



The venom of *Nasonia vitripennis*: involvement in the host-parasitoid interaction and putative biomedical application

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LIST OF ABBREVIATIONS

AA	amino acid
AARE	amino acid deprivation response element
AMP	antimicrobial peptide
Blast	basic local alignment search tool
bp	base pair
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT-enhancer-binding protein
CHOP	C/EBP homologous protien
CRE	cAMP response element
CREB	cAMP response element-binding protein
Ct	treshold cycle
Da	dalton
DEX	dexamethasone
dH ₂ O	distilled water
dsRNA	double-stranded RNA
DTT	dithiothreitol
EIF	eukaryotic translation initiation factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
EST	expressed sequence tags
GC	glucocorticoid
GFP	green fluorescent protein
GO	gene ontology
GR	glucocorticoid receptor
HEK	human embryonal kidney cells
HPLC	high performance liquid chromatography
IL	interleukin
Imd	Immune deficiency
ISB	insect phosphate saline buffer

JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
KOG	eukaryotic orthologous group
MAPK	mitogen-activated protein kinase
MgCl ₂	magnesium chloride
mM	millimolar
mRNA	messenger RNA
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDV	polydnavirus
PEI	polyethilenimine
PO	phenoloxidase
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcriptase-PCR
RT-qPCR	reverse transcription quantitative (real time) PCR
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
Sf9	<i>Spodoptera frugiperda</i> cell line 9
TNF	tumor necrosis factor
TRE	transcriptional response element

SUMMARY

Hymenopteran parasitoids develop at the expense of other arthropods, ultimately killing their host and are thus important regulators of pest arthropods. The ectoparasitoid wasp *Nasonia vitripennis* preferably parasitizes pupae from flesh flies (Sarcophagidae) and blow flies (Calliphoridae). After locating a suitable host, the female wasp injects venom inside the fly pupa and lays her eggs in the space between the pupa and the puparium. The injection of this complex mixture of venom compounds prepares the host to present the best possible environment for the wasp offspring to survive. Host physiology is altered, in which the host development is arrested, its immune system is suppressed and host metabolism is modified so that it is synchronized with the development of parasitoid larvae.

First part of this work focussed on the early alterations of host physiology after parasitization by *N. vitripennis*. Only effects caused by venom without modifications caused by larval feeding were subject of this study. Therefore, *Sarcophaga crassipalpis* hosts were examined by microarray, 3 and 25 hours after parasitization, before wasp eggs hatch. Since we wanted to avoid effects caused by manipulation, the expression of *Nanos* was tested to verify successful parasitization instead of opening host puparium and scavenging for parasitoid eggs. A set of transcripts was selected from the EST database available for this host and used as target for the microarray performed on control and parasitized pupae. Results were validated by RT-qPCR on a set of genes. Clustering the differentially expressed genes, enlightened alterations of key immune, developmental and metabolic pathways due to envenomation. Many of these same pathways have also been implicated in endoparasitoid attack of hosts, suggesting some fundamental, conserved aspects of hostparasitoid interactions to be further investigated.

Bioinformatic and proteomic studies of *N. vitripennis* venom revealed the presence of 80 proteins, of which 23 that show no homology to any known protein. Functionality of this intriguing group of compounds was tackled by 2 different approaches: host injection of recombinant expressed venom protein or knock down of venom protein in the female wasp, both followed by specific bioassays on the host. Since venom proteins appeared to be unglycosylated, recombinant expression of venom proteins was performed in a bacterial

expression system. Due to significant up-regulation of immune genes by bacterial fragments in the solvent of the venom compound, either further purification methods need to be applied, or another expression system needs to be used. The second approach to investigate functionality that was used, included silencing of the venom compound in the parasitoid wasp. Thereby, it became clear that the exact timing of dsRNA injection is crucial to obtain successful knockdown of venom genes. By altering the practical setting, silencing venom transcripts in *N. vitripennis* wasps is still one of the possible approaches to unravel functionalities of venom compounds.

Endoparasitoid wasps have many virulence factors at their disposal, of which polydnaviruses are mostly used to suppress host immunity. Ectoparasitoids, like *N. vitripennis*, are also able to inhibit the host immune reaction, but, in contrast with endoparasitoids, they only contain venom compounds to realize this suppression. As second part of this PhD thesis, the interesting ability to diminish the defence system was tested in mammalian cells. The major central immune pathway in mammals is the NF- κ B signalling cascade, that shows strong homology with the insect Toll and Imd pathways. LPS- and TNF-induced NF- κ B activation was significantly down-regulated by *N. vitripennis* venom as tested by IL-6 ELISA in Raw264.7 macrophages and by NF- κ B luciferase assay in L929sA fibroblasts. Further studies focussed on exploring the specific interaction between venom and this cascade, expanding the search to effects on MAPK and GR signalling.

Deregulation of NF- κ B signalling is involved in several diseases like autoimmune conditions, inflammatory diseases and cancer. The high need for more safe and/or gene-specific anti-inflammatory agents reflects the intensive search for NF- κ B inhibitors. Since *N. vitripennis* venom was proven to have an anti-inflammatory mode of action in the tested cell lines, effects of venom on NF- κ B target genes were investigated by PCR Array on macrophage cells. Several interesting targets involved in inflammatory diseases and cancer were altered by treatment with *N. vitripennis* venom, suggesting its putative biomedical application. Further unravelling the specific interaction of venom on the NF- κ B pathway and identifying the responsible venom compound, are the next steps in investigating the biomedical applications of *N. vitripennis* venom.

SAMENVATTING

Parasitoïde wespen benutten verschillende levensstadia van arthropoden als gastheer, die uiteindelijk gedood worden. Ze hebben een enorm economisch belang als regulator van tal van pestsoorten van voedingsgewassen en dieren. De ectoparasitoïde wesp *Nasonia vitripennis* parasiteert bij voorkeur vleesvliegen (Sarcophagidae) en bromvliegen (Calliphoridae). Hierbij injecteert ze gif in de gastheer om een zo goed mogelijke omgeving te garanderen voor haar nakomelingen. Het gif is in staat om de ontwikkeling van de gastheer af te remmen en het afweersysteem tegen de parasitoïde larven te onderdrukken. Daarnaast wordt het metabolisme op de noden van de parasitoïden afgestemd om van de gastheer een nutriëntrijke omgeving te maken.

In een eerste deel van dit werk, worden vroege veranderingen in fysiologie van de gastheer na parasitering door *N. vitripennis* onderzocht door middel van microarray. Enkel effecten veroorzaakt door het gif zijn doel van deze studie, waarbij effecten door voedende larven worden gemedend. Drie en 25 uren na parasitering werden gekozen als moment van staalname, aangezien hierna parasitoïde larven starten te voeden aan de gastheer. Als uitgangspunt voor de transcriptoom studie werd een zo natuurlijk mogelijke situatie beoogd, wat betekent dat schade door manipulatie van de poppen moest vermeden worden. Daarom werd succesvolle parasitering nagegaan door middel van *Nanos* expressie in plaats van het openen van het puparium om *Nasonia* eitjes te zoeken. Er werd een selectie gemaakt van de beschikbare EST databank voor de gastheer *Sarcophaga crassipalpis*, die als target diende voor de microarray. Ter validatie van de data werd RT-qPCR uitgevoerd op een reeks genen, voordien getest door microarray. Het groeperen van de differentieel geëxprimeerde genen toonde effecten van het gif op verschillende belangrijke pathways aan in verband met immuniteit, ontwikkeling en metabolisme.

Via een bioinformatische benadering en proteoomstudie, werd de aanwezigheid van 80 proteïnen aangetoond in het gif van *N. vitripennis*. Hiervan vertoonden er 23 geen enkele homologie met reeds gekende eiwitten. Om de functionaliteit van deze intrigerende groep eiwitten te onderzoeken, werden 2 benaderingen getest: het recombinant aanmaken van gifeiwitten, of het onderdrukken van transcripten van gifcomponenten in *N. vitripennis*,

beide gevolgd door specifieke bio-assays op hun gastheer. Aangezien de gifproteïnen niet geglycosyleerd bleken te zijn, werd voor de recombinante expressie een bacterieel systeem gekozen. Na injectie van het opgezuiverde solvent in de vleesvliegpoppen, bleek deze reeds een significante opregulatie teweeg te brengen van het immuunsysteem. Een verder doorgedreven opzuivering van de recombinant geëxprimeerde gifcomponent of de productie ervan in een ander expressiesysteem wordt hierbij aanbevolen. Bij de tweede functionaliteitsbenadering worden transcripten van de gifcomponent neergereguleerd door middel van dsRNA injectie in de parasitoïde wesp. Hierbij bleek het exacte moment van de injectie cruciaal te zijn. Na aanpassingen in de praktische setup van dit experiment, blijft deze benadering zinvol om de functionaliteit van gifcomponenten te onderzoeken.

Endoparasitoïde wespen beschikken over verschillende virulentiefactoren om hun gastheer naar hun hand te zetten. Zo blijkt dat vooral het injecteren van polydnavirussen verantwoordelijk is voor het onderdrukken van het immuunsysteem bij de gastheer. Ectoparasitoïden daarentegen gebruiken enkel gif, wat betekent dat een gifcomponent moet verantwoordelijk zijn voor de onderdrukking van het afweersysteem. In een tweede luik van dit werk wordt deze immuunsuppressie van het gif onderzocht op zoogdiercellen. Centraal in het immuunsysteem bij zoogdieren is de NF- κ B pathway, die sterke homologie vertoont met de Toll en Imd pathways bij insecten. Na inductie met LPS en TNF, bleek de activatie van NF- κ B onderdrukt te worden door *N. vitripennis* gif in respectievelijk Raw264.7 macrofagen en L929sA fibroblasten. Verdere onderzoeken trachtten de specifieke interactie van het gif met deze signalisatiecascade te achterhalen.

Ontregeling van de NF- κ B pathway is betrokken in anti-inflammatoire ziekten, autoimmuunziekten en kanker. Door de grote vraag naar anti-inflammatoire componenten, werd onderzocht of het gif van *N. vitripennis* een mogelijke biomedische toepassing heeft. Door middel van PCR Array werd het effect van het gif op een reeks targetgenen van NF- κ B signalisatie onderzocht. Hieruit bleek dat het gif enkele targets van anti-inflammatoire ziekten en kanker significant beïnvloedt, wat een mogelijke biomedische toekomst voor *N. vitripennis* gif biedt. Hiervoor moet echter de specifieke interactie met de NF- κ B pathway uitgeklaard worden en dient de verantwoordelijke component van deze effecten geïdentificeerd te worden.

PART I

INTRODUCTION

CHAPTER 1

General introduction

Parts of this introduction are redrafted from publication in Toxins in 2010:

Venom proteins of the parasitoid wasp *Nasonia vitripennis*: recent discovery of an untapped pharmacopee.

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1. Parasitoids: the 'Trojan insects'

"For, from his home beneath Parnassus, Phocian Epeus, aided by the craft of Pallas, framed a horse to bear within its womb an armed host, and sent it within the battlements, fraught with death; whence in days to come men shall tell of 'the wooden horse,' with its hidden load of warriors."
[Euripides' play 'Trojan women', 415 B.C.]

More than 1,000 years BC, the Greek victoriously won a crucial battle against the Trojans by constructing a wooden horse that hid warriors inside. From within Troy, they could destroy the high-walled city that had been besieged for over 10 years.

Parasitoids are quite similar to the Greek mentioned, in a way that these insects hide their future offspring (warriors) inside the host (enemy), that inevitably loses the battle. Instead of building a wooden horse to get inside the city-walls alive, the parasitoids survival strategy first consists of injecting toxic fluids inside the host in order to present a nutritious environment for its offspring to survive. Only then, the true battle begins: the host is being paralyzed, its development is arrested, the immune response weakened and its behaviour is altered. It is an unfair battle since secondly the host is attacked by larvae that feed on its tissues. Ultimately, the host is completely demolished and the new generation of parasitoids prevail.

