Friedrich Schiller University Jena
Faculty of Biological Sciences
Institute of Ecology and Evolution

Venom characterization of the aquatics bugs *Ilyocoris cimicoides* (Hemiptera: Naucoridae) and *Notonecta glauca* (Hemiptera: Notonectidae)

Master's Thesis

to gain the academic grade as a
Master of Science in the Study Program *Evolution, Ecology, and Systematics* (EES)
(M. Sc.)

submitted by
Sol Andrea Yepes Vivas
born at Colombia

Jena, August 2021

REVIEWERS

1. Dr. Heiko Vogel
   Max Planck for Chemical Ecology / Department of insect symbiosis

2. Prof. Dr. Nicole van Dam
   German Centre for Integrative Biodiversity Research / Molecular Interaction Ecology

TABLE OF CONTENTS

1.1 INTRODUCTION

1.2 Hemiptera

Insects represent at least 60% of all species known until now, making up the majority of eukaryotic species (Grazia & Fernandes, 2012; Walker *et al*., 2018d). Insects were one of the first animal groups adapted to a terrestrial lifestyle that has allowed the success of Hexapoda in different ecological niches and therefore their diversification (Walker *et al*., 2018d). Within Insecta, the order Hemiptera is the only hyperdiverse order of hemimetabolous insects, with at least 100,000 known species worldwide (Wang *et al*., 2019), characterized by their piercing-sucking mouth-parts (Grazia & Fernandes, 2012; Ghamari *et al*., 2014; Wang *et al*., 2019).

The major radiation in Hemiptera occurred in the Permian about 290 to 268 Ma (Wang *et al*., 2019) in the diversification of the monophyletic sub-order Heteroptera or true bugs (Walker *et al*., 2018b). Heteroptera are one of the most diverse clades in terms of habitat (e.g. ant nests, water, spider and Embioptera webs, and mycelia on fungi) (Torres & Boyd, 2009), and feeding ecology, comprising phytophagous, zoophagous, zoophytophagous (plant-feeding carnivores), phytozoophagous (prey-taking herbivores) (Schuh & Slater, 1995) and ectoparasitic species (Swart *et al*., 2006; Wang *et al*., 2019). The true bugs are characterized by the modification of a hemielytron in its anterior wing in which the anterior part is coriaceous and the posterior membranous (Grazia & Fernandes, 2012), and paired metathoracic glands in adults and dorsal abdominal scent glands in nymphs (Wang *et al*., 2019). Morgan *et al.* (2020), states that the evolution of Heteroptera was the product of the diversification of secondary metabolism used to perform digestion or defense (Swart *et al*., 2006). This molecular evolution was accompanied by the adaptation of the food channel of the proboscis, a structure common for all Hemiptera (Walker *et al*., 2017; Denecke *et al*., 2020). Consequently, morphological components as the stylets and barbs reveal differences between phytophagous and zoophagous Heteroptera (Boyd *et al*., 2002).

The sub-order Heteroptera is subdivided into seven monophyletic infra-orders: Dipsocomorpha, Enicocephalomorpha, Gerromorpha, Nepomorpha, Leptopodomorpha, Cimicomorpha, and Pentatomorpha (*sensu* Wang *et al*., 2019). Nevertheless, some phylogenetic hypotheses across the suborder are currently ambiguous (Walker *et al*., 2016). The infra-order Nepomorpha, or true water bugs, is a clade of aquatic bugs, in which the majority of species are confined to the aquatic environment throughout their entire lives

where they either breathe through a siphon, storing atmospheric air, or use a plastron (Ye *et al*., 2020). True water bugs leave the water only occasionally with some exceptions of the families Ochteridae and Gelastocoridae, which are terrestrial and distant from aquatic environments (Walker *et al*., 2016; Ye *et al*., 2020). With the exception of the Antarctic, the infra-order Nepomorpha is distributed worldwide, with at least 2300 species described (Ye *et al*., 2020), the majority of which are predatory (Swart *et al*., 2006; Ye *et al*., 2020).

1.2.1 Heteropterans' feeding apparatus

The mouthparts play an important role in insect evolution and vary according to the nature of their food (Ramm *et al*., 2015). The structural differences are especially significant in the more diverse groups, such as Hemiptera (Cohen, 1993; Ramm *et al*., 2015; Wang *et al.*, 2020). As an example, Cobben (1978) demonstrated that the right maxillary stylets of predatory heteropterans are more serrated and the barbs are pointed toward the head (Boyd *et al*., 2002). The hemipterans' feeding apparatus includes a labium, labrum, and four stylets: two maxillary stylets that form the food and the salivary canal, and two mandibular stylets that are equipped with teeth and rasps (Miles, 1972; Cohen, 1995; Walker *et al*., 2016). These mouthparts form a proboscis able to drill mechanical barriers (e.g. insect cuticles), and then suck up the liquefied food (Cohen, 1995, 1998; Swart *et al*., 2006; Ghamari *et al*., 2014), in a process termed lacerate-and-flush feeding (Miles, 1972). However, the morphology of the stylet became differentiated during evolution, generating differences among different lineages within Hemiptera (Wang *et al.*, 2020).

Heteropterans have a salivary pump that transports enzymes from the labial glands into the food source (Walker *et al*., 2016). These labial glands consist of a main secretory gland with 2 to 4 lobes and an accessory gland (Baptist, 1941; Swart *et al*., 2006; Azevedo *et al*., 2007; Sahayaraj *et al*., 2010; Walker *et al*., 2016). Nevertheless, a large variety of labial glands occurs, even within a single subfamily within Hemiptera (Azevedo *et al*., 2007). These glands are connected to the salivary pump at a junction called the hilus (Cohen, 1993; Azevedo *et al*., 2007; Walker *et al*., 2018c). Azevedo *et al*., (2007), suggested that this morphological arrangement could allow the animal to inject the content of each of the lobes separately, due to the possible differences in the secreted substances between the glands. Nevertheless, some studies in predatory heteropterans showed contradicting results (Edwards, 1961).

1.3 Extra-oral digestion

Digestion is the process by which food molecules are broken into smaller molecules that are absorbed by the gut tissue. The majority of food molecules are polymers and are reduced to their monomers (Terra & Ferreira, 2005). However, even though the gut carries out the absorption of nutrients, some insects realize parts of the digestion outside their body (Terra & Ferreira, 2005; Evangelin *et al.*, 2014). This process is known as extra-oral digestion (EOD). EOD is performed in at least 196 orders within Arthropoda, and it is also found in predatory Heteroptera including the True Water Bugs (Silva-Cardoso *et al.*, 2010*).*

Extra-oral digestion is one of the most important processes in heteropteran predators, which has led to the great diversification within this group since it allows the predator to eat large prey and ingest over 94% of the nutrients present in their prey (Cohen, 1998; Silva-Cardoso *et al.*, 2010). This feeding mode depends on the process of liquefying prey tissues, ingest the liquid, and complete digestion in the gut, to obtain nutrients for their growth and reproduction (Cohen, 1998). There are two classes of EOD (Cohen, 1995; Silva-Cardoso *et al.*, 2010). Type I EOD involves the chemical liquefaction of prey using the prey's cuticle as a container. Type II EOD involves the use of both chemical and mechanical feeding, destroying the prey's cuticle in the process (Cohen, 1998; Silva-Cardoso *et al.*, 2010). Predators that use Type I EOD can be divided into non-refluxing species that use digestive enzymes from external glands, and refluxing species that use digestive enzymes from the gut (Cohen, 1995).

Type I EOD consists of three phases: First, the predator injects digestive enzymes through the prey's cuticle, which decomposes the internal organs of the prey as the first step of the digestion. Then, the liquefied material can be ingested (Cohen, 1998; Azevedo *et al*., 2007; Silva-Cardoso *et al.*, 2010). Also, EOD is a cyclic process in which the predator injects a series of digestive fluids at regular intervals followed by mechanical pauses, during which the liquefaction of the prey material takes place (Azevedo *et al*., 2007). Heteroptera use type I EOD since they inject digestive enzymes (saliva) into intact prey and non-refluxing EOD due to the use of digestive enzymes coming from external glands (Cohen, 1998; Silva-Cardoso *et al.*, 2010).

1.3.1 Biochemistry of extra-oral digestion

At the beginning of this century, science underwent remarkable progress in the biochemistry of insect digestion (Maggio *et al*., 2005). Nevertheless, the majority of studies on this topic have focused on the gut biochemistry of pest insects since it is a plausible starting point to either optimize, reduce or stop the use of chemical insecticides that cause environmental problems (Amino *et al*., 2002; Maggio *et al.*, 2005; Ghamari *et al*., 2014; Ramm *et al*., 2015). Additionally, the gut biochemical profile reflects an interface between the insects' community and their environment (Maggio *et al*., 2005).

The composition and physiology of saliva have been described in different studies, concluding the wide diversity of these molecules within Heteroptera (Cohen, 1998; Maggio *et al*., 2005; Swart *et al*., 2006; Evangelin *et al.*, 2014). These studies suggest the presence of specific digestive enzymes, reflecting the insect's ability to use plant or animal material as food (Swart *et al*., 2006; Azevedo *et al*., 2007; Torres & Boyd, 2009; Sahayaraj *et al*., 2010; Ghamari *et al*., 2014; Walker *et al.*, 2018b), and its distinctive way of feeding (Cobben, 1978; Cohen, 1995; Schuh & Slater, 1995; Maggio *et al*., 2005; Swart *et al*., 2006). Additionally, the enzymes indicate whether saliva can be used for defense (Ghamari *et al*., 2014; Walker *et al*., 2018c). It is well documented that carnivorous bugs inject digestive enzymes from the salivary glands, such as proteases, lipases amylases, and other hydrolases into prey (Sahayaraj *et al*., 2010; Evangelin *et al.*, 2014), while phytophagous bugs inject proteases, lipases, amylases, hydrolases, pectinases, and cellulases into their host plants (Baptist, 1941; Torres & Boyd, 2009; Fialho *et al*., 2012; Evangelin *et al.*, 2014). In addition, some carnivorous Heteroptera (e.g. predatory bugs) have neurotoxin peptides that paralyze their prey (Evangelin *et al.*, 2014; Walker *et al*., 2018c).

Regarding the digestive enzymes, proteases are the most important enzymes in predators that perform EOD (Cohen, 1995; Evangelin *et al.*, 2014). This enzyme class is responsible for attacking several kinds of structural proteins (Cohen, 1998). Lipase breaks down fats into small molecules, such as glycerol (Evangelin *et al.*, 2014). Amylases hydrolyze starch in plant-feeding species (Torres & Boyd, 2009; Ghamari *et al*., 2014) and aid in glycogen digestion (Torres & Boyd, 2009; Evangelin *et al.*, 2014; Ghamari *et al*., 2014). All of these proteins are modulated in different concentrations within Heteroptera and are a reference to the prey choose (Cohen, 1998; Evangelin *et al.*, 2014; Ghamari *et al*., 2014), and are therefore indispensable to perform EOD. Nevertheless, these enzymes cannot be immediately replaced

once the depredator used them. Thus, the enzymatic specificity is determined by the structures that the insect must digest in the EOD (plant or animal source) to be made available for later ingestion (Evangelin *et al.*, 2014).

Peptides with paralytic function have been denoted as a venom component for being able to disrupt prey homeostasis (Terra & Ferreira, 2005), and for being an essential part of many arthropods that practice EOD (Cohen, 1995). Nevertheless, the origin of these peptides has been controversial for many years, since it is considered that many of these originated in structures homologous to the digestive system (Azevedo *et al*., 2007; Evangelin *et al.*, 2014). Most studies have focused on a few species and some assumptions are based on a comparison of species of the same family. Furthermore, the majority of these studies have focused on the gut enzymatic profile only, neglecting the salivary glands of true predators.

1.4 Venom in Hemiptera

Scientists have debated the definition of a venomous animal since variables such as relative lethality are arbitrary (Fry *et al*., 2009). Venom was defined by Fry *et al*. (2009), as a secretion produced in a specialized gland and delivered to a target animal; this secretion must disrupt the normal physiology or biochemical processes of the prey to facilitate feeding or defense. Venom is composed mainly of salts, enzymes, peptides, and proteins that target different systems to incapacitate prey, or deter predators and competitors (Fry *et al*., 2009; Walker *et al.*, 2018a, 2018d). Venom proteins are the result of toxin recruitment events defined by the biology and ecology of the animals that produce them (Fry *et al*., 2009; Walker *et al*., 2016, 2018a; Walker, 2020). Most venomous animals use a stinging apparatus (e.g. proboscis in Hemiptera) to inject their venom into the prey (Maggio *et al*., 2005; Walker *et al*., 2018d).

The majority of studies have focused on the venom systems of medically important clades (e.g. spiders, arachnids, cone snails) (Fry *et al*., 2009; Walker, 2020), and only recently expanded to insects (Hegazi1 *et al*., 2015; Walker *et al*., 2018a, 2018d). This is in part also due to the difficulty of isolating toxins from venom glands of organisms that are typically very small (Walker, 2020). Within Insecta, most studies have focused on Hymenoptera (Hegazi1 *et al*., 2015; Walker *et al*., 2018d). Within Hemiptera, Reduviidae (Corzo *et al*., 2001; Terra & Ferreira, 2005; Swart *et al*., 2006; Sahayaraj *et al*., 2010), the giant water bugs

Belostomatidae (Swart *et al*., 2006; Silva-Cardoso *et al.*, 2010; Walker *et al*., 2018b, 2018c), and some bugs within the family Pentatomidae (Walker *et al*., 2018c) were analysed. These studies showed that Heteroptera venom can cause paralysis, tissue liquefaction, and even death in some invertebrates, while in vertebrates, it can cause pain and induce inflammation (Walker *et al*., 2018d). It has been documented that these molecules are a great source of novel peptides with potential applications in agriculture and medicine (Fry *et al*., 2009; Silva-Cardoso *et al.*, 2010; Evangelin *et al.*, 2014). However, despite the potentially diverse pharmacological use of these molecules, the majority have unknown functions (Corzo *et al*., 2001; Walker *et al*., 2018a).

1.4.1 Venom glands of Heteroptera

The venom system in Hemiptera has evolved through the modification of salivary glands (Evangelin *et al.*, 2014; Walker, 2020), facilitating the production, storage, and injection of venom into other animals (Walker *et al*., 2018c, 2018d). The venom glands of Heteroptera consist of two compartments: the main gland, usually bilobed into the posterior main gland (PMG) and the anterior main gland (AMG); and the vesicular or tubular accessory gland (AG) (Baptist, 1941; Ramm *et al*., 2015; Walker *et al*., 2018d). Walker *et al*., (2018d), characterized the composition of venom of *Pristhesancus plagipennis* and concluded that AMG and PMG are the main secretory tissues of the venom glands in which the proteins from the PMG are used for paralyzing and pre-digesting prey, while AMG venom is used for defense. The AG could have a role in recycling water from the gut to the venom gland. A similar picture emerged in the analysis of venom from *Psytallia horrida* that seems to follow the same strategy (Fischer *et al*., 2020). Nevertheless, other studies have demonstrated fewer functional differences between PMG and AMG (e.g. *Platymeris biguttatus*) (Swart *et al*., 2006, Fischer *et al*, 2020), and even the simplification of the main gland to a single lobe in two Cimicomorphan species (Walker *et al*., 2018c). Within Nepomorpha, the studies have focused on the family Belostomatidae due to its broad range of prey choices and high population density (Swart *et al*., 2006; Silva-Cardoso *et al.*, 2010).

1.4.2 Venom composition of Heteroptera

Venoms are complex mixtures that usually include a combination of proteins used to overwhelm and pre-digest prey and for defense (Fry *et al*., 2009; Walker *et al*., 2018a). Frey *et al*. (2009), suggested generalities between these proteins. First, all are secretory proteins i.e. the precursor peptides contain an N-terminal signal peptide. Second, homologous proteins often perform similar biochemical reactions. Third, the majority of proteins involve one or several physiological processes. In Hemiptera, venom is adapted to the way they hunt, feed, and defend themselves (Walker *et al*., 2016). The above results in venom composition differences in Hemiptera (Walker *et al*., 2016, 2018b). In Nepomorpha, enzymatic assays of Belostomatidae have revealed phospholipase, hyaluronidase, protease, amylase esterase, glucosiminidase, phospholipase, nuclease, and phosphatase and phosphohydrolase activities (Silva-Cardoso *et al.,* 2010; Walker *et al*., 2016).

1.4.3 Ecology of venom

The evolution of venoms have provided extended food sources for many insects (Cohen, 1995), and in others insects, it has influenced the defensive role against depredators (Fry *et al*., 2009; Walker *et al*., 2018a). This evolution has had consequences on other strategies such as aposematism and mimicry in some insects (Sunagar *et al*., 2016). This combination of warning colors or chemical signals with the damage made by the sting provides venomous insects with the ability to defend against potential predators and reduce the risk of being attacked (Haridass & Ananthakrishnan, 1980; Sunagar *et al*. 2016). This complex system has generated slow or static predator behavior that is repeated throughout the animal kingdom and compensates for a quick hunting behavior (Maggio *et al*., 2005; Walker *et a*l., 2016). This conduct is accompanied by morphological structures as the raptorial legs in insects (Grazia & Fernandes, 2012; Walker *et al*., 2016), ambush hunting tactics like the sit and wait of the robber flies (Fisher, 2009), and the development of a venom apparatus (Maggio *et al*., 2005).

