide and pro-peptide.

Figure S2: Redulysin g1038.t1 (contig Phor_Comb_C9529) identified in the LC-MS/MS analysis of *P. horrida* PMG venom conducted in Fischer, et al. [2]. The numbered boxes indicate the bands that were cut out for analysis. The colored boxes highlight the bands where redulysin g1038.t1 (Phor_Comb_C9529) was detected. The colored sequences represent the peptides that were detected in the respective protein band and matched the amino acid sequence of g1038.t1. PMG = PMG extract; M = molecular weight marker. Details on methods of the transcriptomic and proteomic analysis are described in Fischer, et al. [2].

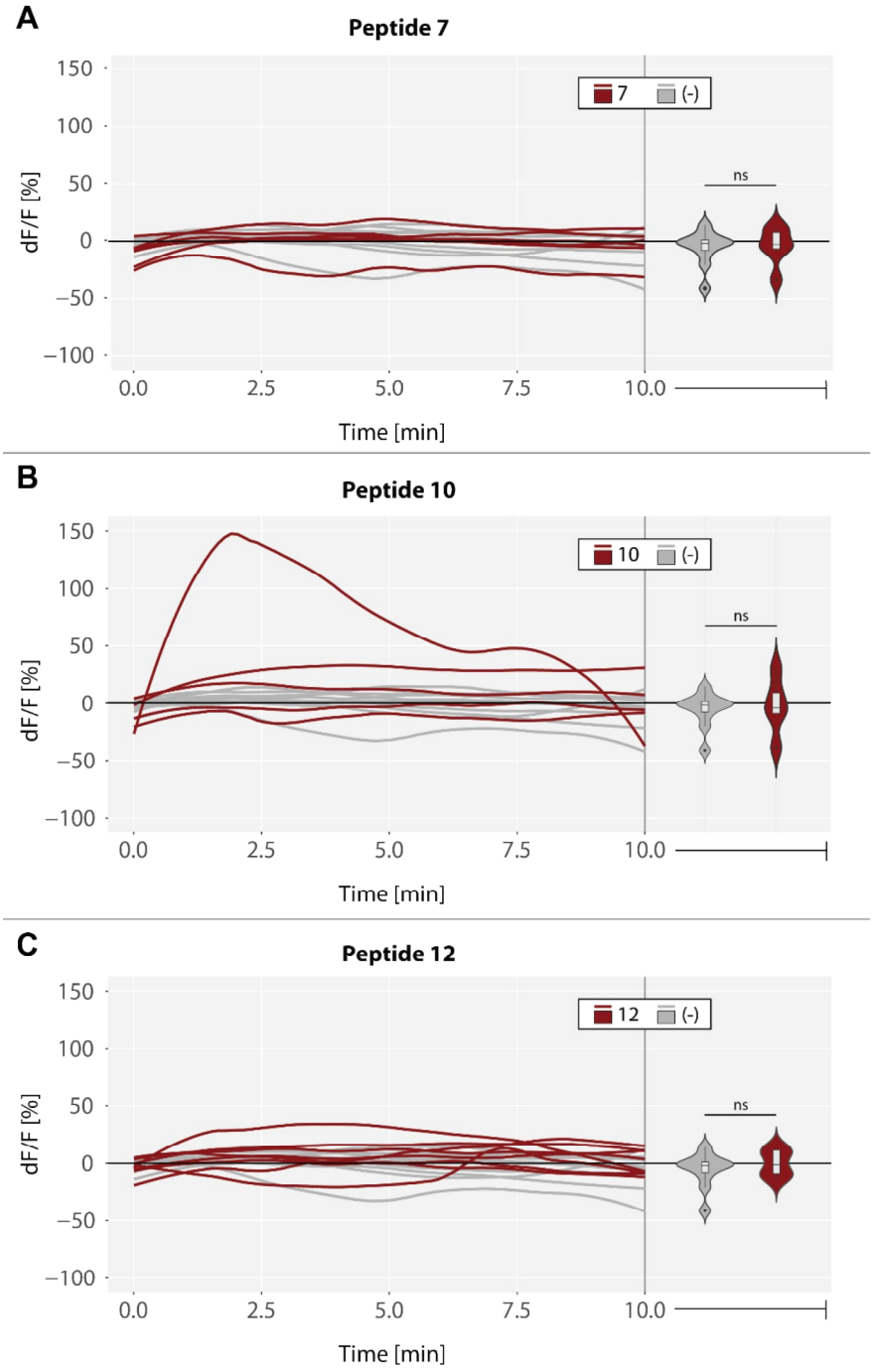


Figure S3: Calcium imaging of *D. melanogaster* antennal lobes after treatment with 100 µM of selected redulysin peptides. Changes of intracellular calcium concentration (represented by dF/F) after treatment with peptide 7 (**A**), peptide 10 (**B**) or peptide 12 (**C**). (-) = 