With its 400,000 species, beetles are commonly known as the most diverse group of insects. Molecular studies, however, have suggested that the 60,000 species of parasitoid wasps that have been described until now (Pennisi, 2010), are only the tip of the iceberg and could be even more diverse than beetles. The fact that these wasps are such picky eaters could partly explain this high diversity. Some parasitoid wasps only lay eggs in Holometabolous insects that have a four-stage life cycle (egg-larva-pupa-adult) and undergo complete metamorphosis, while others are selective for certain life stages of the same host species. Sometimes, even certain sites on the host are preferred so that specific tissues are available for the offspring (Rivers *et al.*, 1993). This specificity makes parasitoids appealing for biological pest control, since they are important regulators of agricultural pest insects (see

3.3.1. Agronomic applications). Next to this, parasitoids also attack significant vectors of human disease, such as house flies, roaches and ticks (Quicke, 1997).

Science-fiction fans may remember the very popular motion picture 'Alien' from 1979 that describes the fate of the occupants of a spaceship infiltrated by a deadly alien life form. Larvae of these creatures entered the bodies of the crew where they developed and grew, finally emerging from the infected human which was inevitably killed. The fact that these events cannot happen to humans on Earth is at least reassuring. Insects, on the other hand, are constantly attacked by parasitoids, resembling the gruesome creature in 'Alien'. The latter can be recognized as an endoparasitoid, which lays its eggs into a mobile and exposed host. The offspring develops inside this host, which in the end is completely consumed by the parasitoids (figure 1A). Ectoparasitoids on the other hand, lay their eggs outside the host (figure 1B). This means that the host needs to be immobilized, which is ensured by the injection of a paralytic venom. Once the larvae are hatched, they use their mouthparts to attach themselves to the host and continue to feed until almost nothing remains. Therefore, parasitoids can be seen as an intermediate between predators and parasites; like predators, they always kill the host they attack, and like parasites, they require a host on which to mature (Godfray, 2004).

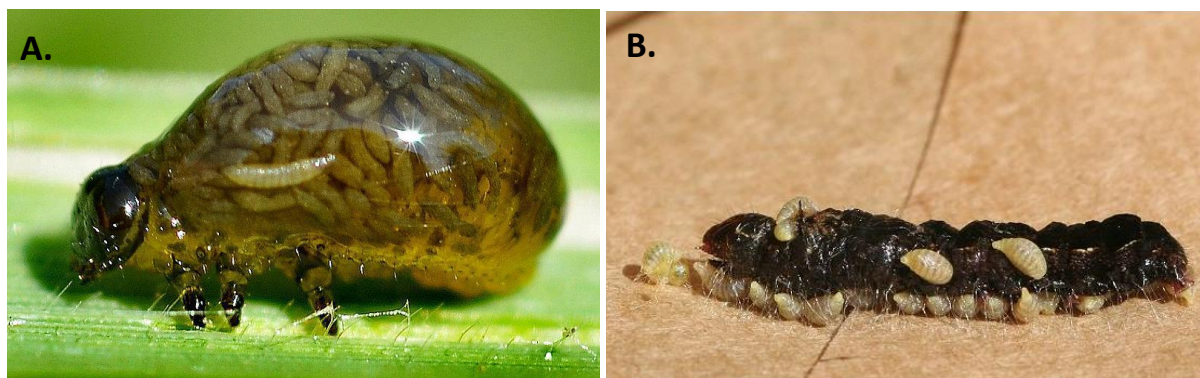


Figure 1: A. Larvae of the endoparasitoid wasp *Tetrastichus julis* inside the Cereal Leaf Beetle, *Oulema melanopus* (©Claude Pilon) B. Larvae of the ectoparasitoid wasp *Euplectrus* sp. on a Noctuidae caterpillar (©Edith Smith).

2. Tiny but mighty: *Nasonia vitripennis*

Nasonia vitripennis can be seen as the Tinkerbell of the animal kingdom: with its size not bigger than 2.5 mm, it received wider recognition since its genome was sequenced in 2010. The genus *Nasonia* consists out of 4 related parasitoid species – *N. vitripennis*, *N. giraulti*, *N. longicornis* and *N. Oneida* – of which *N. vitripennis* is a worldwide distributed ectoparasitoid, that can parasitize the pupal stages of several Dipteran families, including Sarcophagidae and Calliphoridae. Like other parasitic Hymenoptera, *Nasonia* is haplodiploid meaning that females are diploid and develop from fertilized eggs, whereas males are haploid and develop parthenogenetically from unfertilized eggs (The *Nasonia* Genome Working Group, 2010). The availability of the genome together with its ease to work with in the laboratory, makes *Nasonia* a solid model organism in the insect order of Hymenoptera that can keep standing next to heavyweights such as *Caenorhabditis elegans* and *Drosophila*.

2.1. Life cycle

Nasonia has a relatively short generation time of approximately 14 days (figure 2), depending on environmental temperatures and exposure to light. When a female encounters a suitable host, she drills through the puparial wall with her ovipositor. Firstly, she will feed on host hemolymph by producing a tube (with fluids from the alkaline gland) that connects the interior of the pupa with the exterior of the puparium (Whiting, 1967). The wasp feeds on the host fluid drawn up through this feeding tube. Secondly, she injects venom by directly inserting her ovipositor inside the host. Thereafter, she retracts her ovipositor to lay 20 to 60 eggs on the exterior of the developing host, but still within the puparium. *N. vitripennis* therefore is an ectoparasitoid wasp with no need for a paralyzing venom, since the host is already in an immobile state (in contrast to endoparasitoid wasps). The eggs hatch around 24 hours later and the larvae start crawling and feeding on the host. Around 9 days after laying, the larvae pupate and undergo several pupal stages. The adults eclose from pupation in the host and chew their way out through several exit holes. Mating occurs immediately after emergence from the host.

2.2. Hosts

N. vitripennis is known in the field as a generalist that parasitizes a diverse set of hosts. In laboratory choice experiments however, it shows a preference for the flesh fly species *Sarcophaga* (Diptera: Sarcophagidae) (Desjardins *et al.*, 2010). Flesh flies are larviparous, meaning that eggs are not deposited upon full development (figure 2). Instead, the larvae hatch inside of their mother's uterus and are held until a proper host is found. First instar larvae are deposited on varying types of carrion (in laboratory conditions, beef liver is used) where they eat and rapidly develop. Approximately 5 days after larviposition, they leave the host and start pupating. About 10 days later, adult flies emerge. To humans, these flies are usually nuisance pests and they can be vectors of pathogens and bacteria. In agriculture, they are rather beneficial since the larvae of some species can prey on eggs, nymphs or larvae of more harmful insects.

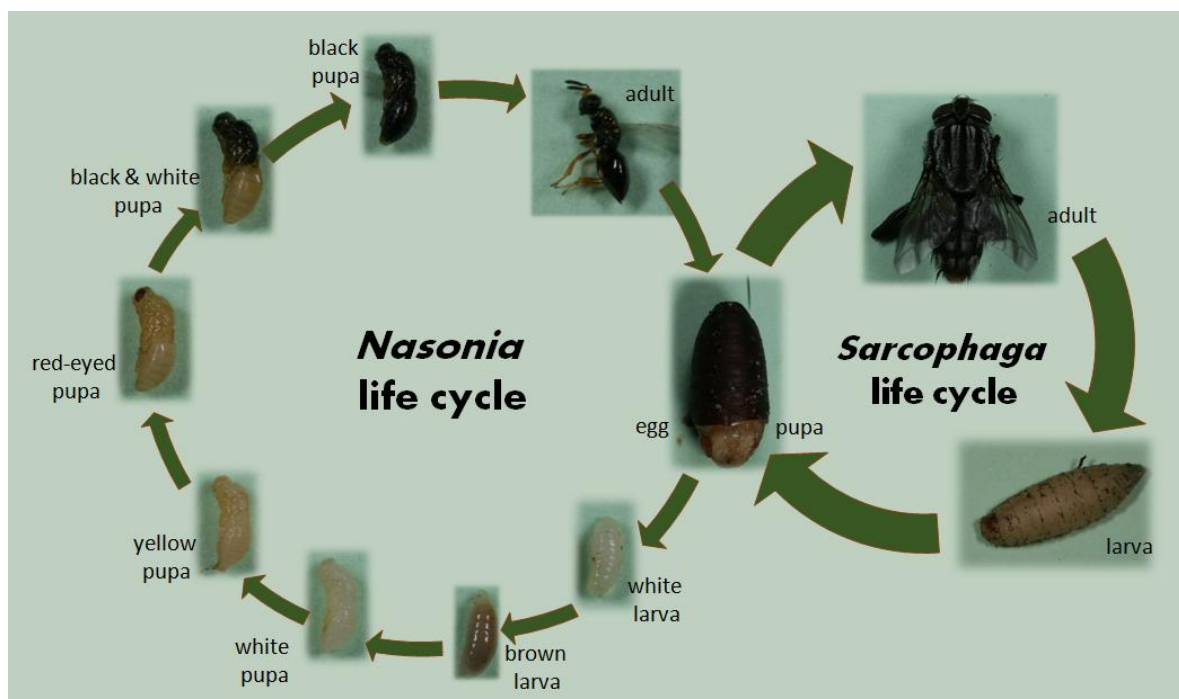


Figure 2: The life cycle of *N. vitripennis* is presented on the left. The eggs are laid on the pupal stage of the host, *S. crassipalpis* (centre of the figure, puparium partly removed). The eggs (difficult to see on the small picture) develop into larvae and go through several instar stages after which they pupate inside the host puparium. The white pupae gradually change in colour starting from the eyes which first become red. Subsequently, the thorax darkens followed by the abdomen until the whole pupa is black. After 14 days, the adults emerge from the host pupa. The life cycle of *S. crassipalpis* is presented on the right. Larvae are laid on the host, beef liver in this case, and start pupating 5 days later. After 10 days, adults emerge.

Sarcophaga crassipalpis is a flesh fly species that is commonly used to study insect pupal diapause (Pavlidis *et al.*, 2011). Under very specific environmental stimuli, the pupae can enter a hibernation-like state where metabolic processes are reduced almost to a complete stop until appropriate environmental conditions return (Denlinger, 1972). In 2009, an EST database became available which makes this species even more suitable for research (Hahn *et al.*, 2009).

3. Parasitism warfare

"You can figure out what the villain fears by his choice of weapons."

[The Bridal Season, Connie Brockway]

The extended plethora of parasitoid effector molecules reflects the hostile environment these wasps need to survive in during their early development (Burke & Strand, 2014). Endoparasitoid eggs come in direct contact with host tissues raising the need for a variety of methods to commander the host. The manipulative capabilities of wasp-injected venom, often together with polydnviruses (PDVs), is essential for wasp survival. These PDVs are genetic symbionts of the wasp and their genome is integrated into the genomic DNA of their wasp carrier (Gundersen-Rindal *et al.*, 2013). They exclusively replicate in the ovaries of the female wasp where they co-mingle with parasitoid eggs. Before or during egg-laying, the female wasp also injects venom into the host, which is traditionally produced and secreted by the acid gland and stored in the venom reservoir until needed (Asgari & Rivers, 2011). After eggs are laid, sometimes teratocytes can be found in the hemolymph of parasitized hosts. These large cells typically originate from the anterior and posterior serosal cells surrounding the developing embryo and are believed to be involved in bypassing immune responses, secretion of proteins and larval nutrient uptake (Andrew *et al.*, 2006; Nakamatsu *et al.*, 2002; Hotta *et al.*, 2001). Even the wasp larvae may secrete products while developing, affecting metamorphosis, development, hemocytes and other immune reactions in the host (Richards & Edwards, 2002; Edwards *et al.*, 2006). This large variety of factors introduced by parasitoids make up their arsenal, empowering them to cause major mortality in host populations.