Venomous species have the advantage to increase their prey choice and reduce their predatory pressure (Haridass & Ananthakrishnan, 1980; Silva-Cardoso *et al.,* 2010). Because of that, these species can expand their niches into more conspicuous environments, and use a large range of prey (Cohen, 1995; Evangelin *et al.,* 2014; Walker *et al.,* 2018b). Hence, several

authors have proposed predatory Heteroptera as ideal candidates for biological control agents in places where a wide variety of pests exists (Evangelin *et al.*, 2014) and in some crops, for which the reduction of insecticides is necessary (Sunagar *et al*., 2016). Nepomorpha species are used as biocontrol agents in aquatic environments for larvae of disease-vectoring mosquitoes (Walker *et al*., 2016). These advantages are explained by the venom's pharmacological enzyme activity properties (Silva-Cardoso *et al.*, 2010).

1.5 Species description

True water bugs (Nepomorpha) are ambush predators that typically prey in submerged vegetation. The family Belostomatidae is the group with most information regarding its venom (Walker *et al*., 2016), while almost nothing has been recorded of the venom of Notonectidae (e.g. *Notonecta glauca*) and Naucoridae (e.g. *Ilyocoris cimicoides*).

I. cimicoides, known as the saucer bug, is the only species representation of the family Naucoridae (Heteroptera - Nepomorpha) (*sensu* Wang *et al.*, 2019) in Germany (Strauss & Niedringhaus, 2014). The species of this family are associated with stones, stems, or static vegetation (Grazia & Fernandes, 2012) where they prey on a huge range of invertebrates (Edward, 1967; Southwood & Lesto, 2005). Although *I. cimicoides* are macropterous, the flight muscles are often not well developed so doubts of its typical dispersion method prevails (Larsen, 1970). As in most cases of the entomofauna in Europe, records about its behavior are scarce, and therefore necessary to carry out more studies in this area.

N. glauca, known as the common backswimmer, is a species of the family Notonectidae (Heteroptera - Nepomorpha) (*sensu* Wang *et al*., 2019) that is widely distributed in Germany (Strauss & Niedringhaus, 2014). The species of this family are characterized by their posterior legs modified for swimming, and by the concentrated hemoglobin in the abdominal segments III to VII (Grazia & Fernandes, 2012). Like *I. cimicoides*, its diet is strictly carnivorous (Southwood & Lesto, 2005), but its biology and ecology are poorly understood. The studies conducted so far have focused on the super-hydrophobic surface chemistry for low friction fluid transport and friction reduction on ship hulls using *N. glauca* as a model (Ditsche-Kuru *et al*., 2011).

Like other predatory heteropterans, *I. cimicoides* and *N. glauca* have a complex venom represented by proteins and peptides able to cause paralysis, tissue liquefaction, and death in

some invertebrates, and pain in vertebrates (Walker *et al*., 2018c). In addition, *I. cimicoides* and *N. glauca* have a complex mouthpart where the mandibles secure the prey's cuticle, and then the maxillary is pushed forward in the EOD process (Cobben, 1978). Nevertheless, there has not been any study about the venom system of *I. cimicoides* and *N. glauca*, and therefore, their venom composition is still unknown.

1.6 Problem description

True Water bugs are well known predatory hemipterans. Despite their ecological function in aquatic environments, the venom composition of water bugs is poorly documented. In the present study, we characterized the biological activity of venom from *I. cimicoides* and *N. glauca* through the interpretation of transcriptomic and proteomic data and by performing bioassays. In addition, we compared our study species with the African assassin bugs *P. biguttatus* and *P. horrida* due to its recent venom characterization carried out by Fischer *et al.* (2020).

1.7 MATERIALS AND METHODS

1.8 Insect collection

We captured *I. cimicoides* and *N. glauca* specimens in the suburbs of Jena (Thuringia - Germany) between July and October 2020. The identification was carried out following the key of Strauss & Niedringhaus (2014). Individuals were housed in containers with cold water for one or two weeks under laboratory conditions and fed regularly with moth larvae (*Galleria mellonella*). The terminology for true water bugs is based on Torres & Boyd (2009).

P. horrida and *P. biguttatus* specimens were obtained from an insectarium and housed in a terrarium at room temperature. The bugs were fed with moth larvae (*G. mellonella*) and Jamaica field crickets (*Gryllus assimilis*).

2.2 Venom collection and preservation

Animals were placed at -20 °C for 5 minutes, then decapitated and dissected under a stereoscope in 1x Phosphate-buffer saline (PBS). Whole glands including PMG, AMG, and AG from several individuals were placed in separate ice-cold 1.5 ml Eppendorf tubes. Later on, the samples were centrifuged for 4 minutes at 4000 g to remove cellular debris. The supernatant was transferred to a new Eppendorf tube and the protein concentration was measured using a Nanophotometer N60 (Implen). The samples were labeled and stored at -20 °C until analysis.

For the identification of stimulus-dependent venom from *I. cimicoides* and *N. glauca*, we prepared a Parafilm dummy (Fig. 1). To obtain defense venom (DV), bugs were first anesthetized at -20 °C for 5 minutes and then restrained by securing the abdomen with a rubber band. Then, gentle harassment by tweezers on the abdomen induced the ejection of venom into the dummy. Next, the content of the dummy was released into an Eppendorf tube and its protein concentration was measured using an N60 Nanophotometer (Implen). The samples were stored at -20 °C until analysis.

Hunting venom (HV) was acquired using a dummy, which was introduced into the water simulating the swimming of a small insect (Appendix video A1). We waited until the bugs hunted the dummy and deposited venom for at least 30 seconds. Afterwards, the dummy was washed in PBS and its content was released into an Eppendorf tube. Protein concentration was measured using an N60 Nanophotometer (Implen). The samples were stored at -20 °C until analysis.

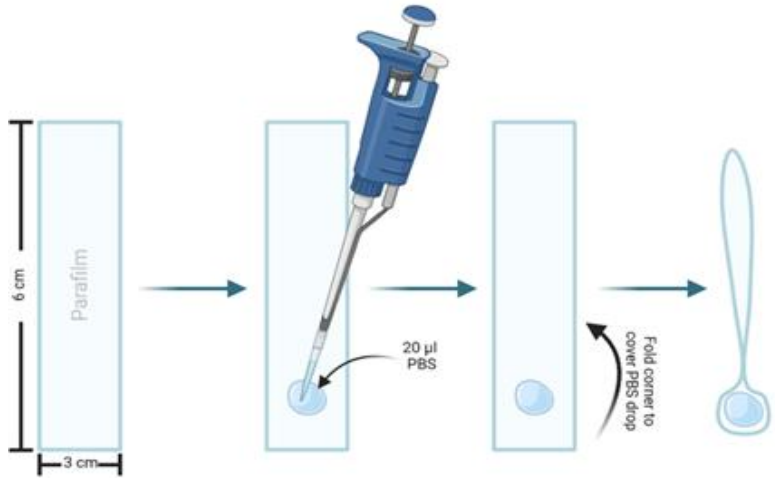

Fig. 1. Schematic of the dummy used for the collection of hunting and defense venom from *I. cimicoides* and *N. glauca.*

2.3 Toxicity assays

2.3.1 Lethality LD_{50}

Jamaica field crickets (*G. assimilis*) and honeycomb moths (*G. mellonella*) were injected with 2 µl (2 µg/µl) PMG and AMG venom or sterile water as a control using a manual microsyringe (World Precision Instrument) (n = 5). Even though the favored sites of injection of some Heteroptera are legs, neck, and antennae (Walker *et al*., 2016), we injected the venom in the second abdominal tergite to avoid the early death of the insects. The syringe was washed between treatments with Millipore water and 70% ethanol to avoid cross-contamination. The number of paralyzed or dead crickets was recorded 1 min, 1 hour, 24 hours, and 48 hours after injection. The LD_{50} was calculated in the statistical program R with the "HelpersMg" package (Girondot, 2020, Version 4.3)

2.3.2 Cell viability assay

We tested the cytotoxic effect of *I. cimicoides* and *N. glauca* venom on *Spodoptera frugiperda Sf*9 cells. Bianca Wurlitzer cultivated the cells at the Max Planck Institute and seeded them into sterile 96-well cell culture plates at approx. 70% confluence. After 24 hours of incubation at 27 °C, the culture medium was replaced by 100 µl of the treatment (10 µl of PMG and AMG [10 µg/µl, 2 µg/µl, 0.1 µg/µl] previously diluted in 990 µl of medium). We used 70 µl Triton x100 diluted in 630 µl medium as a positive control and PBS as a negative control. Additionally, cells A7-12 were empty all the time to later be used as blank in the data measurement. The plate was incubated for 4 hours at 27 °C. Then, the medium was removed and 100 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dilution (5 mg/ml) were added, and incubated for 2 hours at 27 °C. Afterwards, MTT was removed and 50 µl of Dimethyl sulfoxide (DMSO) was added. After 10 minutes of incubation at 27 °C, the plates were briefly vortexed and the absorbance was measured at 540 nm in a plate reader (TECAN. Infinite N200. 609000030).

For analysis, for each value, the average absorption value of DMSO was subtracted and the average of each treatment was plotted.

2.3.3 Calcium imaging of *Drosophila sp.* Neurons

Calcium imaging experiments were performed on 7 day-old *Drosophila* sp. females carrying the UAS_GCamPsu transgene. The dissection was done by Benjamin Fabian at the Max Planck Institute for Chemical Ecology, using previously described methods (Mohamed *et al.*, 2019). Fluorescence corresponding to $[Ca^{2+}]$ was monitored using a 2-photon laser scanning microscope (Zeiss LSM 710 meta NLO) equipped with an infrared Chameleon UltraTM diode-pumped laser (Coherent, Santa Clara, CA, USA) and a 40x water immersion objective lens (W Plan-Apochromat 40x/1.0 DIC M27). The fluorophore of GCaMP was excited at 925 nm. Fluorescence was collected with an internal GaAsP detector. A droplet of 40 ul Ringer solution was placed on the exposed fly brain and baseline fluorescence was monitored for 30 s Then, 5 µl (18 µg/µl) of PMG venom from *I. cimicoides, N. glauca,* or *P. horrida,* were added and the calcium signals were monitored. Flies treated with *I. cimicoides* and *N. glauca* venom were scanned for 20 min, while flies with *P. horrida* venom were scanned for 5 min. After that, we tested whether the insects still responded to the odor 3-hexanone.

All records were corrected for movement and the fluorescence was calculated in ImageJ. The raw fluorescence signals were transformed to $\Delta F/F_0$, where F_0 is the averaged baseline fluorescence. The results were plotted in RStudio.

2.4 Proteomic and transcriptomic analyses

Fischer, M. (2020) provided the proteomic and transcriptomic data from *N. glauca* (Appendix material A1, A2, and A4). For *N. glauca,* we tested whether the presence/absence of a protein in the proteomic set was related to its expression in the transcriptome (Appendix method A1). Additionally, each gland compartment was analyzed separately and we further eliminated proteins with BLAST homology to intracellular process proteins (e.g. RNA polymerase). Fischer, M. (2020) performed the proteomic contrast analysis for *I. cimicoides.* Each data set was manually reviewed. Finally, we grouped the results for both species (Appendix material A4 and A5). The proteins were grouped by homology or function (Table.1), and subsequently plotted against the RPKM values obtained from transcriptomic analysis.

Table 1. Characteristic of proteins grouping by homology or function.

Homology/function	Criteria for inclusion
Carbohydrases	No amylases but other carbohydrases
Hemolysis	Proteins responsible for the lysis of other cells.
Hydrolases	Other hydrolases such as phosphatases, esterase, hyaluronidases, and nucleosides. Group all enzymes that use water for their catalytic activity.
Others	Proteins that play an important role in specimen development. Metabolic proteins present in different developmental stages.
Protease	S1 family, endopeptidases, trypsin precursor, serine-proteases, and peptidases. Proteins with proteolytic function.
Transferase	Enzymes that transfer functional groups across membranes or intercellularly
Uncharacterized	No detectable homology with BLAST and GO searches or proteins without a specific function
Venom protein	Uncharacterized proteins, phospholipases, nucleases, proteases. All protein had the “venom” denomination in the BLAST result.

2.5 SDS-PAGE

Venom samples from *N. glauca* were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-12% Criterion ™ XT Bis-Tris Protein Gel alongside a Novex Sharp Pre-Stained molecular-weight marker (No 57318, Invitrogen). For each treatment, we used 30 µg of venom with a volume of not more than 30 µl and mixed it with XT sample buffer + reducing agent in a 3:1 ratio. The samples were boiled for five minutes at 95 °C before being loaded into the gel. Electrophoresis was carried out with XT MES running buffer at 125 V for one hour. After electrophoresis, the gel was washed with Millipore water three times for 10 min. Then, the gel was stained with 20 ml of Imperial^{Tm} Protein Stain (No 24615, Thermo Fischer Scientific) and 20 ml of Page Blue^{Tm} (No R0571, Fermentas Life

Science) for two hours with gentle shaking. Finally, it was destained with Millipore water for one hour and scanned. Fischer, M. (2020) provided the SDS-PAGE gel from *I. cimicoides*.

2.6 Enzyme activity

Venom gland extracts (PMG and AMG) from *I. cimicoides* and *N. glauca* were evaluated for a variety of quantitative enzyme profile studies (lipase, protease, amylase, and elastase). As a comparison, the venom from *P. biguttatus* and *P. horrida* was analyzed.

For the enzyme activity assays, we subtracted the values of the negative control from the values for other wells. The results we plotted as a relative fluorescence unit (RFU) versus time. For the protease and elastase activity assays were determined the optimal range (10 to 15 minutes) to obtain the initial reaction velocity (V_o). Likewise, in lipase and amylase activity assays the lipase activity was determined by the following formula:

$$\text{Enzyme activity} = \frac{(B * Dilution\ factor)}{(t2 - t1) * V}$$

Where B is the product amount from the standard curve, T_1 is the time for the first reading, T_2 the time for the second reading and V is the sample volume (ml) added into the reaction well.

2.6.1 Protease activity

Protease activity of PMG and AMG venom (except AMG from *I. cimicoides*) of the four species was determined with a protease activity assay kit (No. ab112152, Abcam) with fluorescent casein conjugate as substrate. Venom extract (50 µl with a concentration of 2 µg/µl) was added to 96 well-microplates and mixed with 50 µl of substrate dilution (1:100 protease substrate to assay buffer). Trypsin provided by the kit was used as a positive control (dilution 1:50 Trypsin to water), and PBS was used as a negative control. Fluorescence intensity was read at 490/525 nm (Ex/Em) in a plate reader (TECAN. Infinite N200. 609000030) every five minutes for 60 minutes at 25 °C. The initial reaction velocity (Vo) in

RFU/min was calculated as the amount of product made per unit of time. Each treatment was assayed in triplicates.

2.6.1.1 Zymogram Gel

Novex Zymogram Plus Gels (1 mm, 12 well No. ZY00102BOX, Thermo Fischer Scientific) that use gelatin as substrate were used for the visualization of proteases from *I. cimicoides* and *N. glauca* venom. For each treatment 25 µl of venom with a concentration of 1.2 µg/µl were mixed 1:1 with tris-glycine SDS sample buffer. Electrophoresis was carried out at 125 V for 1.5 hours. The gels were incubated for 30 minutes with 100 ml of 1x zymogram renaturing buffer at room temperature with gentle agitation. Later, the buffer was decanted and 100 ml of 1x zymogram developing buffer were added and incubated for 30 minutes. Next, the buffer was decanted and 50 ml of fresh zymogram developing buffer were added to the gel to incubate it overnight. The gel was stained with 20 ml of Imperial^{Tm} Protein Stain (No 24615, Thermo Fischer Scientific) and 20 ml of Page Blue^{Tm} (No R0571, Fermentas Life Science) for two hours with gentle shaking and destained with Millipore water for one hour. Finally, the gel was incubated with a destaining solution until bands were clearly visible and the gel was scanned.

2.6.2 Elastase activity

Elastase activity of PMG and AMG venom of the four species was determined with an Elastase activity assay kit (No. E-12056, EnzChek). The substrate was DQ elastin. Venom extract (100 µl with a concentration of 2 µg/µl) was added to 96 well-microplates. Additionally, 50 µl of elastin substrate (diluted in the 1x buffer until a final concentration of 0.2 U/ml) and 50 µl of 1x assay buffer were added to each well. An elastase supplied with the assay kit was used as a positive control (100 µl), and 1x buffer and substrate only were used as a negative control. The plate was incubated at 25 °C. Fluorescence was read at 505/515 nm (Ex./Em.) in a plate reader (TECAN. Infinite N200. 609000030) every five minutes for two hours at 21 °C. Since elastin is also digested by proteases other than elastases, we used an elastase inhibitor N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl Ketone to determine whether the proteolytic activity was the result of elastases. The inhibitor was diluted with

buffer (1:500) with the same volume used before. The initial reaction velocity (V_o) in RFU/min was calculated as the amount of product made per unit of time. Each treatment was assayed in duplicates.