20 mM MES pH 5.5. Violin plots represent the fluorescence intensity after 10 min. Statistical test: Kruskal-Wallis test ($\alpha = 0.05$). Boxplots within the violin plots represent median (line), interquartile range (box) and data range (whiskers).

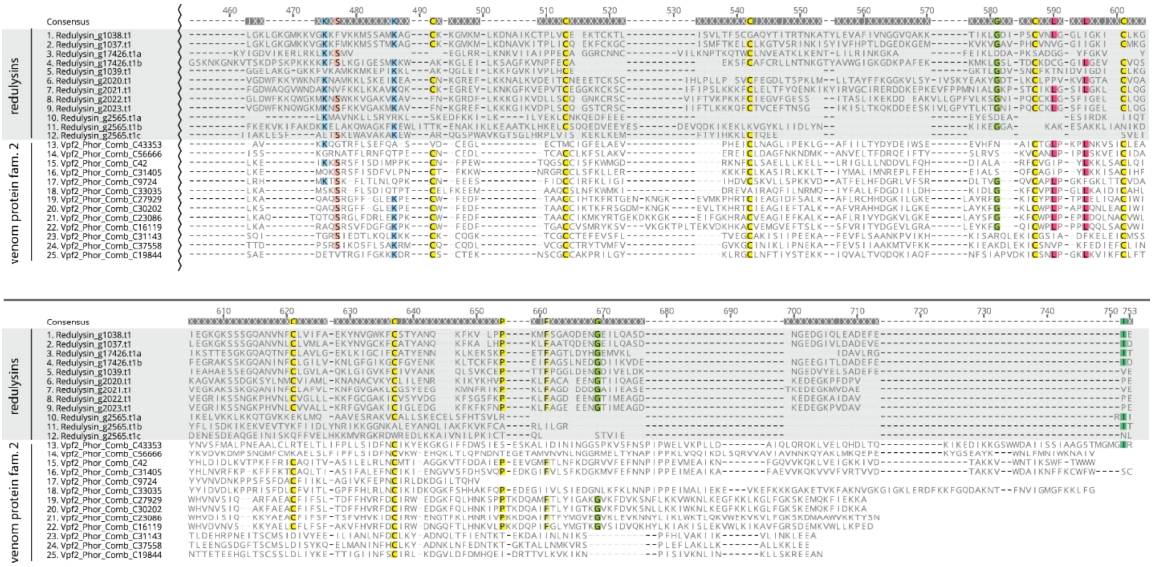


Figure S4: Alignment of the C-terminal region of the *P. horrida* redulysins and venom family 2 proteins. Residues with > 50% agreement to the consensus are highlighted.

Supplementary references

1. Amino R.; Martins, R.M.; Procopio, J.; Hirata, I.Y.; Juliano, M.A.; Schenkman, S. Trialysin, a novel pore-forming protein from saliva of hematophagous insects activated by limited proteolysis. *Journal of Biological Chemistry* **2002**, *277,* 6207-6213.
2. Fischer, M.L.; Wielsch, N.; Heckel, D.G.; Vilcinskas, A; Vogel, H. Context-dependent venom deployment and protein composition in two assassin bugs. *Ecology and evolution* **2020**, *10*, 9932-9947.