3.1. Shared arsenal in parasitoids: venom

Venoms that function as defensive secretions, produced for instance by honeybees, tend to consist of streamlined and conserved components, while predatory venoms, like from parasitoids, are more complex and often highly variable in composition and physiological effects (Casewell *et al.*, 2013). Parasitoid venoms typically comprise a complex cocktail of proteins and peptides (commonly referred to as toxins), salts and organic components, such as amino acids and neurotransmitters (Kapoor, 2010). Most studies have been conducted on the proteinaceous components using mass spectrometry (Formesyn *et al.*, 2011), in *N. vitripennis* resulting in a list of 80 venom proteins (de Graaf *et al.*, 2010; Ye *et al.*, 2010). Since PDVs and teratocytes only aid the parasitization process in endoparasitoids, the arsenal of the ectoparasitoid *N. vitripennis* only consists out of injected venom and secretions from the parasitoid larvae making it an interesting model organism to study venom functionalities.

3.2. Mode of action of the venom

A common strategy in war is to attack on multiple fronts in order to make the enemy more vulnerable. This exact tactic is applied by the parasitoid *N. vitripennis*, modifying host physiology on different levels. The ectoparasitoid wasp needs to create an environment that supports and promotes its own development, but in the end leads to the detriment of the host insect. In order to succeed, *N. vitripennis* needs to weaken the enemies first defence line which means suppressing its immune system (Rivers *et al.*, 2002b). Insects lack adaptive immunity but possess an effective innate immune system that successfully combats infections (Vilmos & Kurucz, 1998). Figure 3 gives an overview of the innate immune system in insects that is (in short) explained below. First, pathogens are recognized (extracellular), after which the humoral and the cellular immune responses become immediately activated and respond in concert to clear the pathogen. The former defence process includes melanisation, clotting of the hemolymph and the synthesis of antimicrobial peptides (AMPs). In cellular reactions, the microorganisms or the apoptotic cells are phagocytised, entrapped by nodule formation or encapsulated by hemocytes. Exogenous factors trigger immune signalling proteins, which transfer the signal across the plasma membrane (now intracellular) and initiate host immune response (Lemaitre & Hoffmann, 2007).

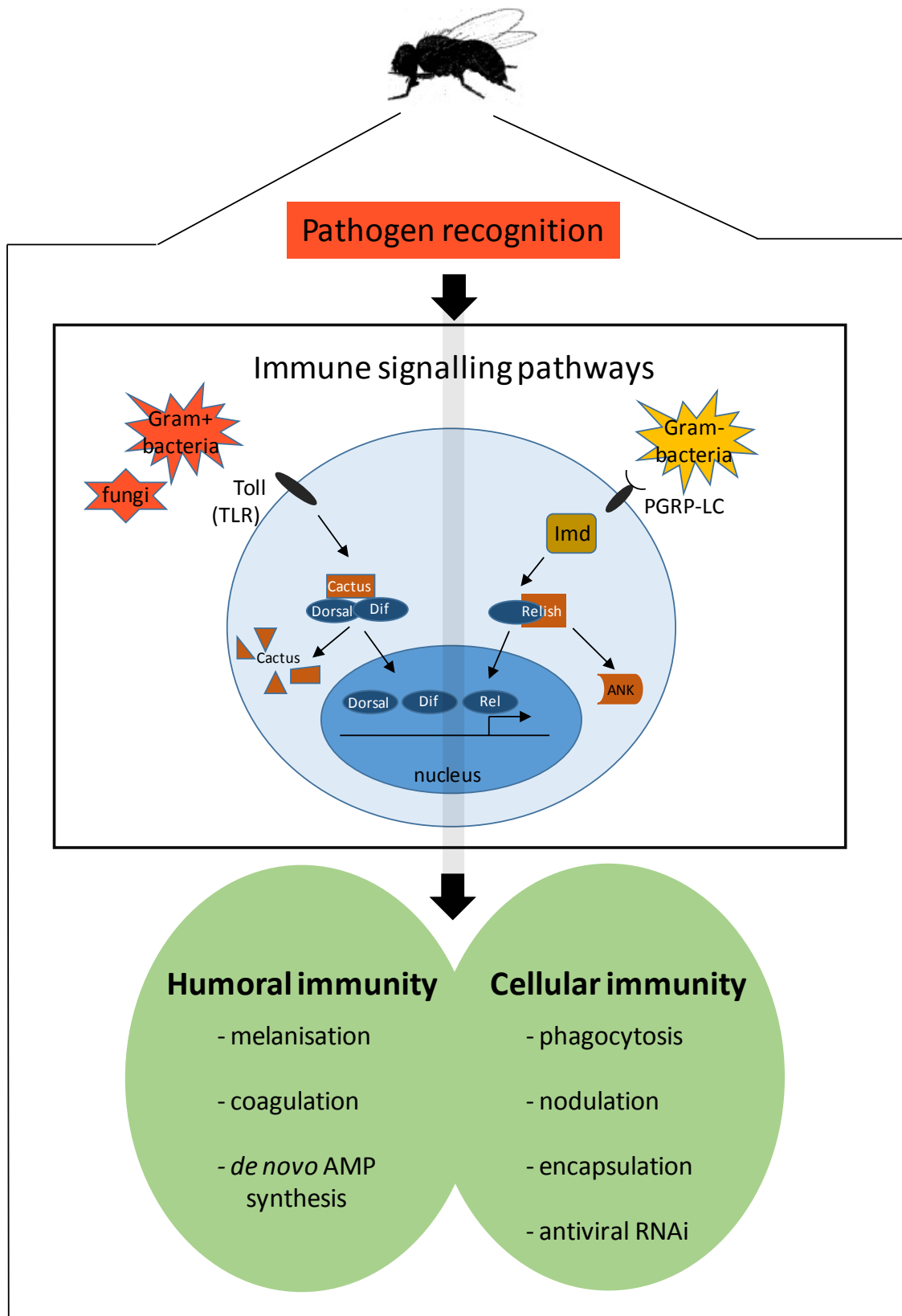


Figure 3: Summary of the innate immune system in insects. Pathogen recognition can directly lead to humoral or cellular immune defence (represented by the vertical grey bar), but can also transfer extracellular signals into intracellular signals via immune signalling pathways. The two major immune signalling cascades, Toll and Imd, result in the production of AMPs.

Defence against Gram-positive bacteria and fungi is driven by the activation of the Toll receptor that leads to a series of intracellular signalling cascades. This leads to the activation of the transactivator Nuclear Factor- κ B (NF- κ B) proteins Dorsal and Dif by the dissociation and degradation of Cactus. Both Dorsal and Dif translocate to the nucleus, resulting in the nuclear transcription of *Drosomycin* and other antimicrobials. Gram-negative bacteria on the other hand, trigger the immune deficiency (Imd) pathway, which also activates a series of signalling cascades in the cytoplasm, this time leading to cleavage of the transcription factor Relish into Rel and ANK domains. After translocation of Rel to the nucleus, transcription of *Diptericin* and other antimicrobials is initiated. Other immune signalling cascades in insects are the JAK/STAT pathway triggered by cytokines (Pastor-Pareja *et al.*, 2008), the Growth Blocking Peptide (GBP) pathway induced by stress (Tsuzuki *et al.*, 2012) and the integrin cascade involved in the initiation of cellular immunity (Irving *et al.*, 2005). Parasitoids have found innovative ways to overcome this wide variety of defence mechanisms. They circumvent the host's encapsulation response and suppress the host cellular immune response by substances introduced into the host's body cavity. Such substances may adversely affect the ability of hemocytes to recognize nonself, proliferate, differentiate, migrate and adhere to foreign surfaces, or prevent the host from synthesizing toxic molecules (Vass & Nappi, 2000).

When the parasitoid venom has crumbled away the host defence walls, further growth and development of the host is arrested (Rivers *et al.*, 1993; Rivers & Denlinger, 1994a), presenting it no choice but to atrophy over time. Contributing to this developmental halt, cell death is elicited by *N. vitripennis* venom injection (Rivers *et al.*, 2011). The loss of membrane integrity and subsequent calcium release disrupt the sensitive balance of calcium homeostasis, causing cells to die by apoptosis or oncosis (Rivers *et al.*, 2006). As if these life-threatening attacks are not enough, complete control over the host is gained by the venom forcing the host to provide lipids and other nutrients in order to satisfy the wasp offspring (Rivers & Denlinger, 1995). Many ecto- and endoparasitoid venoms regulate their host nutritional milieu by digesting fat body cells and stimulating host nutrient synthesis, allowing nutrients to flow into the hemolymph where they can be consumed by the developing larvae (Nakamatsu & Tanaka, 2004). The different fought battles between *N. vitripennis* venom and its host are explained into more detail in the following paragraphs.

3.2.1. Venom targets the host defence system

One major innate defence system in invertebrates is the melanisation of pathogens and damaged tissues. This important process is controlled by phenoloxidase (PO), a multicopper oxidase, which results in the deposition of melanin around the damaged tissue or intruding object. This physical shield prevents or retards its growth and, perhaps even more importantly during melanin formation, will produce highly reactive and toxic quinone intermediates. These quinones are also involved in the production of cytotoxic molecules, such as superoxides and hydroxyl radicals, which could aid in the killing of invading microorganisms. When *N. vitripennis* females parasitize on flesh fly hosts, melanisation occurs within a matter of minutes in tissues penetrated by the ovipositor (Rivers *et al.*, 2002b). Blackening of the tissues remains localized unless the fly is a non-permissive host, in which case melanisation and tissue necrosis may spread rapidly from the site of venom injection (Rivers *et al.*, 2003). In the endoparasitoid wasp *Pimpla hypochondriaca*, three PO proteins were found to be abundantly present in venom (Parkinson *et al.*, 2001). The authors suggested that PO products can bind to the hemocyte plasma membrane and disrupt their function, thus contributing to host immune suppression. Interestingly, *N. vitripennis* venom was also found to possess PO activity that, different from *P. hypochondriaca*, was proven to play an important role in cell death (Abt & Rivers, 2007). This implies that the venom protein is a tyrosinase (EC 1.14.18.1 mono-phenol monooxygenase; L-DOPA:oxygen oxidoreductase) that is known to catalyze synthesis of reactants involved in the biosynthesis of melanin (Mason, 1965). However, in the bioinformatic and proteomic study of *N. vitripennis* venom, only 2 laccases (EC 1.10.3.1 o-diphenol oxidase: 1,2-benzenediol:oxygen oxidoreductase) were found that, just like tyrosinases also catalyse oxidation of L-DOPA, but are mostly involved in tanning of the soft, newly synthesised cuticle rather than in the melanisation pathway (Sugumaran, 1988). The specific venom compound responsible for the PO activity in the host therefore needs to be elucidated.

The PO pathway contributes to the prevention of encapsulation of parasitoid eggs. The capsules trapping invading microorganisms and parasites require activation of series of serine proteinases. This cascade is targeted by several venom compounds of parasitoid wasps as another strategy to suppress the host immune system. These venom compounds known so far are listed in table 1, together with homologues that were found in *N. vitripennis* venom. The function of the latter proteins however still needs to be confirmed.