2.6.3 Amylase activity

Amylase activity of PMG and AMG venom of the four species was determined with an amylase activity kit (No. MAK009, Sigma, St. Louis, MO). The substrate was ethylidene-pNP-G7. Venom extract (50 µl with a concentration of 2 µg/µl) was added to 96 well-microplates. Additionally, 50 µl of the substrate and 50 µl of assay buffer were added to each well. Amylase provided by the kit was used as a positive control (50 µl), and buffer and substrate only were used as a negative control. The plate was incubated at 25 °C. Absorbance was read at 405 nm in a plate reader (TECAN. Infinite N200. 609000030) every five minutes until the value of the most reactive sample was greater than the value of the highest standard. Absorbance was directly related to amylase activity. A standard curve was prepared by adding 0, 2, 4, 6, 8, 10 µl of 2 mM nitrophenol standard into 96 well plates. Then water was added to each well to bring the volume to 50 µl. The relative amylase activity was calculated as absorbance units per milligram of protein. Each treatment was assayed in triplicates.

2.6.3.1 DNS method

Amylase activity was measured by the 3,5-dinitrosalicylic acid (DNS) method. 5 µl (4 µg/µl prepared in 1:1 PBS and amylase 1x assay buffer) of PMG and AMG venom from *I. cimicoides* were incubated in 55 µl of 3:8 glycogen (1% p/v) or starch (1% p/v) and amylase assay buffer (No. MAK009, Sigma, St. Louis, MO) for 4 hours. Amylase provided by the amylase activity kit (No. MAK009, Sigma, St. Louis, MO) was used as a positive control. A mix 1:1 between PBS and amylase buffer was used as a negative control. In addition, inactive venom previously heated at 95 °C for 10 min was used. A mixture of solution 1 (44 mM DNS, 21 mM phenol, 250 mM NaOH) and 2 (400 mM sodium sulfite) in a 99:1 ratio (v/v) was prepared and added to the sample in a 1:1 ratio (v/v) followed by heating for 5 minutes in a PCR cycler at 99 °C. After that, Solution 3 (1.4 M Na/K-tartrate) was added in a 1:6 ratio

(v/v) and 100 µl of each reaction was transferred into a 96 well-plate. The absorbance was measured at 575 nm on a plate reader (TECAN. Infinite N200. 609000030).

2.6.3.2 TLC

Glycogen in water 1% (w/v) and potato starch in water 1% (w/v) were exposed to extracted venom of *I. cimicoides* (1.7 µl with a concentration of 4 µg/µl). The reaction was left overnight at 25 °C. A mix of 1:1 PBS and assay buffer was used as a negative control. Amylase provided by the amylase activity kit (No. MAK009, Sigma, St. Louis, MO) was used as a positive control. In addition, inactive venom previously boiled at 95 °C for 10 minutes was used. Afterwards, 10 µl of the reaction mixtures were separated on a thin-layer-chromatography (TLC) plate (Silica gel 60, 20 x 20 cm, Merck) using a mobile phase of a mixture of ethyl acetate:glacial acetic acid:formic acid:water (9:3:1:4) for about 4 hours. After evaporation of the solvents, the plates were stained by spraying them with 0.2% (w/v) orcinol in methanol:sulfuric acid (9:1) and heated until spots appeared. The reference standard contained maltose, maltotriose (28395.01, Serva), maltotetraos, maltopentaose, maltohexaose (M9153-100MG, Sigma) and maltoheptaose (M7753-50MG, Sigma).

2.6.4 Lipase activity

The lipase activity of PMG and AMG venom of the four species was determined with a lipase assay kit (No. ab118969, Abcam). The substrate was not specified in the kit. Venom extract (50 µl with a concentration of 2 µg/µl) was added to 96 well-microplates. Additionally, 50 µl of the reaction mix (2 µl lipase substrate and 48 µl of assay buffer) were added to each well. Lipase provided by the kit was used as a positive control (50 µl), and buffer and reaction mix only were used as a negative control (50 µl). A standard curve was prepared by adding 0, 2, 4, 6, 8, 10 µl of 10 µM methylresorufin standard solution into 96 well plates. Then, lipase assay buffer was added to each well to bring the volume to 100 µl. The plate was incubated at 37 °C. Fluorescence was read at 529/600 nm in a plate reader (TECAN. Infinite N200. 609000030) every five minutes for one hour. Fluorescence was directly related to lipase activity. The relative lipase activity was calculated as absorbance units per milligram of

protein. The assay was performed two times for each sample, except *P. biguttatus* AMG, in which no measurement was made.

2.7 Hemolytic activity

Hemolytic activity was tested on three different blood agar plates containing human, horse, and sheep erythrocytes. On each plate, seven holes were punched out using a sterile 5 ml pipette tip. The holes were filled with 2 µl of *I. cimicoides* or *N. glauca* venom (PMG = 50 µg/µl, 5µg/µl, and 1 µg/µl, AMG = 5 µg/µl and 1 µg/µl), 2 µl of PBS as the negative control, or 2 µl of Triton-x100 as a positive control. For each blood type and species, triplicates were made. The plates were incubated at 37 °C for 24 hours. In the end, pictures of the plates were taken and analyzed for hemolytic zones.

2.8 Antibacterial Assay

2.8.1 Growth of bacteria

Escherichia coli was grown on Luria-Bertani (LB) agar at 37 °C overnight. Next, a colony was chosen and incubated in 5 ml of LB medium at 37 °C overnight with gentle shaking.

2.8.2 Plate growth antibacterial assay

50 µl of the overnight culture (method 8.1) was diluted in 50 ml of molten LB agar (approximately 50 °C), mixed, and poured into Petri dishes. The plates were cooled down to room temperature for 15 minutes. Then, seven holes were punched out using sterile 5 ml pipette tips. 2.5 µl of *I. cimicoides* and *N. glauca* venom (PMG = 50 µg/µl, 10 µg/µl, and 1 µg/µl, AMG = 10 µg/µl and 1 µg/µl), 2.5 µl PBS, or 2.5 µl of gentamicin were deposited inside the holes. Control plates were spotted with PMG, AMG, and buffer. For each species, triplicates were made. Finally, the plates were incubated overnight at 37 °C and analyzed for

inhibition zones. In the end, pictures of the plates were taken and analyzed for zones with no growth of *E. coli.*

2.8.3 Liquid growth antibacterial assay

50 µl of the overnight culture (method 8.1) was diluted with 5 ml of LB medium. The dilution was incubated at 37 °C for three to four hours with gentle agitation. Afterward, the culture was diluted to give an optical density (OD) of 0.003 A_{600} (Dani *et al*., 2003). In a 96-well plate, 50 µl *I. cimicoides, N. glauca,* and *P. biguttatus* venom were placed and 100 µl of diluted bacterial suspension were added to each well. As a negative control, 50 µl of LB medium were placed (no venom), and a blank with only medium (150 µl of LB medium). For each species, triplicates were made. The OD_{600} of the culture in each well was measured in a plate reader (TECAN. Infinite N200. 609000030) every five minutes for 46 hours at 30 °C with shaking. The blank was subtracted for each well.

2.9 Statistical analysis

Data were analyzed using R statistical software (Version 4.0.3, 2020-10-10). Plots were prepared using the ggplot2 package (Pedersen. T version 3.3.2).

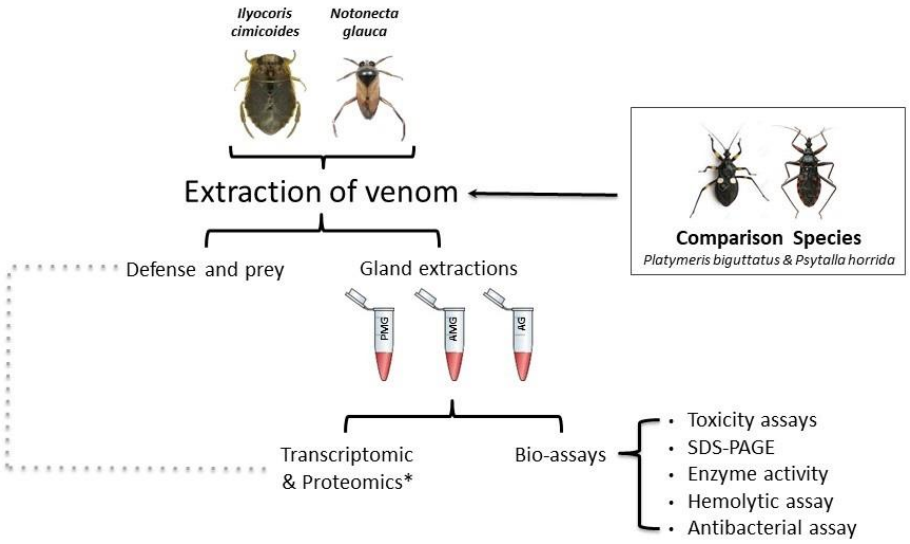

Fig. 2. Schematic workflow to characterize the biological activity of the venom from the species *I. cimicoides* and *N. glauca.* The species *P. biguttatus* and *P. horrida* were used as comparisons.* Data provided at the Max Planck Institute for Chemical Ecology.

3. RESULTS

3.1 Venom system in *I. cimicoides* and *N. glauca*

To obtain venom from the salivary glands complex we dissected several individuals of *I. cimicoides* and *N. glauca*. The salivary glands of both species consisted of a paired multilobed principle gland and an accessory gland (Fig. 3B and C). The anterior gland was always shorter than the posterior gland with a clear constriction between both glands. In *I. cimicoides*, the main gland is located in the prothoracic region extending up to the head. In *N. glauca*, the complex main gland and accessory gland are placed across the postocciput. To compare the total protein concentration per gland and species, we averaged the amount of protein of PMG, AMG, and AG in 10 individuals each. The highest protein content was observed in the PMG *(I. cimicoides* = 144.4 µg, *N. glauca* = 89.68 µg) followed by the AMG (*I. cimicoides* = 16.4 µg, *N. glauca* = 28.42 µg) and AG (*I. cimicoides* = 3.8 µg, *N. glauca* = 5.06 µg) (Fig. 3A).

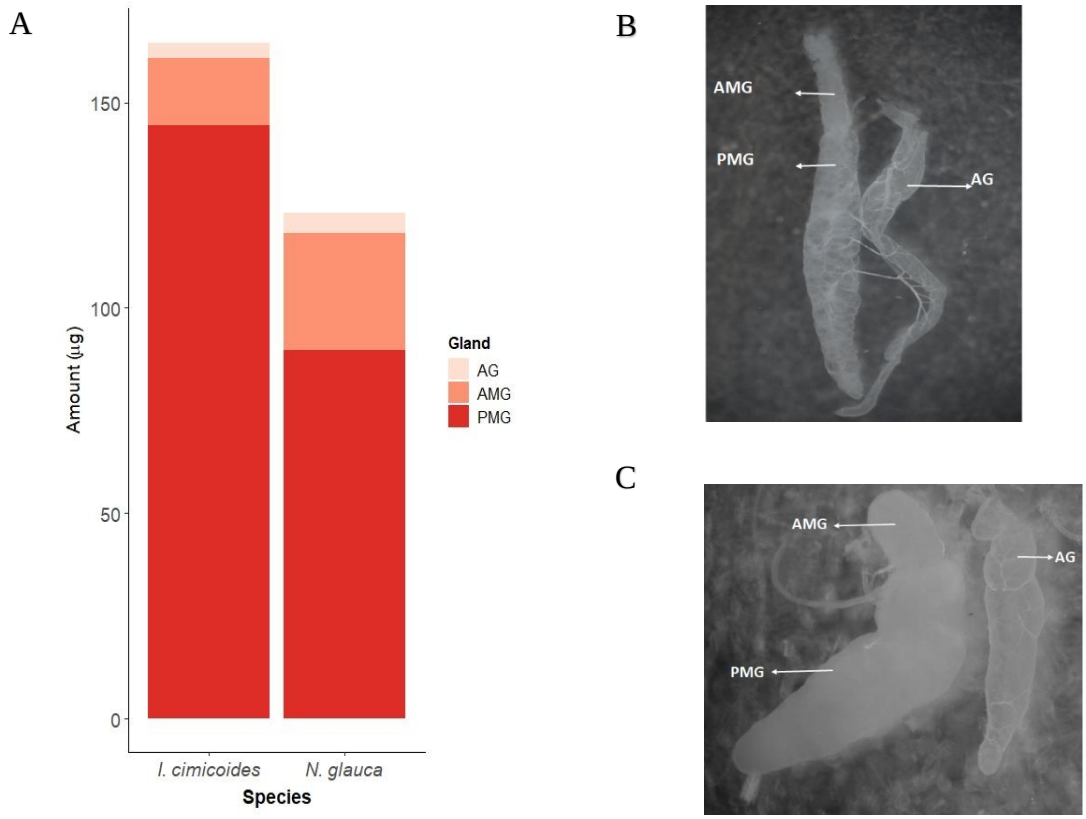

Fig 3. Venom system in *I. cimicoides* and *N. glauca* (A) venom amount per gland of the two species (n = 10) (B) venom glands of *I. cimicoides*. PMG, posterior main gland. AMG, anterior main gland. AG, accessory gland. (C) venom glands of *N. glauca.* Photos taken by Yepes V. 2021

To examine the molecular mass profile of *I. cimicoides* and *N. glauca* venom, we performed SDS-PAGE gel electrophoresis of both species. SDS-PAGE separates a collection of proteins present in a cellular extract, depending on the mass of each protein (Bernot, 2004). In the end, the gels revealed multiple protein bands in the five samples (PMG, AMG, AG, DF, and HV) with a concentration of ~1 µg/µl. In *I. cimicoides,* PMG showed bands ranging from 3.5 to 260 kDa, with especially strong bands of Ma > 160 kDa, 50 kDa, 30kDa, and 20 kDa. AMG showed bands between 260 to 10 kDa, being clear three bands >50, >40, and >30, and AG a less complex protein composition with a Ma between 260 to 15 kDa (Fig. 4A). On the other hand, *N. glauca*, showed proteins between 80 to 15 kDa with an accumulation of proteins between 40 - 20 kDa in the PMG, at the AMG the bands are well distributed between 80 to 15 kDa, and a light band > 260 kDa, and finally, AG showed a collect of bands between 260 and 15 kDa (Fig. 4B).

3.2 Defense and predation yield similar venom to PMG

Since it was previously reported that the lobes PMG and AMG and the AG have different functions in the process of overwhelming prey and defense, we investigated if that was also true for *I. cimicoides* and *N. glauca.* To this end, we collected defense and hunting venom from several individuals of both species, using different venom collection techniques. Unlike electrostimulation (Swart *et al*., 2006; Walker *et al*., 2019), these techniques allow the collection of venom reflecting more natural situations (Fischer et al, 2020). The venoms were analyzed on SDS-PAGE gels. Venom obtained by harassment and simulation of the prey resulted in similar protein bands as the PMG venom (Fig. 4A and B). Nevertheless, the presence of other bands accords with the venom from the AMG (Fig. 4A and B).

3.3 Proteome and transcriptome of *I. cimicoides* and *N. glauca*

The transcriptome is a set of coding and non-coding RNA molecules (transcripts) present in a given sample (Bernot, 2004). Fischer *et al.* (2020) provided the transcriptomic data for *I. cimicoides* and *N. glauca* (unpublished data). The proteome is the collection of proteins produced by tissue at a given moment (Bernot, 2004). The proteome of both species was analysed at the MPI-CE. To determine the protein composition of *I. cimicoides* and *N. glauca*

venom, we generated a library of proteins present in main glands (PMG and AMG) and tested whether the presence/absence of a protein in the proteomic set was associated with its expression in the transcriptome (see methods). The criteria for the homology or function of some proteins are shown in Table 1.