Table 1: Venom compounds from different parasitoid wasps with their effect on the host organism (see reference) that have a homologue venom protein in venom from *N. vitripennis* of which its effect in the host is yet unproven. (Abbreviations: IR = immune response; VP = venom protein; PAP = PO-activating proteinase)

Venom-host interaction	Parasitoid venom compound	Parasitoid wasp	Reference	Homologue <i>N. vitripennis</i> VP
Immune suppression				
inhibition of proPO activation	Vn50, serine protease homologue	<i>Cotesia rubecula</i>	(Zhang <i>et al.</i> , 2004)	serine protease homolog (SPH21)
inhibition of proPO activation	LbSPNy, serine protease inhibitor	<i>Leptopilina boulardi</i>	(Colinet <i>et al.</i> , 2009)	small serine protease inhibitor-like VP
inhibition of proPO activation	serine protease inhibitor	<i>Venturia canescens</i>	(Beck <i>et al.</i> , 2000)	small serine protease inhibitor-like VP
inhibition of proPO activation	cvp1, Kunitz type cysteine rich VP	<i>Pimpla hypochondriaca</i>	(Parkinson <i>et al.</i> , 2004)	cysteine-rich/KU VP
inhibition of proPO activation	cvp2, pacifastin cysteine rich VP	<i>Pimpla hypochondriaca</i>	(Parkinson <i>et al.</i> , 2004)	cysteine-rich/pacifastin VP 1-2
PAP inhibition	Egf1.0, cysteine rich TIL VP	<i>Microplitis demolitor</i>	(Beck & Strand, 2007)	cysteine-rich/TIL VP 1-2
inhibition of host cellular IR	PpCRT, calreticulin	<i>Pteromalus puparum</i>	(Wang <i>et al.</i> , 2013)	calreticulin
inhibition of host cellular IR	CrCRT, calreticulin	<i>Cotesia rubecula</i>	(Zhang <i>et al.</i> , 2006)	calreticulin
breaking physical barriers	PSP, chitinase	<i>Toxoneuron nigriceps</i>	(Consoli <i>et al.</i> , 2005)	chitinase 5
immune stimulation				
antibacterial	ACE, angiotensin converting enzyme	<i>Pimpla hypochondriaca</i>	(Dani <i>et al.</i> , 2003)	angiotensin-converting enzyme
developmental arrest				
retardation of growth	EpMP1-3, metalloproteases	<i>Eulophus pennicornis</i>	(Price <i>et al.</i> , 2009)	metalloprotease
cell death				
Ca ²⁺ release leading to cell death	calreticulin	<i>Pimpla hypochondriaca</i>	(Rivers <i>et al.</i> , 2009)	calreticulin
apoptosis in ovaries	Ae-γ-GT, γ-glutamyl transpeptidase	<i>Aphidius ervi</i>	(Falabella <i>et al.</i> , 2007)	γ-glutamyl transpeptidase-like VP 1-2
nutritional				
catalyze trehalose to provide glucose	tre1, trehalase	<i>Pimpla hypochondriaca</i>	(Parkinson <i>et al.</i> , 2003)	trehalase

Another major innate defence system in invertebrates is the clotting reaction of hemolymph, which is based on a combination of soluble and cell-derived factors. Hemocytes proliferate and differentiate into lamellocytes that carry out a permanent surveillance in circulation. When an invasion occurs, these lamellocytes are massively released to form a multilayered capsule around the particle (Lemaitre & Hoffmann, 2007) being part of the humoral immune response. Some snake venoms have been found to prevent blood clotting, mainly to spread its venom more effectively and to gain a better digestible prey. A similar function can be assigned to parasitoid venom. Remarkably, following oviposition by *N. vitripennis*, no evidence of hemolymph clotting occurred at the wound site. David B. Rivers and colleagues suggested that the female wasp must inject factors into the fly host that retard or inhibit clotting and/or wound healing processes (Rivers *et al.*, 2002b). After the female wasp has injected venom into the host and has laid her eggs, she often starts making a feeding tube (depending on whether the female has oviposited before (Rivers, 1996)), connecting the interior of the pupa with the exterior of the puparium (Whiting, 1967). Inhibition of coagulation is of crucial importance in this process, since the wasp feeds on the host fluid drawn up through the feeding tube. In addition to this, the young parasitoid larvae also feed on the host, grabbing and puncturing the host's integument with their mandibles and taking in the host's body fluids. Richards and Edwards argued that the ectoparasitoid *Eulophus pennicornis* relies entirely on salivary secretions from larvae to knock out host defences (Richards & Edwards, 2000). A similar scenario was reported for the ectoparasitoid *Euplectrus plathypenae* (Coudron *et al.*, 1990). Larval secretions are known to inhibit clotting but they can also contribute to the action of maternal venom that is injected into the host. Next to preventing clotting to keep the host digestible, venom also serves to inhibit accumulation of hemocytes around the parasitoid eggs aiding the suppression of immune defence. The protein calreticulin in venoms of two endoparasitoid wasps, PpCRT in *Pteromalus puparum* and CrCRT in *Cotesia rubecula*, has been demonstrated to inhibit hemocyte spreading behaviour and prevent encapsulation (Wang *et al.*, 2013; Zhang *et al.*, 2006). Calreticulin is also present in *N. vitripennis* venom (table 1), but its possible involvement in host immune suppression still needs to be elucidated.

Both targeted melanisation and coagulation cascades represent direct immune suppression towards the parasitoid eggs. However, since suppression of these cascades typically occurs

with endoparasitic species to protect the eggs/larvae, the exact significance to an ectoparasitoid like *N. vitripennis* still needs to be determined. Furthermore, venom components are also assumed to target the overall defence system of the host next to blocking direct encapsulation or clotting around the invading parasitoids. Chitinase for instance, from teratocytes of the endoparasitoid *Toxoneuron nigriceps* was suggested to be involved in breaking physical barriers and gaining access to the host to initiate an infection process (Consoli *et al.*, 2005; Kramer & Muthukrishnan, 1997). A chitinase 5 protein is also present in *N. vitripennis* venom (table 1), possibly aiding in weakening the host as a whole making it unable to start up a sufficient immune response against intruders.

The suppressive potential towards the immune system of the host is relatively well-known for parasitoid venom (Fang *et al.*, 2010; Labrosse *et al.*, 2003; Schmidt *et al.*, 2001). However, these female wasps face the challenge of conditioning the host to maximize progeny production, while avoiding excessive manipulation that leads to rapid deterioration of the host. If injected venom evokes suppression of host physiology leading to a completely immune-compromised host, unregulated microbial attack may occur. The result is an unfavourable host environment in which the parasitoid's progeny compete directly with microorganisms for host nutrients, and in turn, face direct invasion by the microbes. Thus, one might expect that the parasitoid's venom suppresses host immunity only selectively, for instance by interfering with host melanisation and coagulation responses (see above), yet allowing or even stimulating certain antimicrobial defences. Venom of *P. hypochondriaca*, was found to have antibacterial activity against two Gram-negative bacteria (Dani *et al.*, 2003). Peptidases present in this venom were speculated to be involved in the processing of biologically active peptides, including antibacterial peptides. An angiotensin-converting enzyme (ACE) could be involved in the synthesis of the antibacterial peptide, peptide B, from the C-terminal region of pro-enkephalin Tasiemski *et al.*, 2000). This enzyme was also found in *N. vitripennis* venom (table 1), but its possible antibacterial function has not been investigated yet.

3.2.2. Venom targets the host development

Parasitized insects typically undergo developmental arrest and die some time after the parasitoid has become independent of its host. In endoparasitoids, the developmental arrest

is induced by the venom, polydnviruses or teratocytes (Dahlman *et al.*, 2003; Reed & Beckage, 1997). In ectoparasitoids, paralysis is used to immobilize the host and the developmental arrest is necessary to prevent elimination of the larvae during host moulting. Interestingly, David B. Rivers and colleagues suggested that the venom of *N. vitripennis* is nonparalytic and development of envenomated hosts is either arrested or delayed (Rivers *et al.*, 1993). In several parasitized hosts, developmental arrest is due to ecdysteroid deficiency, which is often the result of a hormone imbalance, caused by a disruption or deterioration of endocrine tissues, or by inhibition of target tissues to respond to hormone signals (Asgari & Rivers, 2011). But unlike many host-parasitoid associations, the arrest in hosts parasitized by *N. vitripennis* seems not to be due to ecdysteroid deficiency (Rivers & Denlinger, 1994a). Interestingly, brain tissues of *Sarcophaga* hosts were also found to be affected by the venom, resulting in cell death and may be involved in the developmental arrest observed in parasitized hosts (Rivers *et al.*, 2011). Recent studies revealed that under this arrest, *N. vitripennis* venom causes incomplete development of bristles and eye pigment deposition (Rivers & Brogan, 2008). When recombinant EpMP3, resembling the venom protein metalloproteinase from *E. pennicornis*, was injected into fifth instar larvae of *Lacanobia oleraceae*, it was found to retard host development (Price *et al.*, 2009). *N. vitripennis* venom contains a similar metalloprotease of which its function until now is unknown (table 1).

3.2.3. Venom causes host cell death

Cell death is a common process in living organisms and plays an important role during developmental processes, tissue homeostasis, immune reactions and wound repair. Under certain pathological conditions, cells can undergo apoptosis, which is often seen in host-parasitoid relationships and associated with immune suppression of the host (Suzuki & Tanaka, 2006; Asgari & Rivers, 2011; Fang *et al.*, 2010). Apoptosis results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cytoskeletal reorganization and formation of apoptotic bodies (Elmore, 2007). Inappropriate regulation of apoptosis leads to many human pathologies, for instance too little apoptosis can result in certain cancers, while overexcessive apoptosis can lead to certain autoimmune diseases.

Upon venom induction by *N. vitripennis*, several events are initiated in the host, starting with the loss of mitochondrial membrane potential (Rivers *et al.*, 1999). The induced changes in

plasma membrane permeability cause an influx of Na^+ , which results in the release of Ca^{2+} from the mitochondria (Rivers *et al.*, 2002a, 2005). The disruption of the calcium homeostasis in cells, may cause them to die by apoptosis. The uncontrolled Ca^{2+} efflux into the cytosol is followed by the increase of cytoplasmic cAMP levels that promote additional Ca^{2+} release from intracellular stores. Excessive Ca^{2+} release then triggers PLA_2 activation which may stimulate fatty acid synthesis in the host fat body. This fatty acid accumulation can become toxic and again induce cell death (Rivers *et al.*, 2002a). When homeostasis of the intracellular environment is disrupted, macromolecules and ions can enter the cell in an unregulated way, followed by water. This process results in membrane blebbing, rounding, swelling and eventually in lysis of the cells. Venom from *N. vitripennis* causes this cellular injury that ultimately culminates in oncotic cell death (Rivers *et al.*, 2006). Phenoloxidase activity has been proposed to be directly linked to cell death, but since only laccases and no tyrosinases were found in the venom (see 3.2.1. Venom targets the host defence system), maybe other venom compounds are involved in this complex process. The protein calreticulin, which was suggested to be involved in the inhibition of hemocyte spreading (see above), was found to mobilize intracellular Ca^{2+} leading to cell death in hosts envenomated by *P. hypochondriaca* (Rivers *et al.*, 2009). The venom compound γ -glutamyl transpeptidase in another endoparasitoid wasp was proven to initiate apoptosis, specifically to the ovaries causing host castration (Falabella *et al.*, 2007). Both calreticulin and γ -glutamyl transpeptidase are also found in *N. vitripennis* venom (table 1), but their function needs to be assigned.

3.2.4. Venom provides host with available nutrients

Parasitoids regulate host physiology in an attempt to maximize mother's fecundity and to provide optimal conditions for the offspring to feed and develop. Therefore, the nutritional and physiological environment of the host is manipulated by venom or other related factors injected by the female wasp. Teratocytes of endoparasitoids for example, have a secretory and nutritive function to ensure a nutritional milieu for the offspring (Nakamatsu *et al.*, 2002), while venom of some ectoparasitoids changes the host metabolism to provide nutrients (Rivers & Denlinger, 1995; Mrinalini *et al.*, 2014). Early after venom injection by *N. vitripennis*, host trehalose levels drop significantly. Since tre1, a trehalase venom protein in *P. hypochondriaca* was found to catalyze trehalose to provide the wasp offspring with

sufficient glucose, a putative role of *N. vitripennis* venom trehalase in providing host nutrients can be proposed (table 1). On the long term, pyruvate levels in the host increase, whereas whole body lipid levels rise during the first 4 days, followed by a decrease 8 days after envenomation (Rivers & Denlinger, 1994b). These high lipid levels seem to be synchronized with the development of the parasitoid larvae. The change in plasma membrane permeability that leads to mitochondrial Ca^{2+} release and finally results in fatty acid synthesis in host fat body (see 3.2.3. Venom causes host cell death), is also a proposed pathway for lipid elevation (Rivers *et al.*, 2002a). Parasitoid larvae have to share the available host lipids with adult *N. vitripennis* wasps, since the latter have lost the ability to synthesize lipids *de novo* and therefore, are completely dependent on the lipids of the host (Rivers *et al.*, 2002a). Next to this, the developing larvae are also competing with host tissues for available nutrients. Teratocytes are able to convert lipids into fatty acids which are specifically delivered to the parasitoid larvae, giving them the lead in the competition for lipids with host tissues (Falabella *et al.*, 2005).

3.3. At war's end: applications

The suite of parasitoid products that is delivered to the host insect is so amazingly potent and innovative in its manipulative ability that we as scientists can still learn immensely from this regulatory warfare. Once these regulatory molecules have been identified and characterized, research activities must be lifted to the next level. More specifically, their potential application must be investigated, either in the agronomic sector or in the biomedical domain.

3.3.1. Agronomic applications

It is estimated that insect pests destroy approximately 14% of the World's crop production (Oerke & Dehne, 2004). In many cases, natural enemies are not sufficient to control pests adequately (Hopper, 2003). Therefore, chemical insecticides are currently the dominant approach for combating crop pests (Vais *et al.*, 2001). However, the perceived ecological and human health risks in combination with the development of insecticide resistance in many pest insect populations, have created an urgent need for improved methods in insect pest control that keeps the pest species below the economic-damage threshold, while leaving the

other fauna species undisturbed. Mechanical methods are used that include barriers or traps to capture pest insects (Hansen *et al.*, 2006), yet biological methods have gained the upper hand. Biological pest control contains the reproduction of natural predators of the host insects (Nagai & Yano, 2000), the interruption of the reproductive process of the pest through the insertion of sterile individuals (Gurr & Kvedaras, 2010), and the use of bio pesticides. For the latter, a promising source of novel insecticidal peptides are the venoms of arthropod predators. These interesting compounds have been optimized via a prey-predator arms race spanning hundreds of millions of years to target specific types of insect ion channels and receptors.

Since parasitoids are known to have the ability to control populations of insect pests, agronomic industrials have already manifested their interest towards parasitoid venom proteins as attested, for example, by the patent application concerning *Bracon hebetor* venom neurotoxins (Quistad & Leisy, 1996). Nonetheless, no parasitoid venom compound has yet been used as a pest control agent. This restriction may come from the fact that these compounds need to be directly delivered into the host hemolymph and they would be degraded after delivery by foliar sprays. Therefore, in order to be effective, the venom proteins or peptides would need to be delivered via a vector, such as entomopathogens. Baculoviruses or fungi can be engineered in such a way that they express transgenes encoding the venomous toxins. This implies that the venom peptides are produced systemically in the insect host after viral/fungal infection and the often difficult delivery by oral uptake is avoided. Another major advantage is the limitation of off-target effects, since the range of affected insects is largely determined by the host range of the entomopathogen (Smith *et al.*, 2013). Another possible deployment of the venom toxins to the pest insects is through transgenic plants. Genetically modified plants can express the venom toxins with a fusion protein so they can be orally delivered to the hemolymph of the insect pests. An insecticidal spider venom neurotoxin has been successfully delivered to the hemolymph of its lepidopteran host by using the mannose-specific snowdrop lectin as a carrier protein (Fitches *et al.*, 2004). The lectin resists gut proteolysis, binds to glycoproteins on the surface of the gut epithelium and can be detected in the hemolymph of orally exposed insects. Also trait stacking of insecticidal venom peptides with δ -endotoxin from *Bacillus thuringiensis* (Bt) that has already proven to be commercially effective (Kumar *et al.*, 2008), might

constitute a promising insect pest control system. Both transgenes have completely different mechanisms of action and are likely to be synergized by *Bt*, which should facilitate movement of venom peptides into the insect hemocoel by virtue of its ability to induce lysis of midgut epithelial cells (Soberon *et al.*, 2007).

3.3.2. Biomedical applications

The most innovative and promising potential of parasitoid venom proteins probably concerns biomedical applications. Many arthropod venoms are known for their antimicrobial and cytolytic peptides (Kuhn-Nentwig, 2003; Ye *et al.*, 2010). Also in the venom of parasitoid wasps, antibacterial activity has been detected (Dani *et al.*, 2003), insinuating a possible use of these compounds as new antibiotics. However, the broad cytolytic effects of many of these venom constituents unfortunately results in difficult discrimination between microorganisms and eukaryotic cells, making them not well suited for antibiotic drug use. On the other hand, more recently, the potential of parasitoid venom compounds in treatments for certain cancers and neurodegenerative and inflammatory diseases seems to be very promising. This is explained in more detail in the following paragraphs.

3.3.2.1. NF- κ B as biomedical target

The central and evolutionary conserved coordinator in these pathologies is the nuclear factor- κ B (NF- κ B) pathway. It controls the expression of genes involved in development, cell cycle regulation (Ledoux & Perkins, 2014), immune regulation, acute and chronic inflammation, cytoskeletal remodelling, cellular adhesion, survival and apoptosis (Baldwin, 2001; Oeckinghaus *et al.*, 2011). When NF- κ B is constitutively active, it drives chronic inflammation, which is essential to the development, maintenance and progression of multiple diseases. These diseases include cancer, arthritis, atherosclerosis, inflammatory bowel disease, degenerative disorders and autoimmune processes. A large number of external stimuli can lead to activation of NF- κ B, such as bacterial and viral infections (e.g. through recognition of microbial products by receptors like the Toll-like receptors), inflammatory cytokines and antigen receptor engagement eventually induced upon physical (UV- or γ -irradiation), physiological or oxidative stresses (Hayden & Ghosh, 2004) (figure 6). In most unstimulated cells, NF- κ B dimers are retained in an inactive form in the cytosol

through their interaction with the inhibitor of NF- κ B proteins (I κ Bs). The majority of the diverse signalling pathways that lead to NF- κ B activation converge on the I κ B kinase (IKK) complex, which is responsible for I κ B phosphorylation that is ubiquitinated and degraded by the IKK proteasome (Karin & Ben-Neriah, 2000). The degradation of I κ B thus allows NF- κ B to translocate into the nucleus where it can act as a transcription factor. NF- κ B then binds to specific elements (κ B-sites) within the promoters of responsive genes to activate their transcription. An extensive list of target genes is controlled by NF- κ B, including central components of the immune response, inflammatory processes (controlled by cytokines, chemokines, cell adhesion molecules, complement cascade factors and acute phase proteins), regulators of apoptosis and proliferation, embryonic development and physiology of the bone, skin and central nervous system (Pahl, 1999; Xing *et al.*, 2013). Figure 6 gives an overview of the most important stimuli and targets of the central NF- κ B pathway, together with a simplified presentation of the canonical NF- κ B cascade. Parallels can be observed when comparing figure 6 that describes the NF- κ B pathway in mammals with figure 3 that overviews the well conserved Toll and Imd pathways in insects.

Because so many key cellular processes such as cell survival, proliferation and immunity are regulated through NF- κ B-dependent transcription, it is not surprising that it is tightly regulated at multiple levels. The primary mechanism for NF- κ B regulation is through I κ B and the kinase that phosphorylates I κ Bs, namely the IKK complex. A number of post-translational modifications also modulate the activity of the I κ B and IKK proteins, as well as the NF- κ B molecules themselves, that generate auto-regulatory feedback loops in the NF- κ B response (Oeckinghaus & Ghosh, 2009). Since the deregulation of the NF- κ B pathway results in severe diseases, there has been (and still is) great pharmacological interest in modulators of NF- κ B activation. Many natural products have appeared to contain therapeutic and preventative effects in certain cancers and inflammatory diseases, most likely due to their ability to inhibit NF- κ B (Gilmore & Herscovitch, 2006). In 2006, over 785 inhibitors of the NF- κ B pathway were already identified, but for most inhibitors, neither the molecular target nor the cell-type specificity was known. Since single molecular components take part in overlapping signalling pathways, it is a challenge to find molecules that block specific pathways leading to NF- κ B activation without interfering with other signalling cascades. Therefore, it remains

interesting to see whether venom compounds from *N. vitripennis* are able to meet with these conditions and could serve as potent therapeutic NF- κ B-inhibitory agents.

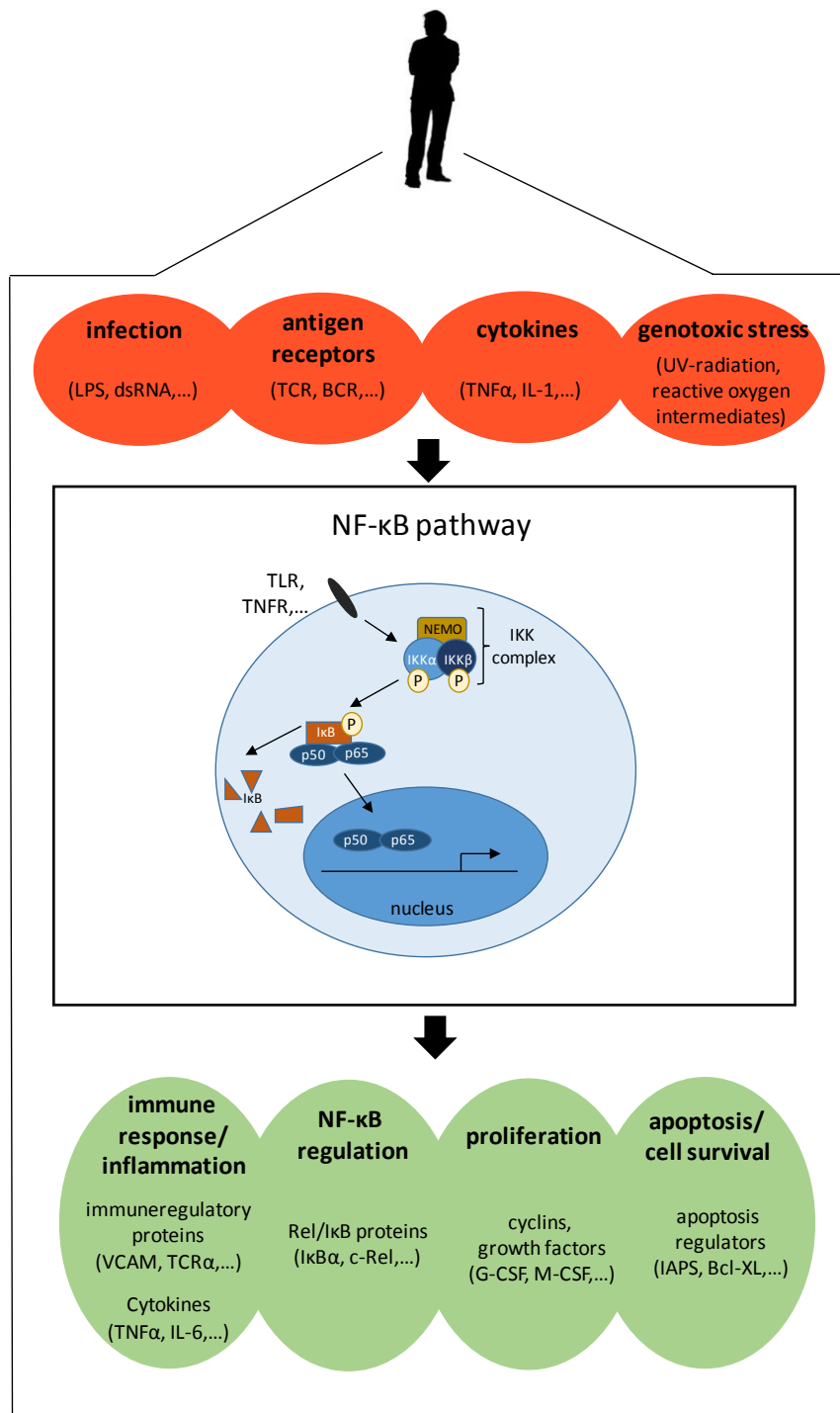


Figure 6: Summary of the NF- κ B pathway in mammals with its stimuli in orange and its targets in green. NF- κ B acts as a central mediator of immune and inflammatory responses, and is involved in stress responses and regulation of cell proliferation and apoptosis. The respective NF- κ B target genes allow the organism to respond effectively to these environmental changes. Representative examples are given for NF- κ B inducers and κ B-dependent target genes.