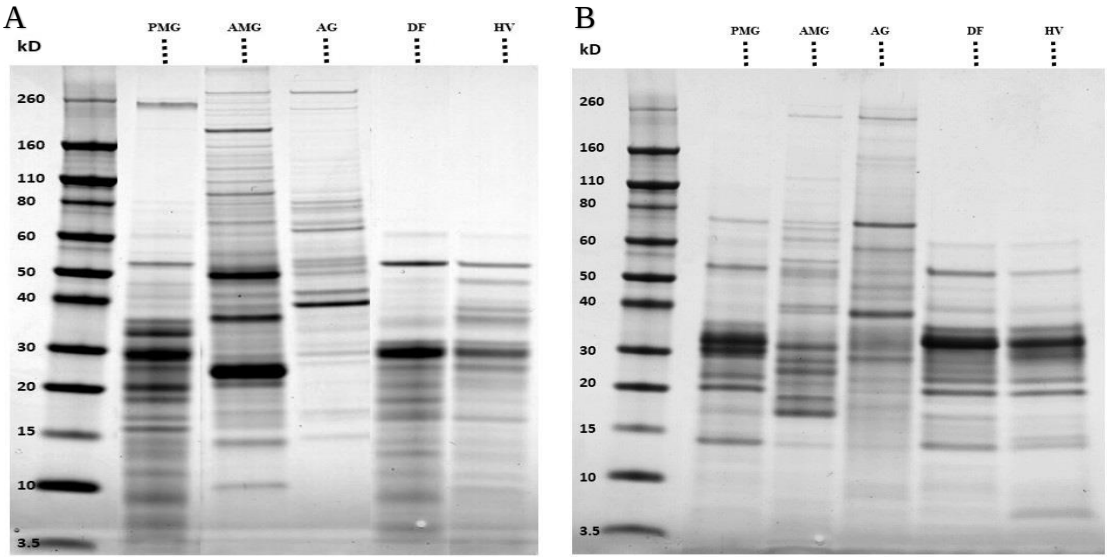

Fig 4. SDS-PAGE of the venom from the three glands (Posterior main gland –PMG-, Anterior main gland –AMG- and Accessory gland –AG-) of *I. cimicoides* (A) and *N. glauca* (B) and the comparison with the venom of defense (DV) and hunting behavior (HV).

For *I. cimicoides* 139 predicted secreted proteins were transcribed in the main gland (PMG = 86, AMG = 534). For *N. glauca* 276 proteins were transcribed (PMG = 129, AMG = 147). We grouped the peptides and proteins into 12 groups according to their homology or putative functional activity. We observed differences in terms of the enrichment of proteins between the two glands (PMG and AMG) for both species. At the homology and functional level, the most abundant encoded transcripts of *I. cimicoides* were proteases (PMG = 376.59 RPKM, AMG = 115.96 RPKM) followed by venom protein family members (PMG = 124.04 RPKM, AMG = 88.02 RPKM) (Fig 5A). In contrast, for *N, glauca* the most abundant proteins were members of the venom protein family (PMG = 186.55 RPKM, AMG = 80.30 RPKM), followed by proteases (PMG = 88.55 RPKM, AMG = 30.74 RPKM) (Fig. 5B). Besides, *I. cimicoides* included unique proteins as amylases, hemolysin-like proteins, and lyase. On the other side, transcriptomic and proteomic data of *N. glauca* resulted in the identification of carbohydrases other than amylases and serine protease inhibitors. The data demonstrates that the venom of both species was enriched in a number of enzymes that might help the insects to feed on other animals.

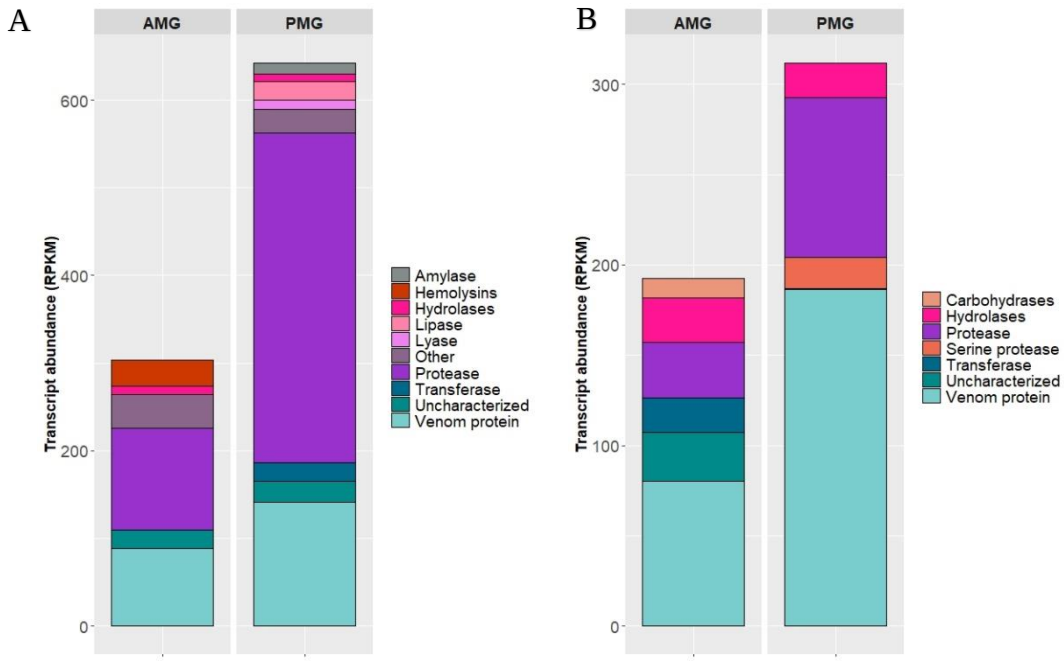

Fig 5. Transcript abundance of the major classes of proteins in the posterior main gland (PMG) and the anterior main gland (AMG) of *I. cimicoides* (A) and *N. glauca* (B).

3.4 Enzyme activity in the water bugs venom

3.4.1 Qualitative enzyme activity

To evaluate the enzymatic activity from the PMG and AMG venom of four species of Heteroptera, we performed four different enzymatic activity assays (protease, elastase, amylase, and lipase activity assays). Each of the assays provides the speed and sensitivity for measuring the enzyme activity in each treatment. The enzymatic activity assays yielded a positive reaction for three enzymes in the salivary gland extracted from *I. cimicoides* (proteases, lipases, and amylases) and two for *N. glauca* (proteases and lipases) (Table 2). Both comparison species yielded positive activity for the protease and lipase assay (Table 2). The PMG venom of *I. cimicoides* obtained the highest values for amylase (21.49 Umol/ml), and protease (22.23 Umol/ml) activity, and the PMG venom of *P. biguttatus* gained 21.51 Umol/ml for lipase activity.

Table 2. Digestive enzyme activity in four Heteroptera species. One unit is the amount of enzyme that hydrolyses the substrate to yield 1.0 µmol of product per minute. For protease activity, the units are RFU/min and for the other two assays are Umol/ml.

Enzyme Activity	*Ilyocoris cimicoides*		*Notonecta glauca*		*Platymeris biguttatus*		*Psytalla horrida*	
	PMG	**AMG**	**PMG**	**AMG**	**PMG**	**AMG**	**PMG**	**AMG**
Protease	21.49	-	18.47	1.12	21.85	5.83	14.70	3.37
Elastase	0	0	0	0	0	0	0	0
Amylase	22.23	12.52	0	0	0	0	0	0
Lipase	15.34	10.50	17.46	6.46	21.51	-	18.17	4.02

3.4.2 Protease detection assays

Proteases are enzymes present in all species, from bacteria to vertebrates, and are involved in almost all fundamental processes in the cell (Cohen, 1998). In addition to the wide range of cellular housekeeping roles, these proteins are probably the most important liquefaction enzymes for predators since they attack several structural proteins. Since protease activity has been documented in heteropteran predators (Cohen, 1998; Boyd *et al*., 2002; Fischer *et al.*, 2020), we performed a protease activity assay for four heteropteran species. Protease activity was detected in both glandular lobes of the four species with a higher value in the PMGs (50 µl with a concentration of 2 µg/µl) (Fig. 6A). Of the venom treatments, yielded neither a similar nor a higher activity than the positive control (dilution 1:50 trypsin to water) (Fig. 6A). To visualize the protease activity of the PMG and AMG venom from *I. cimicoides* and *N. glauca*, we separated the proteins on a zymogram gel. These gels are used to detect proteases that utilize gelatin as a substrate. The zymogram gels showed a large variety of proteases in the PMG of *I. cimicoides* and a very clear band in *N. glauca* (Fig. 6B and C). The AMG bands of both species were not as clear as from the PMG (Fig. 6B and C).

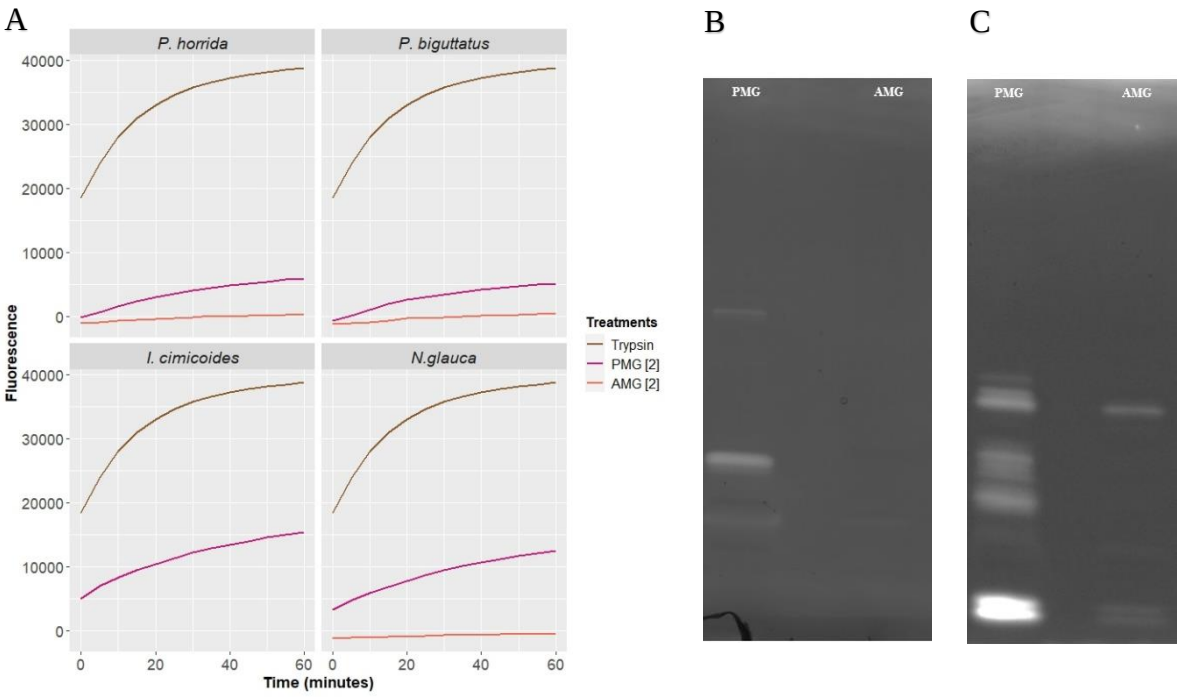

Fig 6. Protease activity assays (A) protease activity in fluorescence (490/525 nm) vs time (60 min) in four Heteroptera species. Zymogram gel of *I. cimicoides* (B), and *N. glauca* (C).

3.4.3 Lipase activity assay

Lipids are an essential structural component of insects' cell membranes and cuticles. Predaceous insects need to break down these lipids through hydrolytic enzymes defined as lipases (Rockstein, 1978). To identify lipases in PMG and AMG venom from the study species, we performed a lipase activity assay. The assay allows us to identify enzymes that catalyze a specific substrate to generate a fluorometric product. In the lipase activity assay, all species showed a positive activity of PMG and AMG venom (Fig. 7). Nevertheless, the enzymatic activity was higher in the PMG venom (Table. 2). For the AMG of *P. biguttatus* there was not enough venom to perform the assay.

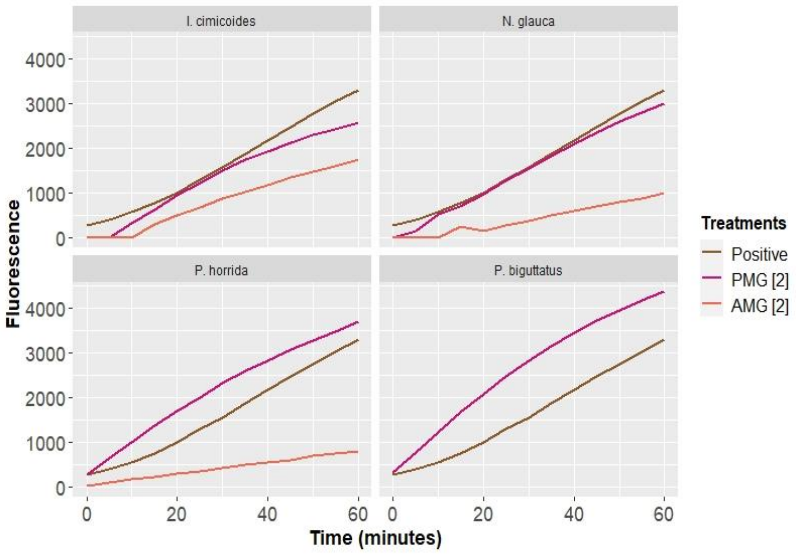

Fig7. Lipase activity in fluorescence (529/600 nm) vs time (60 minutes) in four Heteroptera species.

3.4.4 Elastase detection assay

Elastase is a protease that breaks down proteins of connective tissue as elastin (Christeller *et al*., 1990). The elastase assay measures elastase activity by elastin digestion. The digestion of elastin provided by the elastase activity assay showed a positive result for the PMG (100 µl with a concentration of 2 µg/µl) venom of *I. cimicoides* and *N. glauca* (Fig. 8A). Nevertheless, in presence of the elastase inhibitor N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl Ketone (1:500, inhibitor (10 mg/ml): 1x buffer), both species successfully degraded the substrate, suggesting that the digestion without inhibitor resulted from other proteases (Fig. 8B). Therefore, for none of the four species, elastase activity could be confirmed.

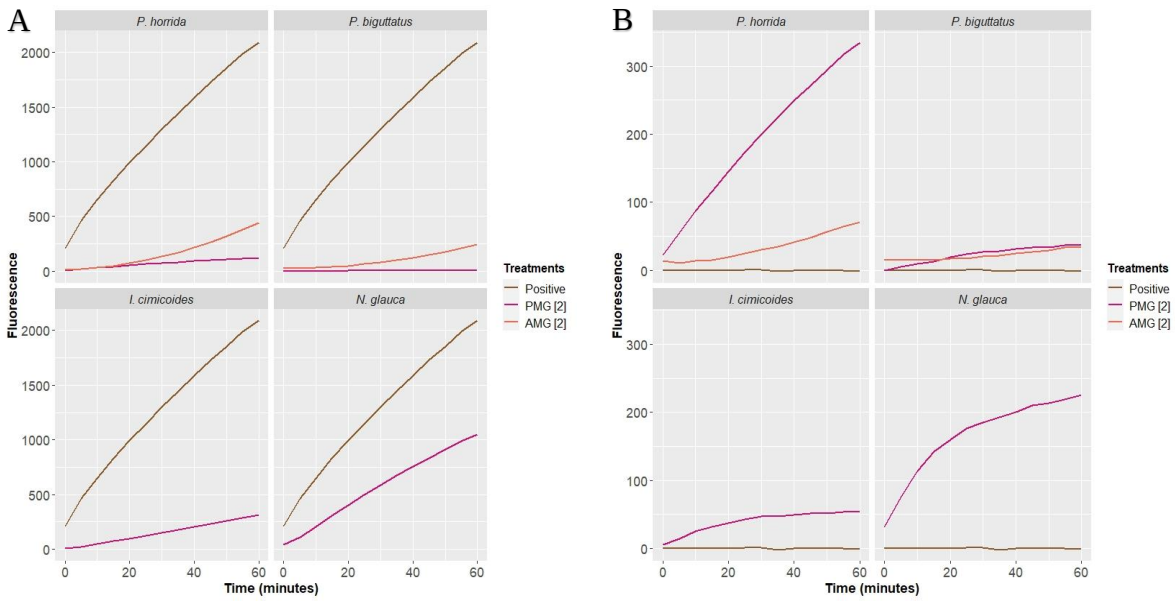

Fig 8. Elastase activity assays. (A) Elastase activity in fluorescence vs time in four Heteroptera species. (B) Elastase activity assay with the presence of elastase inhibitor *N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl Ketone.*

3.4.5 Amylase detection assays

Amylase enzymes are hydrolases that break down glucan linkages in polysaccharides such as starch in plants or glycogen in animals (Ghamari *et al*., 2014). The amylase activity assay kit quantifies amylase activity by a colorimetric product proportional to the amount of substrate cleaved by the amylase. *I. cimicoides* was the only species with positive results in the amylase activity assay for both glands (Fig. 9A). The previous results were corroborated first by the DNS method (data not shown). Afterwards, the TLC showed a degradation of starch and glycogen to their trisaccharides from the AMG (Fig. 9B) and disaccharides from the PMG (Fig. 9C). For the other three species, the TLC did not show any clear spot in the gel, suggesting no degradation of the two substrates (Fig. 9B).