3.3.2.2. Insect compounds targeting mammalian immune systems

The innate immune system confers broad protection against pathogens and its response is essentially instantaneous. Interestingly, most multicellular organisms exclusively depend on this fundamental immune system (Kimbrell & Beutler, 2001). Many insect proteins that are important compounds in this innate immune system, have been tested for potential therapeutical use in mammalian pathologies. For instance, several insect hemolymph peptides have shown to display specific tumoricidal activity by disrupting the membrane of cancer cells. Some AMPs like cecropins and defensins have the ability to form ion-permeable channels subsequently resulting in cell depolarization, irreversible cytolysis and finally cell death. Interestingly, these antibacterial compounds in insects also exert tumoricidal activity by selective cytotoxicity against several mammalian cancer cell lines (Iwasaki *et al.*, 2009; Lehmann *et al.*, 2006; Hui *et al.*, 2002; Winder *et al.*, 1998). Furthermore, hemolymph from *Heliothis virescens* contains a N-myristoylated peptide, which was found to have antitumor activity against 34 human tumour cell lines by inhibiting their cell growth (Ourth, 2011).

The major mediator of immune signalling in mammals, the NF- κ B pathway, has a paralogous system in insects, defined by two distinct control pathways, the Toll and the Imd pathways (see 3.2. Mode of action of the venom) (Lemaitre *et al.*, 1995). Dorsal, Dif and Rel are related to NF- κ B, whereas Cactus is a homologue of I κ B. The high conservation has led to the biomedical paradigm in which insect proteins are tested for potential therapeutical use in mammalian diseases caused by dysregulation of the NF- κ B pathway. More specifically, venom from several insects has been extensively studied for its biomedical functions in human auto inflammatory diseases and oncology (Heinen & da Veiga, 2011; Dkhil *et al.*, 2010; Son *et al.*, 2007). For instance melittin from honeybees induces cell death in cancer cells, using cytotoxicity as its tumoricidal strategy (Orsolic, 2012). More interestingly, instead of killing the mammalian tumour cells with toxic compounds, several parasitoids have shown to directly inhibit NF- κ B activation in the host, appointing their virulence factors as perfect candidates for NF- κ B inhibitors in the treatment of inflammatory diseases and cancer. PDVs that are symbiotically associated with parasitoid wasps, encode a family of genes with homology to I κ B proteins from insects and mammals. Two of these genes expressed by the

bracovirus from the endoparasitoid wasp *Microplitis demolitor* greatly reduced the expression of AMPs, which are under NF- κ B regulation through the Toll and Imd pathways, suggesting their involvement in the suppression of the host immune system (Thoetkiattikul *et al.*, 2005). Moreover, both bracoviruses and ichnoviruses from parasitoid wasps in the Braconidae and Ichneumonidae families respectively, express several genes belonging to the viral ankryrin (vankyrin) gene family (Kroemer & Webb, 2005; Gundersen-Rindal & Pedroni, 2006). Vankyrin proteins are homologous to I κ B proteins, but lack sequences for regulated degradation, suggesting that these proteins effectively inhibit NF- κ B signalling in parasitized insects by perturbing the NF- κ B-I κ B interaction in the host. Later, vankyrins were proven to suppress host antiviral response (Bae & Kim, 2009) and aid the parasitic wasp survival by selective inhibition of immune NF- κ B signalling (Gueguen *et al.*, 2013). These genes were primarily studied to gain a better understanding of insect immune systems and of the counterstrategies pathogens have evolved to circumvent host defences. Interestingly, it seems promising to investigate the potential of these polydnal viral genes and other parasitoid venom compounds as therapeutic agent by inhibiting NF- κ B in human diseases.

CHAPTER 2

Aims and scope

N. vitripennis can quite deserved be called the “lab rat” of the Hymenoptera. Unlike many other hymenopteran species, these parasitoid wasps are easy to work with in the laboratory. Due to short generation time (2 weeks in 25°C) and large family size (more than 500 offspring per female), *N. vitripennis* can be cultivated the whole year around. They can easily be sexed in the immobile pupal stage and the adults can be handled without anesthetization, resulting in many advantages for laboratory experiments. Next to its laboratory tractability, its haplodiploidy and the presence of closely related interfertile species makes *N. vitripennis* the perfect model for complex genetic studies. But maybe the most important reasons to study this little wasp, are the many benefits it holds towards human health. They are parasitoids of houseflies and other filth flies, that can harbour over 100 human pathogens and are vectors of many human diseases. *N. vitripennis* venom is known to be highly toxic to several mosquitoes that are vectors of diseases such as malaria, encephalitis, yellow fever and West Nile. Its venom contains a wealth of compounds with unique chemistries and modes of action, that may serve as leads for developing new classes of synthetic chemical insecticides and therapeutic compounds affecting cell physiology.

In 2010, the genome of *N. vitripennis* became publically available and more research facilities started working on this small ectoparasitoid wasp. But already two decades before that breakthrough, investigations on the venom advanced quite far. Bioassays revealed the many alterations in host physiology caused by the maternally derived venom: arrestment of development, alteration in growth and metabolism, suppression of immune responses, induction of paralysis, oncosis or apoptosis, and alteration of host behaviour (Quicke, 1997). However, only suggestions could be made with regard to specific functions of the large number of venom compounds.

This dissertation encloses two major parts. The first part studies the host-parasitoid interaction from a fundamental point of view. An in depth investigation of the alterations in host physiology caused by the venom from *N. vitripennis*, creates a better knowledge on the processes involved in host development, immunity and metabolism and how the parasitoid changes these to its own advantage. This fundamental research soon leads to the more applied approach, where more specifically the immune suppressive action of the venom was investigated. The availability of a lot more well described techniques and the possibility of

discovering a biomedical application for *N. vitripennis* venom, led us to study this inhibitory effect on immunity in mammalian cells, which is included in part 2 of this dissertation. The aims and scope of the several chapters in both parts are described below.

To fully explore the precise modes of action of this complex venom mixture regarding to its interactions with the host, we decided to tackle this quest by a large transcriptomic study. The aim of the specific experimental setup used, was to closely represent the natural parasitization. Two conditions needed to be fulfilled: sampling must be performed before *Nasonia* larvae start to feed on the host and actual venom injection and egg-laying needs to be verified without harming the flesh fly pupae. Three and 25 hours, 2 relatively short time points after parasitization, were chosen since after that larvae start to develop mouth parts and begin to feed on the host. Microarray analysis on whole bodies of flesh flies was chosen to investigate possible interactions between venom compounds and all host tissues. The results are described in chapter 3 of this dissertation.

Since parasitization involves the injection of a complex venom mixture, the important question remains what specific compound is causing the effects. Therefore, functions of venom proteins in the host organism were investigated by using two different approaches. One is the injection of the venom protein, recombinantly expressed in *E. coli*, into the host. To assess what processes are altered by the recombinant venom protein, bioassays need to be performed on the host. The second approach is to silence the venom compound in female *N. vitripennis*. After envenomation in the host, possible caused effects must be analysed by bioassays on the host organism. Both approaches were used, but due to several practical difficulties, further experiments needed to be postponed. Nonetheless, the performed preliminary investigations are described in Addendum to chapter 3.

A collaboration between L-MEB and LEGEST led to the exciting opportunity to investigate whether the immune suppressive action of *N. vitripennis* venom on its host may have comparable effects in mammalian cells. The insect immune pathways Toll and Imd are known to have high homology to the NF- κ B cascade in mammals. First assays with extracted venom from *N. vitripennis* females on murine cells were very promising. Together with the high need for NF- κ B inhibitors in biomedicine, the clear inhibition of NF- κ B activation by the

Aims and scope

venom intrigued us to dig deeper into its possible interactions with this major immune pathway in mammals. Effects of venom on other pathways, that have inhibitory effects on NF- κ B signalling, and feedback loops were included and the results of this research are explained in chapter 4. Probably the most described pathway that shows negative regulation of NF- κ B signalling is the glucocorticoid receptor (GR) signalling cascade. Since *N. vitripennis* venom was shown to increase two GR regulated target genes, involvement of venom on this pathway was further investigated. Latter results are added as an addendum to chapter 4.

Since the slightest alterations in the NF- κ B signalling cascade can have large implications relating to human pathology, we decided to further investigate the consequences of the inhibitory action of the venom on NF- κ B activation relating to its targets. Since such an extended number of NF- κ B target genes have been described, we decided to perform a PCR array that already contains a sub-set of these genes. The interesting potential of *N. vitripennis* venom as therapeutic agent in several diseases is discussed in chapter 5.

PART II

MOLECULAR TRAITS OF THE HOST- PARASITOID INTERACTION

CHAPTER 3

Early changes in the pupal transcriptome of the flesh fly *Sarcophaga crassipalpis* to parasitization by the ectoparasitic wasp, *Nasonia vitripennis*

Personal contribution: Insect cultures
 Implementation of the parasitization experiments
 Sample preparation and PCR
 Microarray validation by RT-qPCR
 Functional analysis of the dataset
 Writing the manuscript (together with Dr. Ellen Formesyn)

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Early changes in the pupal transcriptome of the flesh fly *Sarcophaga crassipalpis* to parasitization by the ectoparasitic wasp, *Nasonia vitripennis*.

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1. Abstract

We investigated changes in the pupal transcriptome of the flesh fly *Sarcophaga crassipalpis*, 3 and 25 hours after parasitization by the ectoparasitoid wasp, *Nasonia vitripennis*. These time points are prior to hatching of the wasp eggs, thus the results document host responses to venom injection, rather than feeding by the wasp larvae. Only a single gene appeared to be differentially expressed 3 hours after parasitization. However, by 25 hours, 128 genes were differentially expressed and expression patterns of a subsample of these genes were verified using RT-qPCR. Among the responsive genes were clusters of genes that altered the fly's metabolism, development, induced immune responses, elicited detoxification responses, and promoted programmed cell death. Envenomation thus clearly alters the metabolic landscape and developmental fate of the fly host prior to subsequent penetration of the pupal cuticle by the wasp larva. Overall, this study provides new insights into the specific action of ectoparasitoid venoms.



Figure 1: A female *N. vitripennis* wasp injecting venom inside a pupal host (*S. crassipalpis*). The insertion of the stinger through the puparium is visible.

2. Introduction

Endoparasitoids, wasps that deposit their eggs inside the body of their arthropod hosts, have developed sophisticated mechanisms to disable the host's defensive responses. An array of virulence factors including polydnviruses, venoms, virus-like particles, ovarian fluids and teratocytes are used to combat the host's defence system, thus enabling the wasp larvae to freely manipulate and devour the host (Glatz *et al.*, 2004; Labrosse *et al.*, 2003; Pennacchio & Strand, 2006). Ectoparasitoids, wasps that deposit their eggs on the surface of the host, appear to lack many of the virulence factors known from endoparasitoids, yet they too are capable of manipulating the host, mainly through the use of the venom that they inject (Rivers *et al.*, 1999).

Transcriptomic approaches have pinpointed host pathways that are targeted during parasitization, as demonstrated in recent studies that have probed endoparasitoid-host relationships including work completed on *Drosophila melanogaster*-*Asobara tabida* (Wertheim *et al.*, 2005; Wertheim *et al.*, 2011), *D. melanogaster*-*Leptopilina* spp. (Schlenke *et al.*, 2007), *Bemisia tabaci*-*Eretmocerus mundus* (Mahadav *et al.*, 2008), *Pieris rapae*-*Pteromalus puparum* (Fang *et al.*, 2010), *Spodoptera frugiperda*-*Hyposoter didymator*, *Ichnovirus/Microplitis demolitor* *Bracovirus* (Provost *et al.*, 2011), *Plutella xylostella*-*Diadegma semiclausum* (Etebari *et al.*, 2011), and *Pseudoplusia includes*-*M. demolitor* (Bitra *et al.*, 2011). In spite of this burst of recent work, none of the above analyses have used a transcriptomic approach to examine similar responses elicited by an ectoparasitoid. In this study we examine the response elicited by an ectoparasitoid, *N. vitripennis*, on one of its favoured hosts (Whiting, 1967), the flesh fly *S. crassipalpis*.

Although *N. vitripennis* larvae are capable of developing on late larvae or even pharate adults of their hosts, parasitoid survival is highest when parasitizing hosts that are in the pupal stage (Rivers *et al.*, 2012). Thus, *N. vitripennis* is attracted to sites where fly larvae are wandering and preparing for pupariation so that it may parasitize freshly pupated hosts. The wasp inserts its ovipositor through the puparium, envenomating the fly pupa and then depositing its egg on the surface of the pupal body. The eggs are lodged within the space between the puparium and the pupal cuticle, thus they are encased within the puparium but

remain on the external surface of the fly pupa. The wasp's venom arrests development in the host (Rivers & Denlinger, 1994a), alters host metabolism (Rivers & Denlinger, 1994b), and induces paralysis and apoptosis (Rivers *et al.*, 1999), thus ensuring a suitable environment for growth and development of the parasitoid. Although *N. vitripennis* lacks some of the virulence factors commonly documented in endoparasitoids, like teratocytes or symbiotic polydnviruses, the venom proteome shows high similarity to the venoms of endoparasitoids (Werren *et al.*, 2010; de Graaf *et al.*, 2010). This means that the early steps of host manipulation are solely the result of the venomous mixture injected by *N. vitripennis*, clearly differentiating this ectoparasitoid from endoparasitoids that employ teratocytes or symbiotic viruses.

The recent EST dataset available for *S. crassipalpis* (Hahn *et al.*, 2009) has enabled us to examine transcriptomic responses of the flesh fly to envenomation by the ectoparasitoid, *N. vitripennis*. Using microarrays we examine transcript expression in the host 3 hours and 25 hours after envenomation. The sampling points selected are both prior to hatching of the wasp larva, thus the response we document here is the response to injection of the venom rather than later responses associated with feeding by the wasp larvae. We report little response at the transcript level by 3 hours after envenomation, but by 25 hours after envenomation expression patterns of key immune, developmental, and metabolic pathways have clearly been altered by envenomation. We use these data to build hypotheses for the molecular underpinnings of the *Sarcophaga* host response to envenomation by *N. vitripennis*, and we compare current results from this endoparasitoid with molecular patterns observed in other systems involving host attack by endoparasitoids.

3. Materials and methods

3.1. Preparation of parasitized and non-parasitized flesh fly pupae

3.1.1. Insect strains

The laboratory strain *N. vitripennis* Asym C was kindly provided by Prof. Dr. L. W. Beukeboom from Evolutionary Genetics, Centre for Ecological and Evolutionary Studies in The Netherlands. This wild-type line collected in The Netherlands was cured from *Wolbachia*

infection and maintained in the laboratory since 1971 (Van den Assem & Jachmann, 1999). Wasps were reared at 25°C under a 16:8 light:dark cycle. The flesh flies (*S. crassipalpis*) were provided from a culture maintained at the University of Florida, and cultured in the laboratory as described by Denlinger (Denlinger, 1972). Larvae were fed on beef liver at 25°C and exposed to a 16:8 light:dark cycle.

3.1.2. Collection of parasitized and control hosts

Before the start of the experiment, female wasps were exposed to flesh fly puparia for 6 hours to condition them to oviposit. The next day, new *S. crassipalpis* puparia (5 days after puparium formation) were placed in a culture tube. Holes the size of a flesh fly puparium were made in the stopper of the culture tube, so only the posterior region of the puparium was accessible to the parasitoids. The experienced *N. vitripennis* females and the pupae, 5 days after pupariation, were placed together in a tube in a 3:1 ratio, to promote parasitization. After 2 hours, females of *N. vitripennis* were removed. Flies were sampled 2 or 24 hours after parasitoids were removed from the tube, snap-frozen in liquid nitrogen, and then stored at -80°C until RNA extraction. Because the *N. vitripennis* females had 2 hours to parasitize the pupae, the sampling points correspond on average to 3 and 25 hours post-parasitization. Control pupae were treated identically except that they were not exposed to the parasitoid.

3.2. Sample preparation for the microarray and validation experiment

3.2.1. RNA isolation and cDNA synthesis

RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen). From each treatment (3 hours control, 3 hours parasitized, 25 hours control, and 25 hours parasitized), 4 replicates consisting of a single fly pupa each were analysed. Due to the hard puparium, the Precellys® 24 Homogenizer (Bertin Technologies, Montigny le Bretonneux, France) was used after adding one stainless steel bead (2.3 mm mean diameter) and ¼ of a PCR tube of zirconia/silica beads (0.1 mm mean diameter) to an individual pupa. An on-column DNase I treatment with the RNase-free DNase set (Qiagen) was performed. RNA was eluted twice, first with 30 µl RNase free water, then with 20 µl RNase free water, and then stored at -80°C. Five µg of total RNA from each sample was converted to cDNA using Oligo(dT)₁₈ primers (0.5

Early changes in the pupal transcriptome of *S. crassipalpis* to parasitization by *N. vitripennis*

µg/µl) and was carried out according to the RevertAid H Minus First strand cDNA Synthesis kit protocol (Fermentas).

3.2.2. Checking parasitized pupae for the nanos gene

To ensure that the pupae were parasitized, samples were checked for the presence of *N. vitripennis nanos (nos)* (NM_001134922.1) (Olesnický & Desplan, 2007). This early embryonic protein is present in eggs of *N. vitripennis* but not in pupae of *S. crassipalpis*, thus enabling us to distinguish parasitized from non-parasitized fly pupae. The following primer set was used for reverse transcriptase PCR: 5'-TGGCAAGATTCTTTGTCCTAT-3' and 3'-AGAAACAGGTAACTGTCCGC-5'. The obtained amplicon has a length of 264 base pairs and was loaded on a 0.8% agarose gel and visualized by ethidium bromide staining.

3.3. Microarray study of *S. crassipalpis* pupae transcriptional response to parasitization by *N. vitripennis*

3.3.1. Selection of the EST dataset

An EST dataset for *S. crassipalpis* (whole bodies of different life stages including pupae as well as protein-fed and protein-starved males and females) became available in 2009. It was produced by parallel pyrosequencing on the Roche 454-FLX platform and identified approximately 11,000 independent transcripts that are a representative sample of roughly 75% of the expected transcriptome (Hahn *et al.*, 2009). A sub-set of these sequence data was made by blasting the sequences against the protein sequences of *D. melanogaster*. The sequences that showed the best homology to known genes were retained, resulting in a dataset of 10,129 EST sequences. Probes were designed and spotted on a custom 8 x 15k Agilent array developed with Agilent eArray software.

3.3.2. Microarray experimental procedures

RNA concentration and purity were determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and RNA integrity was assessed using a Bioanalyser 2100 (Agilent). RNA concentrations varied between 1.9 and 8.6 µg/µl, and the RNA Integrity Numbers and the RNA ratios indicated high quality of the RNA samples (table S1, p.66). Per sample, 100 ng of total RNA spiked with 10 viral polyA transcript controls

(Agilent) was converted to double stranded cDNA in a reverse transcription reaction. Subsequently the sample was converted to antisense cRNA, amplified and labeled with Cyanine 3-CTP (Cy3) or Cyanine 5-CTP (Cy5) in an *in vitro* transcription reaction according to the manufacturer's protocol (Agilent). A mixture of purified and labeled cRNA (Cy3 label: 300ng; Cy5 label: 300ng) was hybridized on the custom Agilent array followed by (manual) washing, according to the manufacturer's procedures. To assess the raw probe signal intensities, arrays were scanned using the Agilent DNA MicroArray Scanner with surescan High-Resolution Technology, and probe signals were quantified using Agilent's Feature Extraction software (version 10.7.3.1). The microarray data were deposited in the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GPL15391 (microarray including detailed annotation) and GSE36996.

3.3.3. Microarray data quality control and statistical analysis

Statistical data analysis was performed on the processed Cy3 and Cy5 intensities, as provided by the Feature Extraction Software version 10.7. Further analysis was performed in an R programming environment using a series of Bioconductor packages (<http://www.bioconductor.org>; Gentleman *et al.*, 2004). Differential expression between the parasitized and non-parasitized hosts, as well as time comparisons within each group was assessed via the moderated t-statistic, described in Smyth (2004) and implemented in the limma package of Bioconductor. This moderated t-statistic applies an empirical Bayesian strategy to compute the gene-wise residual standard deviations and thereby increases the power of the test, which is especially suitable for smaller data sets. To control the false discovery rate (FDR), multiple testing correction was performed (Benjamini & Hochberg, 1995) and probes were called differentially expressed in case of a corrected p-value below 0.05 and an absolute fold change larger than 1.25.

3.4. Validation experiment

3.4.1. Reference gene selection and primer design

Because no reference genes for RT-qPCR were available for *S. crassipalpis*, candidate reference genes were chosen due to their stable expression in other studies that had exposed either *D. melanogaster* or *Apis mellifera* to a bacterial challenge (Ling & Salvaterra,

2011;Scharlaken *et al.*, 2008). Primers with product size-ranges of 80 to 150 bp, were designed with Primer3Plus (Untergasser et al., 2007), using the default settings (table S5).

3.4.2. RT-qPCR reaction mixture and cycling program

RT-qPCR was executed in opaque white 96 well microtiter plates (Hard-Shell 96 Well PCR plates, Bio-Rad), sealed with Microseal 'B' seals (Bio-Rad), using the CFX96 Real-Time PCR Detection System (Bio-Rad). Each 15 µl reaction consisted of 7.5 µl 2x Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.2 µM forward and 0.2 µM reverse primers (Integrated DNA Technologies), 6.5 µl Milli-Q and 1 µl cDNA template.

Each sample was run in triplicate using a PCR program with the following conditions: 50 °C 2 minutes; 95 °C 2 minutes; and 40 cycles of a combined denaturation (95 °C 20 seconds) and annealing (60 °C 40 seconds) step. Fluorescence was measured after each cycle. At the end of the program, a melt curve was generated by measuring fluorescence after each temperature increase of 0.5 °C for 5 seconds over a range from 65 °C to 95 °C.

3.4.3. Computational selection of reference genes

Primer efficiencies, R^2 values and melt curves were calculated with CFX Manager Software (Bio-Rad). Reference gene stability was analyzed using the geNorm^{PLUS} algorithm within the qBase^{PLUS} environment (Biogazelle NV). Default settings were kept, except that target specific amplification efficiencies were used.

3.5. Microarray data analysis

S. crassipalpis genes that were differentially expressed across these treatments were functionally annotated with Blast2GO (Conesa *et al.*, 2005). After GO term annotation, an enrichment analysis (two-tailed Fisher's exact test with default settings) within the Blast2GO environment was undertaken (table 1). Clusters of Orthologous Groups (COG) functional categories were assigned with COGNITOR and stand-alone PSI-BLAST using the Eukaryotic Orthologous Groups (KOG) database (Tatusov et al., 2000). Further, genes were manually clustered by searching for groups of genes with the same GO-terms, or by putative functions of homologue genes in other species.