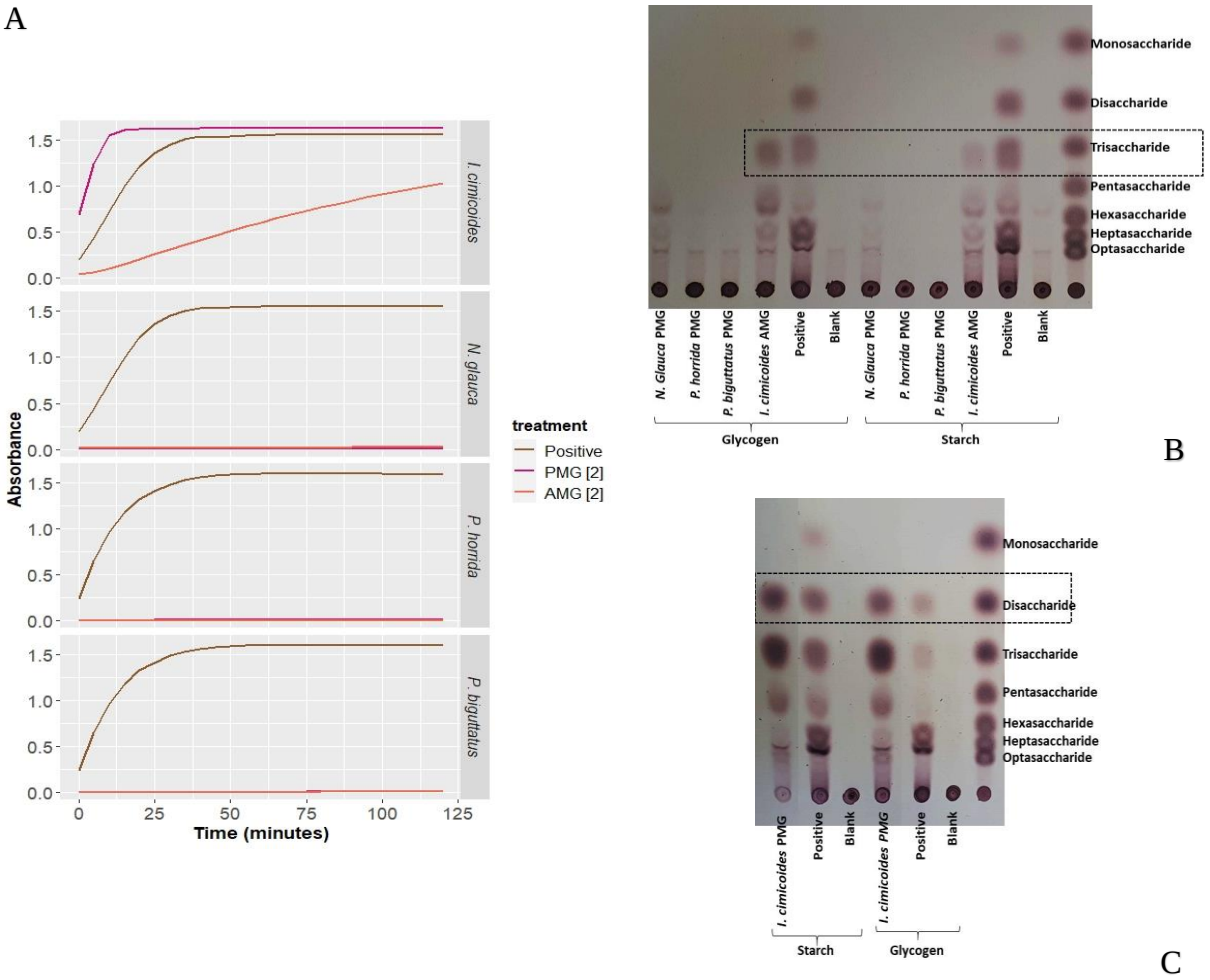

Fig 9. Amylase activity assays. (A) Amylase activity in absorbance (405 nm) vs time (120 min). (B) TLC of the PMG venom from *P. biguttatus*, *P. horrida*, and *N. glauca*, and the AMG of *I.* cimicoides. (C) TLC of the PMG venom from *I. cimicoides.*

3.4.6 Hemolysis detection

Each milliliter of vertebrate blood contains numerous erythrocytes or red blood cells. Mammalian erythrocytes are flat, disc-shaped cells without nuclei that serve primarily to transport oxygen (Sherwood *et al*., 2013). To visualize if our species are able to lysis mammal erythrocytes we performed a hemolysis detection assay in three different blood agars: human, horse and sheep erythrocyte. *I. cimicoides* showed positive hemolysis activity with a concentration of 50 µg/µl of the PMG venom in the human and horse blood agar. (Fig. 10B and C). However, *N. glauca* did not show any positive result for the hemolysis activity for both the AMG and PMG (Fig. 10E, F, and G).

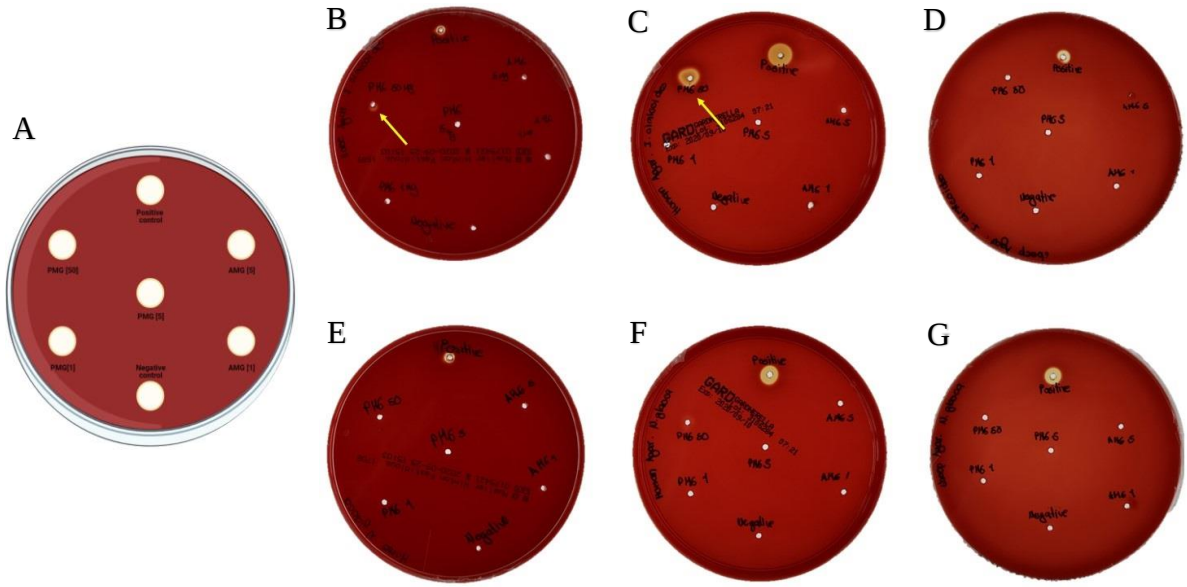

Fig. 10. Hemolysis detection of the venom from *I. cimicoides* and *N. glauca.* (A) Diagram of the treatment per each blood agar. *I. cimicoides* horse blood agar (B), human blood agar (C), and sheep blood agar (D). *N. glauca* horse blood agar (E), human blood agar (F), and sheep blood agar (G). The yellow arrows represent the lysis of the blood agar in the venom treatments.

3.4.7 Antibacterial proteins

The complex mixture of proteins and peptides in venomous animals can also exhibit a wide range of pharmacological activities including antibacterial activity. Some venoms from insects such as bees and wasps have previously been shown to be effective against bacteria (Dani *et al*., 2003). Because of that, we elaborated a series of experiments to identify potential antibacterial activity of *I. cimicoides* and *N. glauca* venom. Both *I. cimicoides* and *N. glauca* did not show antimicrobial activity against *E. coli* in the plate growth assay (data not shown). Since the liquid assay is more sensitive (Dani *et al*., 2003), we evaluated the antibacterial activity of *I. cimicoides*, *N. glauca*, and *P. biguttatus* venom against *E. coli*. *P. biguttatus* venom (PMG = 100 ug), inhibited the growth of the gram-negative bacteria for the first 30 hours, but the venom from the other two species did not show any growth inhibition (Fig. 11).

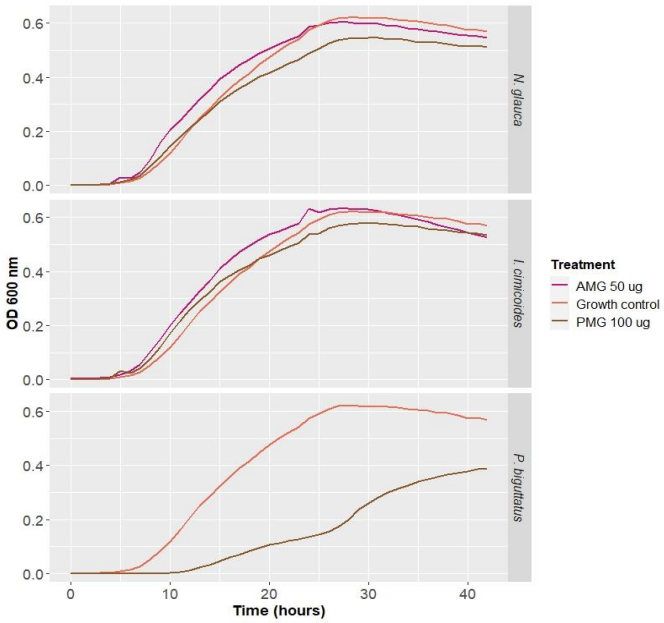

Fig. 11. Inhibition of *E. coli* growth by *I. cimicoides, N. glauca,* and *P. biguttatus* venom. The absorbance (600 nm) was recorded every five minutes for 44 hours.

3.5 Venom bioactivity

To investigate the biological activity of *I. cimicoides* and *N. glauca*, different concentrations of PMG (10 µg/µl, 20 µg/µl, and 50 µg/µl) and AMG (2 µg/µl and 10 µg/µl) venom were injected into Jamaica field cricket (*G. assimilis*) and honeycomb moth larvae (*G. mellonella*). The behavior of the larvae did not change across the experiment with either of the two species venom (data not shown). The behavior of crickets at the first minute injected with 2 µl water was unaltered, whereas crickets injected with 2 µl PMG venom (estimated concentration of 20 µg/µl and 50 µg/µl) from *N. glauca* exhibited a reduced motor activity and muscle spasms within the first seconds, reversible after one minute.

To corroborate our results we performed the colourimetric MTT assay. This assay allowed us to evaluate the cell viability of *S. frugiperda Sf9* cells. The metabolic active cells reduce the MTT to an insoluble violet-blue formazan, which can be measured using spectrometry. Therefore, the amount of MMT formazan is directly proportional to the number of living cells (Stockert *et al.*, 2011). The result did not show a clear difference between the treatments and the negative control (Fig, 12A and, B). Nevertheless, to corroborate the result we performed a pairwise test in which the values between the negative control and the different treatments. Only two treatments in *I. cimicoides* (AMG [0.2 and 20]) showed a significant difference with

95% confidence. Nevertheless, *N. glauca* venom only had one significant difference below the negative controls with the AMG venom at a concentration of 20 µg/µl. The concentration at 0.2 and 20 µg/µl showed significant differences above the negative control showing a major viability of the cells in these two treatments.

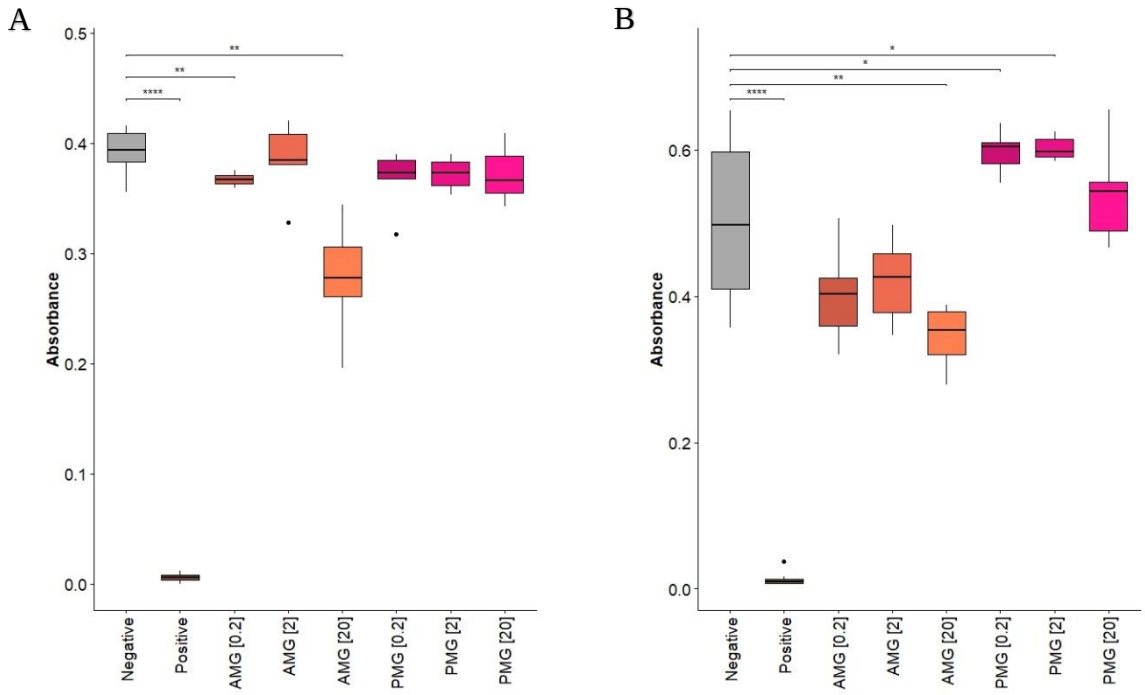

Fig. 12. MTT measure in absorbance (540 nm) of the venom from *I. cimicoides* (A), and *N. glauca* (B). The orange results refer to AMG concentrations and the pink color indicates PMG concentrations. For *I. cimicoides* the treatments AMG [20] and AMG [0.2] showed a significant difference compared to the negative control (p = 0.01347, P = 0.000111, respectively). For *N. glauca* AMG [20] had a significant difference compared to the negative control (p = 0.00474), showing a mean below the negative.

Finally, we tested the effects of venom of *I. cimicoides* and *N. glauca* on *Drosophila* sp. female neurons. The genetically encoded calcium indicator (GECIs) allows scientists to visualize the calcium dynamics in the animal target due to a conformational change that induces fluorescence in the fluorescent protein (Barykina *et al*., 2017; Mohamed *et al.*, 2019). For the *N. glauca* venom, a decrease in intracellular calcium concentration was observed in the first 10 minutes (Fig. 13F and H)but *I. cimicoides* did not show any significant changes (Fig. 13 A, B, C, and D) in the calcium response assays.

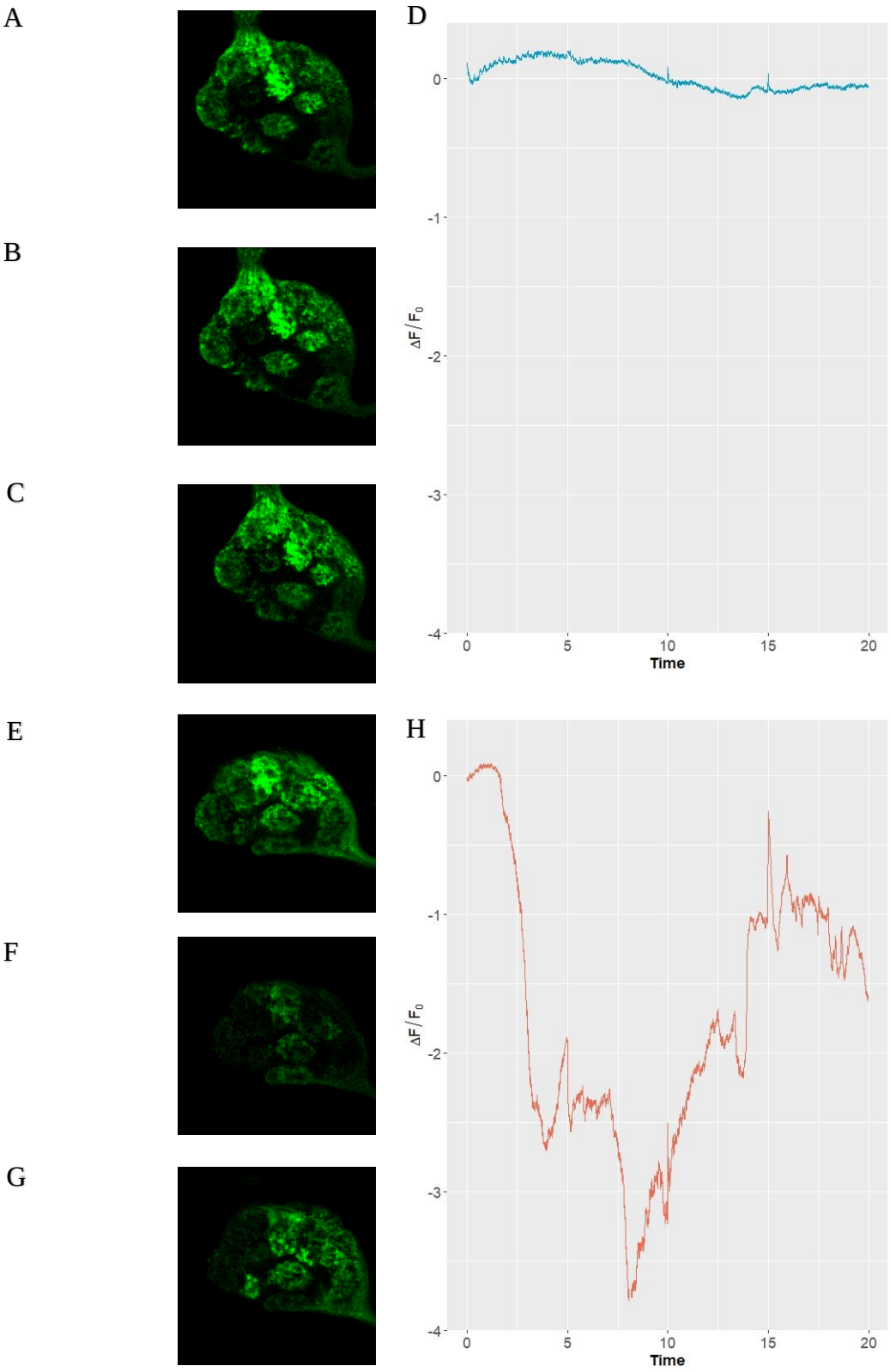

Fig. 13. Representative calcium responses in *Drosophila* sp. neurons after treatment with PMG venom from *I. cimicoides* (A, B, C and D) and *N. glauca* (E, F, G, and H). A and E represent the antennal lobe of the fly before treatment. B and F represent the highest $\Delta F/F_0$ value (*I. cimicoides* = +0.23 at 5 minutes, *N. glauca* = -0.27 at 15 minutes). C and G indicate the lowest $\Delta F/F_0$ value (*I. cimicoides* = -0.14 at 13.6 minutes, *N. glauca* = -3.86 at 8.14 minutes). D and H are the graphic representation of the $\Delta F/F_0$ versus time, within 20 minutes for the PMG venom.

4. Discussion

The high species diversity within Heteroptera can at least in parts also be attributed to the numerous feeding habits (Swart *et al*., 2006). Although *I. cimicoides* and *N. glauca* both belong to the true water bugs (Nepomorpha), and utilize similar habitats, we showed that their suites of digestive enzymes and other venom-related effectors are distinctly different. We characterized biological activity of venom from the species *I. cimicoides* and *N. glauca* through the interpretation of transcriptomic and proteomic data, and by performing bioassays. This is the first study that analyses the venom composition and its activity of both species with bimolecular approaches.

4.1 Venom glands in *I. cimicoides* and *N. glauca*

Baptist (1941) reported that *I. cimicoides* and *N. glauca* have a separation of the main salivary glands into two lobes: PMG and AMG, and a tubular accessory gland. Some authors (Cohen, 1993; Sahayaraj *et al*., 2010; Fischer *et al*, 2020) suggest a differential use of those lobes involving division of labor for predation and defense. In this study, we corroborated the compartmentalization of the main gland into two lobes, PMG and AMG. In addition, we verified the presence of an accessory gland near the main gland (Fig. 3B and C). Importantly, we observed differences in protein composition between the two lobes of the main gland (PMG and AMG) (Fig. 3A). Although the protein composition of defense and hunting venom was similar to the venom composition of the PMG on the SDS-PAGE gel, some bands were present in the AMG too (Fig. 4A and B). This may be due to higher venom concentrations in the PMG in comparison to the AMG and the complexity of the system involved in regulating venom injection. Moreover, some proteins could be the result of an overlap between enzymes in the extraction and the analytic procedures. Since both strategies seem to yield similar protein compositions of the PMG and AMG, we conclude that both glands are activated in the EOD process and each contributes in a specific way to that process, but we could not clarify which of these glands plays a specific role in specific ecological strategies. Notwithstanding the above, it is necessary proteomic and transcriptomic comparison of defense and hunting venom against the PMG and AMG venom.

4.2 Enzymatic profile in *I. cimicoides* and *N. glauca*

Predatory heteropterans use EOD as a mode of feeding in which they inject digestive enzymes from the venom glands into their preys' cuticle (Cohen, 1998; Evangelin *et al.*, 2014, Walker *et al*., 2016). Accordingly, the venom of predatory bugs must contain a complex enzyme cocktail capable of disrupting extra and intracellular matrices ensuring feeding success (Cohen, 1998; Silva-Cardoso *et al.*, 2010; Evangelin *et al.*, 2014). Therefore, we analyzed the enzyme complexity of *I. cimicoides* and *N. glauca* venom by analyzing transcriptomic and proteomic data. We identified a wide range of proteins (12 protein groups by homology and function) with diverse putative functions and characteristics (Fig. 5A and B). Many of these proteins are putatively involved in membrane disruption and cytolysis (hemolysin-like proteins, venom proteins families), enzymatic activity (proteases, lipases, amylases, transferases), and paralytic activity (venom protein-like proteins) (Cohen, 1998; Azevedo *et al*., 2007; Sahayaraj *et al*., 2010; Walker *et al*., 2019; Wait *et al*., 2020). Thus, the proteins present in both species appear well suited to helping in the EOD process, previously described for Hemiptera (Swart *et al*., 2006; Walker *et al*., 2017; Wait *et al*., 2020), and the paralytic activity of some heteropterans (Fischer *et al*, 2020; Wait *et al*., 2020). Nevertheless, some enzymes could not be assigned to a specific function since they are not characterized yet, and the protein inference problem is not exempt from our study.

To corroborate the enzymatic activity of the venom of *I. cimicoides* and *N. glauca*, we performed different enzymatic assays (protease, elastase, amylase, and lipase). The preys' structural proteins are hydrolyzed by endopeptidases (proteases), while cell membranes and storage tissues are liquefied by phospholipases, lipases, and α-amylase (Cohen, 1998; Azevedo *et al*., 2007; Sahayaraj *et al*., 2010). These processes are important in the process of EOD (Cohen, 1998). Additionally, some of these proteins can be used to characterize the feeding habits of insects (Swart *et al*., 2006, Azevedo *et al*., 2007; Sahayaraj *et al*., 2010). For example, proteases, lipases, and amylases are mainly associated with phytophagous insects (Boyd *et al*., 2002; Torres & Boyd, 2009; Sahayaraj *et al*., 2010). Likewise, the presence of only proteases and lipases is associated with strict predators (Torres & Boyd, 2009).

Proteases are the main group of digestive enzymes in predatory animals (Sahayaraj *et al*., 2010; Evangelin *et al.*, 2014; Walker *et al*., 2017), including aquatic Heteroptera (Swart *et al*., 2006). Sahayaraj *et al*. (2010), demonstrated how proteases act in the EOD of the predaceous Reduvid *Catamirus brevipennis* and their importance in the acquisition of proteins.

Additionally, (Fischer *et al*, 2020) demonstrated the activity of endopeptidases during extra oral digestion in *P. biguttatus* and *P. horrida.* These enzymes catalyze the release of peptides and amino acids from a protein substrate (Sahayaraj *et al*., 2010). They can attack proteins membrane and release the nutrient-rich content of cells (Cohen, 1998). Due to the predatory habit of *I. cimicoides* and *N. glauca* (non-refluxing EOD), we expected protease activity in the PMG and AMG venom. Our results showed activity in the PMG and AMG of the four species studied (Fig. 6A). However, the PMG of all species always showed higher activity compared to AMG. Proteases were one of the most highly expressed proteins in the PMG and AMG of *I. cimicoides* and *N. glauca*, thus corroborating the enzymatic activity. Additionally, both species had a large number of different hits in both the transcriptomic and proteomic data that were assigned to the protease group. Furthermore, the presence of proteases must be accompanied by a similar expression of phosphatases due to their importance in the transport of material through membranes (Sahayaraj *et al*., 2010). These phosphatases were grouped in the family of other hydrolases; however, their presence in both species is undeniable.

Elastase breaks down proteins such as elastin present in connective tissues in vertebrates (Christeller *et al*., 1990). In this study for none of the four species studied, we could verify the presence of elastase activity (Fig. 8A and B). The lack of elastase is common for Heteroptera predators (Boyd *et al*., 2002), since the main protein in the connective tissue of arthropods is resilin (Su *et al*., 2014). As the name implies, resilin is highly elastic and contributes to the mechanical properties of insects (e.g. energy storage to jumping in fleas) (Su *et al*., 2014). Therefore, a more specific enzymatic activity assay is necessary to identify enzymes able to digest resilin.

α-Amylases are a group of glycoside hydrolases widely distributed in animal tissues that catalyze the hydrolysis of α-D- (1-4) glucan linkages in starch and glycogen and consequently release maltose, maltotriose, or branched oligosaccharides (Ghamari *et al*., 2014, Brust *et al*., 2020). These polysaccharides are broken down during EOD in arthropods. Previous research found amylases in the saliva of phytophagous species (Azevedo *et al*., 2007), and zoophytophagous (plant-feeding carnivores) species (Swart *et al*., 2006; Torres & Boyd, 2009), such as the true water bug *Belastoma lutarium* (Swart *et al*., 2006; Ghamari *et al*., 2014). Nevertheless, in strict predators (e.g. *I. cimicoides* and *N. glauca*), the presence of amylases has been reported only in the midgut (Boyd *et al*., 2002; Sahayaraj *et al*., 2010). Our results showed that venom from the PMG and AMG of *I. cimicoides* have amylase activity, which is a new finding (Fig. 9A). The transcriptomic and proteomic data corroborated the

presence of α-amylase in the PMG of *I. cimicoides* (Icim_MCn_C21361). Nevertheless, the lack of amylases in the transcriptomic and protease data of the AMG could be due to missing data or an error in the data processing pipeline and it is therefore necessary to revise the data.

To identify what substrate the α-amylase of the PMG and AMG venom was able to cleave we used starch and glycogen. Both polymers are storage carbohydrates, but starch is only found in photosynthetic eukaryotes and glycogen is found in the majority of animals, fungi, bacteria and archaea (Ball & Morell, 2003). While both, starch and glycogen, are chemically identical, major differences are related to the molecular organization of glucan chains compared to starch, resulting in differences in their water solubility (Brust *et al*., 2020). In our study the α-amylases in the PMG venom were able to hydrolyze starch and glycogen to their disaccharides, and the AMG venom to their trisaccharides (Fig. 9B and C).

The presence of α-amylases in *I. cimicoides* could be because this species is not strictly zoophagous, using amylases to obtain other substances such as sugar from plants or from herbivorous insect species (Swart *et al*., 2006; Azevedo *et al*., 2007; Torres & Boyd, 2009). For zoophagous species the protease activity by the saliva is less in comparison with the amylase activity (Sahayaraj *et al*., 2010; López-Rodríguez *et al*., 2012). Nevertheless, the above did not hold true for *I. cimicoides*, since this species showed a high protease activity in comparison to the other three studied species. Moreover, Torres & Boyd (2009), conclude that Hemipterans who have access to plant material have greater survival, larger body weight, live longer, and have a shorter developmental time. In the present study, we did not evidence either in the field or over laboratory conditions these characteristics. One explanation is that *I. cimicoides* is able to exploit the plant material already ingested by their prey. Also, due to the ability to cleave glycogen in our studies, *I. cimicoides* could use this α-amylase to digest glycogen from animal prey. Nevertheless, due the lack of studies in the aquatic Heteroptera and the novelty of our results we suggest a thorough research about amylase activity among Nepomorpha.

Lipases catalyze the hydrolysis of fats, an essential structural component of the cell membrane and storage compounds (Cohen, 1995; Evangelin *et al.*, 2014). Torres & Boyd, (2009) suggested a major amount of lipases in the midgut of predatory heteropterans, since the digestion of phospholipids takes place in the gut (Fischer *et al.*, 2020). The presence of lipases indicates the ability to digest food of animal origin where the lipids are the main nutritional components (López-Rodríguez *et al*., 2012). Fischer *et al.* (2020), identified lipases in the species *P. biguttatus* and *P. horrida* suggesting that EOD prioritizes the

predigestion of storage lipids while the main lipid digestion takes place in the gut. We corroborated the lipase activity from the PMG of *P. biguttatus* and *P. horrida* (Fig. 7). In our study, *I. cimicoides* and *N. glauca* showed enzymatic activity in the PMG (Fig. 7). This activity was supported with the transcriptomic data for *N. glauca* and with proteomic data for *I. cimicoides.*

Heteropterans perform non-refluxing EOD and therefore secrete a complex mixture of enzymes that can differ depending on the food source (Azevedo *et al*., 2007). Therefore, the digestive activity of the venom has to be higher compared to other venomous animals (Walker *et al*., 2017), to maximize the possibility of successful hunting (Silva-Cardoso *et al.,* 2010; Walker *et al*., 2016). Besides, the mouthparts only can suck liquid material and therefore the acquisition of the food has to be small enough to pass the alimentary canal (Walker *et al*., 2016, 2017). The biochemical composition of venom from *I. cimicoides* and *N. glauca* was unique in comparison to other invertebrates such as spiders and scorpions. In both species, the majority of proteins found in the proteomic analyses were enzymes like proteases and uncharacterized venom proteins. Additionally, the enzymatic profile showed the activity of proteases, lipases, and in *I. cimicoides* of amylases. These enzymes are an important component of extra-oral digestion since they liquefy the organs in the initial digestion process and reduce the viscosity of prey fluids (Azevedo *et al*., 2007; Ghamari *et al*., 2014). Proteases and lipases are associated with predatory insects (Baptist, 1941; Azevedo *et al*., 2007; Sahayaraj *et al*., 2010), and because of the digestion of glycogen by the α-amylases in *I. cimicoides* we corroborate the predatory habit of both species. Nevertheless, it is important to point out that the enzymatic content of the saliva cannot be assumed constant since it could present some variation through the developmental stages, the season, and the starvation time (Miles, 1972; Torres & Boyd, 2009). Additionally, the function of many *I. cimicoides* and *N. glauca* venom proteins are still unknown, showing a similar result with previous studies (Maggio *et al.,* 2005; Walker *et al*., 2018a; Fischer *et al*., 2020).

4.3 Effect of venom on the prey

The venom of predaceous species is used also to immobilize and kill their prey (Swart *et al*., 2006; Walker *et al*., 2016, 2018b). This prey paralysis is part of the EOD and is common in Heteroptera predators that feed on large prey (Azevedo *et al*., 2007; Walker *et al*., 2017), since more efficient prey paralysis allows the ingestion of larger and therefore more nutrient-

rich prey (Sahayaraj *et al*., 2010). Fischer *et al.* (2020), noted that venoms from *P. biguttatus* and *P. horrida* are able to produce paralysis in the honeycomb moth. Other bugs such as the Belostomatidae are even able to kill vertebrates by a single envenomation (Swart *et al*., 2006; Walker *et al*., 2016, 2018b). These results suggest the presence of neurotoxins in Heteroptera venom (Walker *et al*., 2016). However, (neuro)toxins can be difficult to distinguish from digestive enzymes, because they can also act as enzymes (Azevedo *et al*., 2007) and it is therefore unclear which proteins are involved in this behavior (Wait *et al*., 2020). Thus, the prey paralysis may also simply result from damage of neurons, muscle and storage tissue of the prey by certain digestive enzyme (Walker *et al*., 2018c; Wait *et al*., 2020; Ye *et al*., 2020). Additionally, evidence of paralytic activity has been obtained from the venom of just a few families, mainly Reduviidae (Walker *et al*., 2016, 2017, 2018c; Wait *et al*., 2020).

Because venom is used for predation, competition, or defense, it must have a rapid effect, such as fast immobilization of prey, inhibition of blood coagulation, or quick induction of pain (Fry *et al*., 2009). Therefore, to corroborate the paralytic activity and lethality of *I. cimicoides* and *N. glauca* venom, we observed the effects of PMG venom when injected into *G. assimilis* and *G. mellonella.* Nevertheless, in the present study, venom of neither *I. cimicoides* nor *N. glauca* had a significant paralytic and/or killing activity in Jamaica field crickets or the honeycomb month. When applied to neurological activity assays using insect neurons, only *N. glauca* venom showed a decrease in intracellular calcium concentration (Fig. 13H). It is documented that toxins from assassin bugs target Ca^{2+} channels (Fry *et al*., 2009; Walker *et al*., 2018a, 2019). The toxin Ptu1 from the assassin bug *Peirates turpis* is the only heteropteran toxin with a known neurotoxic activity (Bernard *et al*., 2001; Walker *et al.*, 2018d; Wait *et al*., 2020). Other proteins have been suggested to have a paralytic activity (Walker *et al*., 2017, 2018b), such as redulysin (Walker *et al*., 2018d), tryalysin (Amino *et al*., 2002), cystatin (Fry *et al*., 2009; Wait *et al*., 2020), lysophospholipids (Fry *et al*., 2009; Silva-Cardoso *et al.*, 2010; Walker *et al.*, 2018b), phospholipases (Tan *et al*., 2011; Evangelin *et al.*, 2014), and some hemolysis-like proteins (Walker *et al.*, 2018b). However, it is not possible to assign any putative function based on amino acid sequences alone (Walker *et al*., 2018d), and therefore the function of the majority of venom proteins within Heteroptera is still unknown (Walker *et al*., 2017). In the PMG of *I. cimicoides*, we only identified one peptide homologous to *P. turpis* Ptu1 (Icim_Mcn_C44371). Nevertheless, as Wait *et al*. (2020) demonstrate, injection of Ptu family peptides did not result in paralysis of the prey, suggesting that other components are responsible for the paralytic effect of assassin bug venom.

A suitable explanation for the lack of paralytic activity of both species is their environment. These species are strict aquatic insects and prey drowning could facilitate their prey capture. Besides, it has been demonstrated that both species present a large complex mouthpart system, in which the mandibles and the maxillae mix the host´s contents, a behavior termed as lacerate-and-flush feeding (Cobben, 1978; Evangelin *et al.*, 2014). This system could have a stronger participation in the EOD process than in other studied species. Therefore, we suggest a more thorough morphological study for both species.

4.4 The cytotoxic activity of *I. cimicoides* and *N. glauca* venom

Recent research on venoms has focused on the identification and isolation of proteins with antimicrobial activity (Bulet *et al*., 1993; Dani *et al*., 2003). These proteins were suggested to be a defense mechanism against infections that might arise due to the digestion of prey or to keep the homeostasis in the host of parasitoids insects (e.g. ichneumon wasps) (Bulet *et al*., 1993; Dani *et al*., 2003). In predatory hemipterans, the PMG venom of *P. horrida* and *P. biguttatus* showed a reduction in the growth of *E. coli* (Fischer *et al*, 2020). Nevertheless, proteins responsible for the antibacterial activity remain unknown. In our study, neither *I. cimicoides* nor *N. glauca* venom showed inhibitory activity against *E. coli* (Fig. 11). However, in the transcriptomic analysis data of the AMG of *N. glauca* we noted the presence of a defensin (Nglau_LC_C223). Defensin is an antibacterial peptide directed against Gram-positive bacteria that acts through membrane permeabilization (Bulet *et al*., 1993). Previous studies suggest that Gram-negative bacteria seem to be least sensitive to venom, as was shown for *Apis mellifera* venom (Hegazi1 *et al*., 2015). Therefore, we did not conclude antimicrobial activity in *I. cimicoides* and *N. glauca* since it is necessary to carry out more studies with Gram-positive bacteria.

Hematophagous insects take up hemoglobin and serum albumin, the two major blood protein components (Dorrah *et al.*, 2021). The salivary protein involved in the feeding process includes antihistamine, a sialidase, a serine protease, a sodium channel blocker, immunosuppressants, and pore-forming molecules (Kato *et al*., 2010). In predatory insects only 1 to 10% of the key nutrients are in the hemolymph, while the remainder can be found in solid tissues (Cohen, 1998). Previous studies showed the hemolysis of erythrocytes by venom from the heteropteran families such as Reduviidae (Fischer *et al.*, 2020), and Belostomatidae (Walker *et al.*, 2018b). Despite these findings, little is known about the enzymes that lyse

erythrocytes (Gooding, 1977), and the ecological implication for predatory insects. Our results showed that PMG extract from *I. cimicoides* was able to lyse human and horse erythrocytes (Fig. 10 A and B) while neither PMG nor AMG venom from *N. glauca* showed hemolysis of any erythrocyte type. In the transcriptomic data, AMG venom from *I. cimicoides* expressed two proteins homologous to hemolysin (Icim_MCn_C43543, Icim_MCn265). Hemolysin is a pore-forming toxin found in the blood-sucking bug *Triatoma dimidiata* (Kato *et al*., 2010), but is also often described in microorganisms (Gouaux, 1998). Gouaux (1998) showed that the sensitivity of different cells to this protein varies across organisms (e. g human erythrocytes require 1µ for lysis). In our study the experiment with AMG were carried out with low venom concentrations (5 µg/µl and 1 µg/µl), and therefore it might not be enough venom to result in cell. The PMG from *I. cimicoides* expressed a homologous protein to serpin (Icim_MCn_C35927). Serpin generally functions as an inhibitor of several fundamental biological processes such as platelet adhesion, coagulation, and fibrinolysis but does not have a known cytolytic activity (Kato *et al*., 2010). Thus, the molecular mechanisms inducing hemolysis are variable and some uncharacterized proteins may be involved in this process (Fischer *et al*, 2020).

In addition to hemolytic assays, we also tested the effects of venom extracts from *I. cimicoides* and *N. glauca* on the cell viability of *S. frugiperda* cells. These results were unique since the AMG venom from both species showed a significant difference from the negative control. Other studies had reported the lysis of insect cells by PMG venom (Fischer *et al.* 2020), but by AMG venom. Nevertheless, the proteins responsible for this activity remain unknown since it is unclear if the mechanism that causes cell death is because of a specific lytic protein or because of cell apoptosis. Our results were intriguing since the PMG venom from *N. glauca* showed significant differences with the negative control but they showed higher viability of the cells (Fig. 12B).

Fischer *et al*., (2020) showed the toxic activity of *P. biguttatus* and *P. horrida* venom. In their study, the authors demonstrated inhibition of bacterial growth (*E. coli*), lysis of erythrocytes (human and horse), and the effects of venom on insects' cell viability (*S. frugiperda Sf9*). In the same study, the authors suggested the activity of pore-forming proteins to be responsible for the toxic activity in the assassin bugs venom. Previous studies have shown the presence of a pore-forming protein called tryalysin in the saliva of *Triatoma infestans* (Bernard *et al*., 2001; Walker *et al*., 2018d, 2019). This protein is part of the innate defense mechanism in insects against microorganisms (Amino *et al*., 2002; Walker *et al*., 2019). Tryalysin forms

negative voltage-dependent pores in the lipid bilayer of bacteria, protozoa, and mammalian cells (Amino *et al*., 2002). This and previous described proteins could play a role in the cytotoxic activity of heteropteran venom. However, predatory heteropteran species showed a high variety of uncharacterized proteins. These proteins could have a role in the cytotoxicity of their prey as occurred in previous research (Walker *et al*., 2018a; Fischer *et al*, 2020).

5. CONCLUSIONS AND PERSPECTIVE

Although *I. cimicoides* and *N. glauca* are both from the sub-order Nepomorpha, are present in similar habitats and both are strict predatory aquatic insects (Strauss & Niedringhaus, 2014), we show that their group of digestive enzymes are distinctly different. The present study shows a high number of proteases, uncharacterized venom family proteins, and other uncharacterized transcripts in the venom composition of *I. cimicoides.* Overall, *N. glauca* venom components were similar to *I. cimicoides* venom. *I. cimicoides* showed protease, lipase, and amylase activity. Besides, we observed hemolytic activity of PMG venom. Venom from *N. glauca* displayed protease and lipase activity and had a neurological activity in the *Drosophila* neuronal assay. Although we identified a large number of proteins in both species, and we demonstrated the bioactivity in its venoms the model of digestion is still unknown. Furthermore, the venom of both species needs to be analyzed in more detail through fractionation and the further characterization of venom components.

The differences in the venom composition of the two species could be a representation of the generalist predation habit of *N. glauca* (Southwood & Lesto, 2005). It was shown that this species waits on the surface of the water for any kind of prey. In contrast, for *I. cimicoides* a much more specific prey selection strategy was reported (Southwood & Lesto, 2005). The wide variety of digestive proteins suggest that both species can feed on a wider variety of prey species, which increases their importance in shaping prey population dynamics in their environment. Nevertheless, the comparison between the species is incomplete due to a significant lack of research conducted on their ecology and behavior. In addition, histological studies should be done to provide more details on the salivary glands' capacity to produce and store enzymes between meals.

The bioactivity and composition of *N. glauca* and *I. cimicoides* venom assist further efforts to characterize the function and pharmacological activity of venom toxins of insects and to

understand the complex biomolecular processes carried out by true water bugs. In addition, we demonstrate the importance of combining molecular and enzymatic profiling experiments to characterize the complexity of the venom systems in novel taxa. Our results provide a solid foundation for understanding the role of insect venom composition in prey capture and the extra-oral digestion process.

Summary

The saucer bug *Ilyocoris cimicoides* and the common backswimmer *Notonecta glauca* are heteropteran predators of a wide variety of arthropods. They secrete venomous saliva, which they inject into their prey to perform extra-oral digestion. Although several studies have characterized the venom composition of true bugs, most of them focused on species belonging to the Reduviidae or Belastomatidae family. In this study, we analyzed transcriptomic and proteomic data of the aquatic bugs *I. cimicoides* and *N. glauca* and performed bioassays to characterize their venom activity. In both species, the salivary glands secreted protein mixtures containing, among others, proteases, venom protein family proteins, carbohydrases, lipases and many uncharacterized proteins. While *I. cimicoides* secreted hemolysin-like proteins and lyases *N. glauca* venom also contained serine protease inhibitors. There were considerable differences in enzyme activity and bioactivity between the two species. While venom extracts from both insects exhibited protease and lipase activity, only venom from *I. cimicoides* showed amylase and hemolytic activity and venom from *N. glauca* showed neurotoxicity activity in *Drosophila* sp neurons. The amylase present in *I. cimicoides* was able to cleave both starch and glycogen, an unexpected result due to its strict predatory behavior. The bioactivity and composition of *N. glauca* and *I. cimicoides* venom assist further efforts to characterize the function and pharmacological activity of insect venom and to understand the complex biomolecular process carried on by true water bugs. Here we demonstrate the importance of combining molecular and enzymatic profiling experiments to characterize the complexity of the venom systems in novel taxa. Our results provide a solid foundation for understanding the role of insect venom composition in prey capture and the extra-oral digestion process.

Zusammenfassung

Die Schwimmwanze *Ilyocoris cimicoides* und der gemeine Rückenschwimmer *Notonecta glauca* sind räuberische Wanzen, die sich von einer Vielzahl von Arthropoden ernähren. Sie sekretieren giftigen Speichel, den sie in ihre Beute injizieren um diese extraoral zu verdauen. Obwohl bereits mehrere Studien zur Giftzusammensetzung echter Wanzen existieren, konzentrierten sich die meisten auf Arten, die zur Familie der Reduviidae oder

Belastomatidae gehören. In dieser Studie analysierten wir transkriptomische und proteomische Daten der Wasserwanzen *I. cimicoides* und *N. glauca* und führten Bioassays durch, um die Aktivität deren Gifte zu charakterisieren. Bei beiden Arten sekretierten die Speicheldrüsen Proteingemische, die unter anderem Proteasen, Proteine der sogenannten „Venom protein families“, Carbohydrasen, Lipasen und viele uncharakterisierte Proteine enthielten. Während *I. cimicoides* Hämolysin-ähnliche Proteine und Lyasen produzierte, enthielt das Gift von *N. glauca* auch Serinprotease-Inhibitoren. Es gab erhebliche Unterschiede in der Enzymaktivität und Bioaktivität zwischen den beiden Wanzenarten. Während die Giftextrakte von beiden Insekten Protease- und Lipaseaktivität zeigten, hatte nur das Gift von *I. cimicoides* Amylase- und hämolytische Aktivität. Dagegen zeigte das Gift von *N. glauca* neurotoxische Aktivität auf *Drosophila* sp. Neuronen. Die in *I. cimicoides* vorhandene Amylase war in der Lage, sowohl Stärke als auch Glykogen zu spalten, was aufgrund der strikten räuberischen Lebensweise ein unerwartetes Ergebnis war. Die Analyse der Bioaktivität und Zusammensetzung der Gifte von *N. glauca* und *I. cimicoides* unterstützt weitere Bemühungen, die Funktion und pharmakologische Aktivität von Insektengiften zu charakterisieren und den damit verbundenen komplexen biomolekularen Prozess zu verstehen. Hier zeigen wir, wie wichtig es ist, molekulare und enzymatische Profiling-Experimente zu kombinieren, um die Komplexität der Giftsysteme in neuartigen Taxa zu charakterisieren. Unsere Ergebnisse bieten eine solide Grundlage für das Verständnis der Rolle der Insektengiftzusammensetzung beim Beutefang und dem extraoralen Verdauungsprozess.

Acknowledgements

Firstly, I would like to thank my supervisor, Maike Fischer, whose expertise and advice was invaluable in formulating the research questions and methodology. Your insightful feedback pushed me to sharpen my thinking and brought my work to a higher level. Also, I would like to thanks my supervisors Dr. Heiko Vogel and Prof. Dr. Nicole van Dam for their valuable guidance throughout my thesis. In addition, I would like to thank the Max Planck Institute for Chemical Ecology (Jena - Germany) to provide me with funds and materials to develop my thesis.

I would also like to thank Dr. Roy Kirsch (Department of Insect Symbiosis) and Benjamin Fabian (Research group olfactory coding) at the Max Planck Institute for chemical ecology for helped me in various essential experiments for my project.

Finally, I want to thank my family and friends for their support and unconditional motivation through the development of this project.

LIST OF REFERENCES

Amino, R., Martins, R. M., Procopio, J., Hirata, I. Y., Juliano, M. A., & Schenkman, S. (2002). Trialysin, a novel pore-forming protein from saliva of hematophagous insects activated by limited proteolysis. Journal of Biological Chemistry, 277(8), 6207–6213. https://doi.org/10.1074/jbc.M109874200

Azevedo, D. O., Cola Zanuncio, J., Salazar Zanuncio, J., Ferreira Martins, G., Marques-Silva, S., Sossai, M. F., & Serrão, J. E. (2007). Biochemical and morphological aspects of salivary glands of the predator *Brontocoris tabidus* (Heteroptera: Pentatomidae). Brazilian Archives of Biology and Technology, 50(3), 469–477. https://doi.org/10.1590/s1516-89132007000300013

Ball, S. G., & Morell, M. K. (2003). From Bacterial Glycogen to Starch: Understanding the Biogenesis of the Plant Starch Granule. Annual Review of Plant Biology, 54, 207–233. https://doi.org/10.1146/annurev.arplant.54.031902.134927

Baptist, B. A. (1941). The Morphology and Physiology of the Salivary Glands of Hemiptera-Heteroptera. In Journal of Cell Science (Vols. s2-83, Issue 329). https://doi.org/10.1242/jcs.s2-83.329.91

Barykina, N. V., Subach, O. M., Piatkevich, K. D., Jung, E. E., Malyshev, A. Y., Smirnov, I. V., Bogorodskiy, A. O., Borshchevskiy, V. I., Varizhuk, A. M., Pozmogova, G. E., Boyden, E. S., Anokhin, K. V., Enikolopov, G. N., & Subach, F. V. (2017). Green fluorescent genetically encoded calcium indicator based on calmodulin/M13-peptide from fungi. PLoS ONE, 12(8), 1–27. https://doi.org/10.1371/journal.pone.0183757

Bernard, C., Corzo, G., Mosbah, A., Nakajima, T., & Darbon, H. (2001). Solution structure of Ptu1, a toxin from the assassin bug *Peirates turpis* that blocks the voltage-sensitive calcium channel n-type. Biochemistry, 40(43), 12795–12800. https://doi.org/10.1021/bi015537j

Bernot. A (2004). Genome, Transcriptome, and Proteome Analysis. John Wiley & Sons. Ltd

Boyd, D. W., Cohen, A. C., & Alverson, D. R. (2002). Digestive enzymes and stylet morphology of *Deraeocoris nebulosus* (Hemiptera: Miridae), a predacious plant bug. Annals of the Entomological Society of America, 95(3), 395–401. https://doi.org/10.1603/0013-8746(2002)095[0395:DEASMO]2.0.CO;2

Brust, H., Orzechowski, S., & Fettke, J. (2020). Starch and glycogen analyses: Methods and techniques. Biomolecules, 10(7), 1–26. https://doi.org/10.3390/biom10071020

Bulet, P., Dimarcq, J. L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Van Dorsselaer, A., & Hoffmann, J. A. (1993). A novel inducible antibacterial peptide of Drosophila carries an O- glycosylated substitution. Journal of Biological Chemistry, 268(20), 14893–14897. https://doi.org/10.1016/s0021-9258(18)82417-6

Cobben (1978). Evolutionary trends in Heteroptera. Part II. Mouthpart-structure and feeding strategies. Mededelingen Landbouwhogeschool wageningen. Nederland. 78-5

Cohen, A. C. (1993). Organization of digestion and preliminary characterization of salivary trypsin-like enzymes in a predaceous heteropteran, *Zelus renardii*. Journal of Insect Physiology, 39(10), 823–829. https://doi.org/10.1016/0022-1910(93)90114-7

Cohen, A. C. (1995). Extra-oral digestion in predaceous terrestrial Arthropoda. Annual Review of Entomology, 40, 85–103. https://doi.org/10.1146/annurev.en.40.010195.000505

Cohen, A. C. (1998). Solid-to-liquid feeding: The inside(s) story of extra-oral digestion in predaceous Arthropoda. American Entomologist, 44(2), 103–117. https://doi.org/10.1093/ae/44.2.103

Corzo, G., Adachi-Akahane, S., Nagao, T., Kusui, Y., & Nakajima, T. (2001). Novel peptides from assassin bugs (Hemiptera: Reduviidae): Isolation, chemical and biological characterization. FEBS Letters, 499(3), 256–261. https://doi.org/10.1016/S0014-5793(01)02558-3

Christeller, J. T., Laing, W. A., Shaw, B. D., & Burgess, E. P. J. (1990). Characterization and partial purification of the digestive proteases of the black field cricket, Teleogryllus commodus (Walker): Elastase is a major component. Insect Biochemistry, 20(2), 157–164. https://doi.org/10.1016/0020-1790(90)90008-I

Dani, M. P., Richards, E. H., Isaac, R. E., & Edwards, J. P. (2003). Antibacterial and proteolytic activity in venom from the endoparasitic wasp *Pimpla hypochondriaca* (Hymenoptera: Ichneumonidae). Journal of Insect Physiology, 49(10), 945–954. https://doi.org/10.1016/S0022-1910(03)00163-X

Denecke, S., Ioannidis, P., Buer, B., Ilias, A., Douris, V., Topalis, P., Nauen, R., Geibel, S., & Vontas, J. (2020). A transcriptomic and proteomic atlas of expression in the *Nezara viridula* (Heteroptera: Pentatomidae) midgut suggests the compartmentalization of xenobiotic metabolism and nutrient digestion. BMC Genomics, 21(1), 1–15. https://doi.org/10.1186/s12864-020-6459-6

Ditsche-Kuru, P; Schneider, E; Melskotte, J; Brede, M; Leder, A & Barthlott W. (2011). Superhydrophobic surfaces of the water bug *Notonecta glauca*: a model for friction reduction and air retention. Beilstein J. Nanotechnol. Pg. 137–144

Dorrah, M., Bensaoud, C., Mohamed, A. A., Sojka, D., Bassal, T. T. M., & Kotsyfakis, M. (2021). Comparison of the hemolysis machinery in two evolutionarily distant blood-feeding arthropod vectors of human diseases. PLoS Neglected Tropical Diseases, 15(2), 1–19. https://doi.org/10.1371/journal.pntd.0009151

Edwards, J (1961). The action and composition of the saliva of an assassin bug *Platymeris rhadamanthus* Gaerst (Hemiptera, Reduviidae). Exp. Biol. 38, 61-67

Evangelin, G., Horne, B., Muthupandi, M., & S, J. W. (2014). Venomous Saliva of Non-*Haematophagous Reduviid* Bugs (Heteroptera : Reduviidae): a Review. Biolife, 2(2), 615–626.

Fialho, M. C. Q., Moreira, N. R., Zanuncio, J. C., Ribeiro, A. F., Terra, W. R., & Serrão, J. E. (2012). Prey digestion in the midgut of the predatory bug *Podisus nigrispinus* (Hemiptera: Pentatomidae). Journal of Insect Physiology, 58(6), 850–856. https://doi.org/10.1016/j.jinsphys.2012.03.009

Fisher, E. (2009) Asilidae (Robber flies, assassin flies, moscas cazadoras, moscas ladronas). *In:* Brown, B.V., Borkent, A., Cumming, J.M., Wood, D.M., Woodley, N.E. & Zumbado, M.A. (Eds.), *Manual of central American Diptera. Vol 1*. NRC Research Press, Ottawa, pp. 585–632.

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. https://doi.org/10.1002/ece3.6652

Fry, B. G., Roelants, K., Champagne, D. E., Scheib, H., Tyndall, J. D. A., King, G. F., Nevalainen, T. J., Norman, J. A., Lewis, R. J., Norton, R. S., Renjifo, C., & Rodríguez De La Vega, R. C. (2009). The toxicogenomic multiverse: Convergent recruitment of proteins into animal venoms. Annual Review of Genomics and Human Genetics, 10, 483–511. https://doi.org/10.1146/annurev.genom.9.081307.164356

Ghamari, M., Hosseininaveh, V., Darvishzadeh, A., & Chougule, N. P. (2014). Carbohydrases in the digestive system of the spined soldier bug, *Podisus maculiventris* (say) (hemiptera: Pentatomidae). Archives of Insect Biochemistry and Physiology, 85(4), 195–215. https://doi.org/10.1002/arch.21153

Gooding. R. H (1997) Digestive processes of haematophagous insects. XIV. Haemolytic activity in the midgut of Glossina morsitans morsitans Westwood (Diptera: Glossinidae). Canadian Journal of Zoology. 55(11): 1899-1905. https://doi.org/10.1139/z77-243

Gouaux, E. (1998). α-Hemolysin from Staphylococcus aureus: An archetype of β-barrel, channel-forming toxins. Journal of Structural Biology, 121(2), 110–122. https://doi.org/10.1006/jsbi.1998.3959

Grazia, J. & Fernandes. J (2012). Subordem Heteroptera. In: Rafael. J., Melo, G., Carvahlo, C., Casari, S. & Constantino, R.; Insectos do Brasil - Diversidade e Taxonomia. Holos, Editora, Brasil. 369 – 405

Haridass, E. T. & Ananthakrishnan, T. N. (1980). Models for the predatory behavior of some reduviids from Southern India (Insecta – Heteroptera - Reduviidae). Indian Acad. Sci. 89(4), 387-402

Hegazi1, A. G., EL-Feel M., A., Abdel-Rahman, E. H., & A, A. A.-F. M. (2015). Original Research Article Antibacterial Activity of Bee Venom Collected from Apis mellifera Carniolan Pure and Hybrid Races by Two Collection Methods. 4(4), 141–149.

Kato, H., Jochim, R. C., Gomez, E. A., Sakoda, R., Iwata, H., Valenzuela, J. G., & Hashiguchi, Y. (2010). A repertoire of the dominant transcripts from the salivary glands of the blood-sucking bug, *Triatoma dimidiata*, a vector of Chagas disease. Infection, Genetics and Evolution, 10(2), 184–191. https://doi.org/10.1016/j.meegid.2009.10.012

Larsen, O (1970). The flight organs of *Ilyocoris cimicoides* L (Hem, Naucoridae). Insect Systematics & Evolution. Vol 1. Issue 3. 227 – 235. DOI: https://doi.org/10.1163/187631270X00078

López-Rodríguez, M. J., Trenzado, C. E., Tierno de Figueroa, J. M., & Sanz, A. (2012). Digestive enzyme activity and trophic behavior in two predator aquatic insects (Plecoptera, Perlidae). A comparative study. Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 162(1), 31–35. https://doi.org/10.1016/j.cbpa.2012.01.020

Maggio, F., Sollod, B., Tedford, H., & Harvey, W. (2005). Spider toxins and their potential for insect control. In: Gilbert, L., Iatrou, K. & Gill, Sarjeet; Comprehensive Molecular Insect Science; Volume five; First Edition; Elsevier BV. Oxford. UK. 221 - 238

Miles, P. W. (1972). The Saliva of Hemiptera. Advances in Insect Physiology, 9(C), 183–255. https://doi.org/10.1016/S0065-2806(08)60277-5

Mohamed, A. A. M., Retzke, T., Das Chakraborty, S., Fabian, B., Hansson, B. S., Knaden, M., & Sachse, S. (2019). Odor mixtures of opposing valence unveil inter-glomerular crosstalk in the Drosophila antennal lobe. Nature Communications, 10(1), 1–17. https://doi.org/10.1038/s41467-019-09069-1

Ramm, C., Wayadande, A., Baird, L., Nandakumar, R., Madayiputhiya, N., Amundsen, K., Donze-Reiner, T., Baxendale, F., Sarath, G., & Heng-Moss, T. (2015). Morphology and Proteome Characterization of the Salivary Glands of the Western Chinch Bug (Hemiptera: Blissidae). Journal of Economic Entomology, 108(4), 2055–2064. https://doi.org/10.1093/jee/tov149

Rockstein, M. (1978). Biochemistry of Insects. Academic Press Inc. pp. 661

Sahayaraj, K., Kanna, A. V., & Kumar, S. M. (2010). Gross morphology of feeding canal, salivary apparatus and digestive enzymes of salivary gland of catamirus brevipennis (Servile) (Hemiptera: Reduviidae). Journal of the Entomological Research Society, 12(2), 37–50.

Schuh, R., & Slater, J. (1995). True bugs of the world. In Phymatinae (Vol. 151).

Sherwood, L., Klandorf, H., & Yancey, P. H. (2013). Animal Physiology: From Genes to Organisms, Second Edition.

Silva-Cardoso, L., Caccin, P., Magnabosco, A., Patrón, M., Targino, M., Fuly, A., Oliveira, G. A., Pereira, M. H., Do Carmo, M. D. G. T., Souza, A. S., Silva-Neto, M. A. C., Montecucco, C., & Atella, G. C. (2010). Paralytic activity of lysophosphatidylcholine from saliva of the waterbug belostoma anurum. Journal of Experimental Biology, 213(19), 3305–3310. https://doi.org/10.1242/jeb.041954

Southwood, T., & Lesto, D. (2005). Land and Water Bugs of the British Isles. Frederick Warne & Co Pg. 450.

Stockert, J. C., Blazquez-Castro, A., Canete, M., Horobin, R. W., & Villanueva, A. (2011). MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. Acta Histochemica, 114, 785-796. https://doi.org/10.1016/j.acthis.2012.01.006

Strauss, G. & Niedringhaus, R (2014). Die Wasserwanzen Deutschlands: Bestimmungsschlüssel für alle Nepo- und Gerromorpha; Fründ; Germany; 66 pg

Su, R. S. C., Kim, Y., & Liu, J. C. (2014). Resilin: Protein-based elastomeric biomaterials. Acta Biomaterialia, 10(4), 1601–1611. https://doi.org/10.1016/j.actbio.2013.06.038

Sunagar, K., Morgenstern, D., Reitzel, A. M., & Moran, Y. (2016). Ecological venomics: How genomics, transcriptomics and proteomics can shed new light on the ecology and evolution of venom. *Journal of Proteomics*, *135*, 62–72. https://doi.org/10.1016/j.jprot.2015.09.015

Swart, C. C., Deaton, L. E., & Felgenhauer, B. E. (2006). The salivary gland and salivary enzymes of the giant waterbugs (Heteroptera; Belostomatidae). Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 145(1), 114–122. https://doi.org/10.1016/j.cbpa.2006.05.010

Tan, C. H., Sim, S. M., Gnanathasan, C. A., Fung, S. Y., Ponnudurai, G., Pailoor, J., & Tan, N. H. (2011). Enzymatic and toxinological activities of *Hypnale hypnale* (hump-nosed pit viper) venom and its fractionation by ion exchange high performance liquid chromatography. Journal of Venomous Animals and Toxins Including Tropical Diseases, 17(4), 473–485. https://doi.org/10.1590/S1678-91992011000400015

Terra, W. & Ferreira, C. (2005). Biochemistry of Digestion. In: Gilbert, L., Iatrou, K. & Gill, Sarjeet; Comprehensive Molecular Insect Science; Volume four; First Edition; Elsevier BV. Oxford. UK. 171 – 224

Torres, J. B., & Boyd, D. W. (2009). Zoophytophagy in predatory Hemiptera. Brazilian Archives of Biology and Technology, 52(5), 1199–1208. https://doi.org/10.1590/S1516-89132009000500018

Wait, L. C., Walker, A. A., & King, G. F. (2020). Crouching Tiger, Hidden Protein: Searching for Insecticidal Toxins in Venom of the Red Tiger Assassin Bug (Havinthus rufovarius). Toxins, 13(1). https://doi.org/10.3390/toxins13010003

Walker, A. A., Weirauch, C., Fry, B. G., & King, G. F. (2016). Venoms of heteropteran insects: A treasure trove of diverse pharmacological toolkits. Toxins, 8(2), 1–32. https://doi.org/10.3390/TOXINS8020043

Walker, A. A., Madio, B., Jin, J., Undheim, E. A. B., Fry, B. G., & King, G. F. (2017). Melt with this kiss: Paralyzing and liquefying venom of the Assassin bug *Pristhesancus plagipennis* (Hemiptera: Reduviidae). Molecular and Cellular Proteomics, 16(4), 552–566. https://doi.org/10.1074/mcp.M116.063321

[a]Walker, A. A., Dobson, J., Jin, J., Robinson, S. D., Herzig, V., Vetter, I., King, G. F., & Fry, B. G. (2018). Buzz kill: Function and proteomic composition of venom from the giant assassin fly *Dolopus genitalis* (Diptera: Asilidae). Toxins, 10(11). https://doi.org/10.3390/toxins10110456

[b]Walker, A. A., Hernández-Vargas, M. J., Corzo, G., Fry, B. G., & King, G. F. (2018). Giant fish-killing water bug reveals ancient and dynamic venom evolution in Heteroptera. Cellular and Molecular Life Sciences, 75(17), 3215–3229. https://doi.org/10.1007/s00018-018-2768-1

[c]Walker, A. A., Mayhew, M. L., Jin, J., Herzig, V., Undheim, E. A. B., Sombke, A., Fry, B. G., Meritt, D. J., & King, G. F. (2018). The assassin bug *Pristhesancus plagipennis* produces two distinct venoms in separate gland lumens. Nature Communications, 9(1), 1–10. https://doi.org/10.1038/s41467-018-03091-5

[d]Walker, A. A., Robinson, S. D., Yeates, D. K., Jin, J., Baumann, K., Dobson, J., Fry, B. G., & King, G. F. (2018). Entomo-venomics: The evolution, biology and biochemistry of insect venoms. Toxicon, 154(July), 15–27. https://doi.org/10.1016/j.toxicon.2018.09.004

Walker, A. A., Robinson, S. D., Undheim, E. A. B., Jin, J., Han, X., Fry, B. G., Vetter, I., & King, G. F. (2019). Missiles of mass disruption: Composition and glandular origin of venom used as a projectile defensive weapon by the assassin bug *Platymeris rhadamanthus*. Toxins, 11(11). https://doi.org/10.3390/toxins11110673

Walker, A. A. (2020). The evolutionary dynamics of venom toxins made by insects and other animals. Biochemical Society Transactions, 48(4), 1353–1365. https://doi.org/10.1042/BST20190820

Wang, Y. H., Wu, H. Y., Rédei, D., Xie, Q., Chen, Y., Chen, P. P., Dong, Z. E., Dang, K., Damgaard, J., Štys, P., Wu, Y. Z., Luo, J. Y., Sun, X. Y., Hartung, V., Kuechler, S. M., Liu, Y., Liu, H. X., & Bu, W. J. (2019). When did the ancestor of true bugs become stinky? Disentangling the phylogenomics of Hemiptera–Heteroptera. Cladistics, 35(1), 42–66. https://doi.org/10.1111/cla.12232

Wang, Y., Brożek, J., & Dai, W. (2020). Comparative morphology of the mouthparts in three predatory stink bugs (Heteroptera: Asopinae) reveals feeding specialization of stylets and sensilla. Insects, 11(11), 1–23. https://doi.org/10.3390/insects11110762

Ye, Z., Damgaard, J., Yang, H., Hebsgaard, M. B., Weir, T., & Bu, W. (2020). Phylogeny and diversification of the true water bugs (Insecta: Hemiptera: Heteroptera: Nepomorpha). Cladistics, 36(1), 72–87. https://doi.org/10.1111/cla.12383

Appendix

The following documents are attached: Appendix File A1: spreadsheet containing bioinformatics identification for all peptides and proteins found in the transcriptome of *N. glauca.* Appendix File A2: spreadsheet containing identification bioinformatics identification for all peptides and proteins found in the proteomic data from the PMG of *N. glauca.* Appendix File A3: spreadsheet containing bioinformatics identification for all peptides and proteins identified in the proteomic data from the AMG of *N. glauca.* Appendix File A4: spreadsheet containing the results from the transcriptomic analyses of *I. cimicoides* and *N. glauca.* Appendix File A5: spreadsheet containing the proteomic and transcriptomic comparison from both species. Appendix Method A1: comparison of detected protein sequences in the transcriptomic and proteomic data set of *N. glauca.* Appendix video A1: venom acquisition of *I. cimicoides* through a dummy made with paraffin.

Declaration of Self-Dependence

Herewith I declare that I prepared this thesis on my own, that I did not use any other sources and resources than those that are specified, that all arguments and ideas that were literally or analogously taken from other sources are sufficiently identified, and that the thesis in identical or similar form has not been use as part of an earlier course achievement or examination procedure.

....

Place, Date Signature

- **Note:** A plagiarism is given if the above Declaration of Self-Dependence is violated, i.e. if ideas or arguments or even complete text sections are taken from other sources without a proper indication and a reference to the source. Plagiarisms are categorically marked with 5.0 and in serious cases even revision is not permitted!