4. Results and Discussion

4.1. Verifying parasitization

Before conducting the microarray experiment, we verified whether individual flesh flies were parasitized by screening for transcripts of *nanos* (*nos*), a developmental patterning gene that should be abundant in early embryos of *N. vitripennis*, but not in pupae of *S. crassipalpis*. Only individual RNA samples that showed a clear band at 264 nucleotides indicating *N. vitripennis nos* expression were used for further experiments (figure 2).

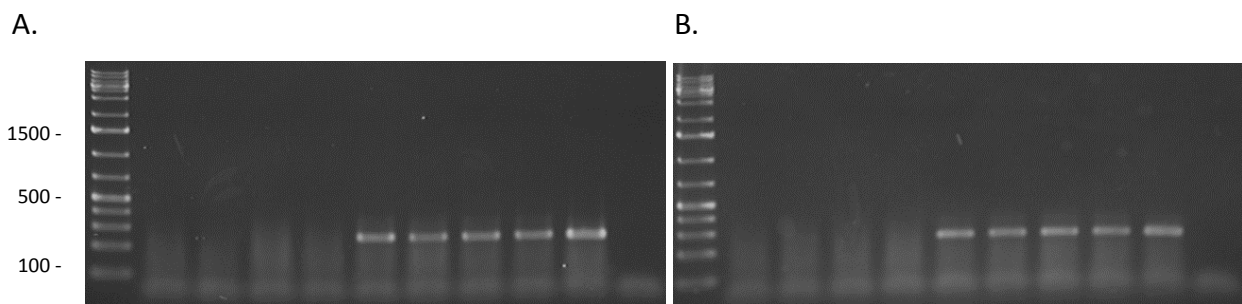


Figure 2: Gel electrophoresis analysis for the presence of *nos* in test samples for microarray and validation experiment. A. Samples at 3 hours parasitization. B. Samples at 25 hours parasitization. From left to right: 1 kb ladder (Fermentas), 4 control samples, 4 parasitized samples, positive control (abdomen from *N. vitripennis* female), negative control

4.2. Overview of differential gene expression

In current analyses, we considered a transcript to be differentially expressed between envenomated and non-parasitized control samples if the FDR-adjusted p-value was < 0.05 and the fold change was > 1.25 . When comparing all four treatments to each other (Control 3 h, Parasitized 3 h, Control 25 h, and Parasitized 25 h) hundreds of genes were differentially expressed between the 3 hours and 25 hours samples in both control and parasitized samples (figure 3). The large changes in transcript profiles observed through time do not reflect the effects of envenomation on the transcriptome, but rather reflect the dynamic nature of development as *S. crassipalpis* pupae undergo metamorphosis and transition into pharate adult development (Denlinger & Zaarek, 1994; Ragland *et al.*, 2010).

A

	Down-regulated		Up-regulated	
	p-val < 0.05 and FC > 2	p-val < 0.05 and FC > 1	p-val < 0.05 and FC > 2	p-val < 0.05 and FC > 1
Contr_3h versus Contr_25h	286	978	185	753
Paras_3h versus Paras_25h	749	1722	199	1428
Paras_3h versus Contr_3h	0	1	0	0
Paras_25h versus Contr_25h	6	19	51	109

B

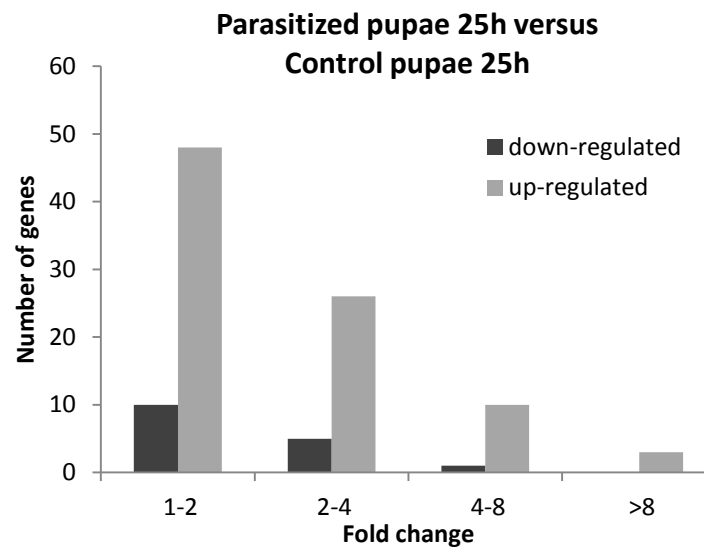


Figure 3: General statistics on the differentially regulated genes in response to parasitization. A. The 4 comparisons with their respective amounts of up- and down-regulated genes that were identified by microarray hybridization according to the following selection criteria: p-value < 0.05 and fold change ≥ 2 , or p-value < 0.05 and fold change ≥ 1 . B. Distribution of induced (grey bars) and repressed (dark bars) genes based on their fold change for the comparison of control and parasitized pupae after 25 hours.

Reinforcing the effect that envenomation has on host physiology, the numbers of differentially expressed transcripts between the 3 and 25 hours parasitized pupae were greater than the numbers of differentially expressed transcripts between the 3 and 25 hours control pupae. When comparing solely the time-matched parasitized and control samples, only 1 transcript was differentially expressed 3 hours after envenomation and 128 transcripts were differentially expressed 25 hours after envenomation (table S2). The 1 differentially regulated transcript at 3 hours after envenomation was a leucine rich repeat protein that was slightly down-regulated (-0.74 fold change). By 25 hours after

envenomation, 19 transcripts were down-regulated and 109 were up-regulated (figure 3). We found fewer transcripts differentially regulated than several other studies of parasitic wasp-host interactions (Wertheim *et al.*, 2005; Fang *et al.*, 2010; Bitra *et al.*, 2011; Etebari *et al.*, 2011; Provost *et al.*, 2011; Wertheim *et al.*, 2011). Unlike the previous studies that focused on endoparasitoids, *N. vitripennis* is an ectoparasitoid and we sampled fairly early during the interaction, before the developing parasitoid larvae began to interact with the host. Thus, the results represent only the earliest stages of the host response to envenomation.

4.3. Validation by RT-qPCR

Twenty ESTs were selected for validation with RT-qPCR, including the single transcript found in the 3 hours group (*Irr-pr*) (table S4). Ten candidate reference genes were tested for their stable expression levels across all conditions (3 and 25 hours, control and parasitized), resulting in two selected reference genes (table S5, figure S1). EIF or eukaryotic translation initiation factor 1 has 97.15% homology to eIF-1A that has been used as a reference target for expression profiles of *D. melanogaster*. Ubq or ubiquitin-conjugating enzyme displays 92.8% homology to Ubi from *D. melanogaster*, which has a function in protein degradation. In all except two cases, RT-qPCR revealed the same log ratio trends as found in the microarray study. Nine of them gave significant differences in expression pattern including 5 that were up-regulated and 4 that were down-regulated (figure 4).

4.4. Gene-Ontology analysis of microarray data

Using Blast2GO, we assigned Gene Ontology (GO) terms to the *S. crassipalpis* EST sequences that were used for the microarray analysis, successfully annotating 8,618 out of the 10,129 ESTs (85%). We then performed GO term enrichment analyses on 4 sets of data Parasitized 3 h vs Parasitized 25 h, Control 3 h vs Control 25 h, Control 3 h vs Parasitized 3 h and Control 25 h vs Parasitized 25 h. GO term enrichment analyses of the two sets that compared 3 hours versus 25 hours samples showed enrichment of GO terms associated with energy metabolism (table 1).

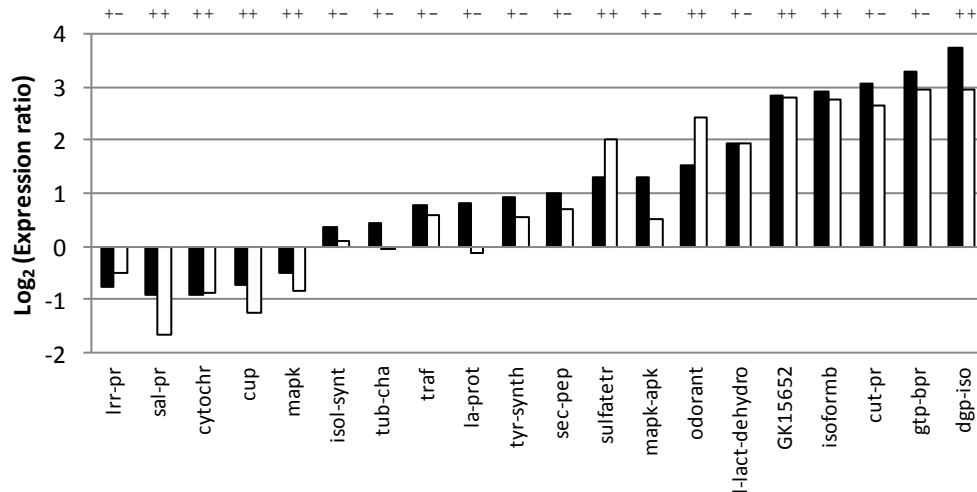


Figure 4: Validation of microarray data with RT-qPCR. Log₂-transformed expression ratio of Paras_3h compared to Contr_3h and of Paras_25h compared to Contr_25h. White bars: RT-qPCR experiment. Black bars: microarray experiment. T-tests were used to statistically compare parasitized versus unparasitized samples. +: significant differential expression ($p < 0.05$). -: no significant expression ($p \geq 0.05$).

As with the time comparisons in the transcript-by-transcript level analyses above, the enrichment in energy metabolism categories likely reflects the metabolic demands that have been documented to occur at the pupal-pharate adult metamorphic molt (Denlinger & Zaarek, 1994; Ragland *et al.*, 2010).

To further explore the effects of envenomation at levels above single ESTs we performed a second set of analyses where we tested for enrichment across categories in the COG (Clusters of Orthologous Groups from eukaryotic genomes) functional classification using Blast2GO (figure 5). We observed enrichment that suggested differential regulation in categories associated with growth (including replication, transcription, and translation), cell signalling, intermediary metabolism, and defensive mechanisms. In a complementary analysis, we also manually clustered 103 differentially expressed EST's in control versus envenomated pupae at 25 hours into 8 GO clusters (25 ESTs out of 128 could not be annotated by Blast2GO) representing genetic information processing, metabolism, development, programmed cell death, detoxification, immune system, sensory system and transporters (table S3 and figure 6).

Table 1: Selected biological process GO-terms which were enriched among the EST's that were differentially expressed in control and parasitized pupae, 3 hours and 25 hours after parasitization.

GO-nr	GO biological process term	Control 3 hours versus 25 hours				Parasitized 3 hours versus 25 hours			
		FDR ^a	p-value	NS ^b	FE ^c	FDR ^a	p-value	NS ^b	FE ^c
GO:0006119	oxidative phosphorylation	4.32E-05	4.78E-08	14	7.53	5.09E-07	5.54E-10	20	6.87
GO:0022900	electron transport chain	6.53E-05	8.83E-08	15	6.53	4.79E-05	1.06E-07	19	6.70
GO:0042773	ATP synthesis coupled electron transport	8.21E-05	1.21E-07	13	7.64	3.46E-06	5.37E-09	18	5.04
GO:0022904	respiratory electron transport chain	8.71E-05	1.39E-07	14	6.83	4.41E-05	9.20E-08	18	6.71
GO:0045333	cellular respiration	1.29E-04	2.21E-07	16	5.61	3.46E-06	5.53E-09	23	4.31
GO:0006091	generation of precursor metabolites and energy	2.24E-04	4.42E-07	19	4.48	5.78E-05	1.38E-07	26	5.39
GO:0042775	mitochondrial ATP synthesis coupled electron transport	2.24E-04	4.68E-07	12	7.43	7.41E-06	1.37E-08	17	5.04
GO:0015980	energy derivation by oxidation of organic compounds	4.40E-04	1.30E-06	16	4.84	3.47E-05	6.83E-08	23	3.73

^aFalse Discovery Rate

^bNumber of genes associated with GO-term which were differentially expressed

^cFold Enrichment

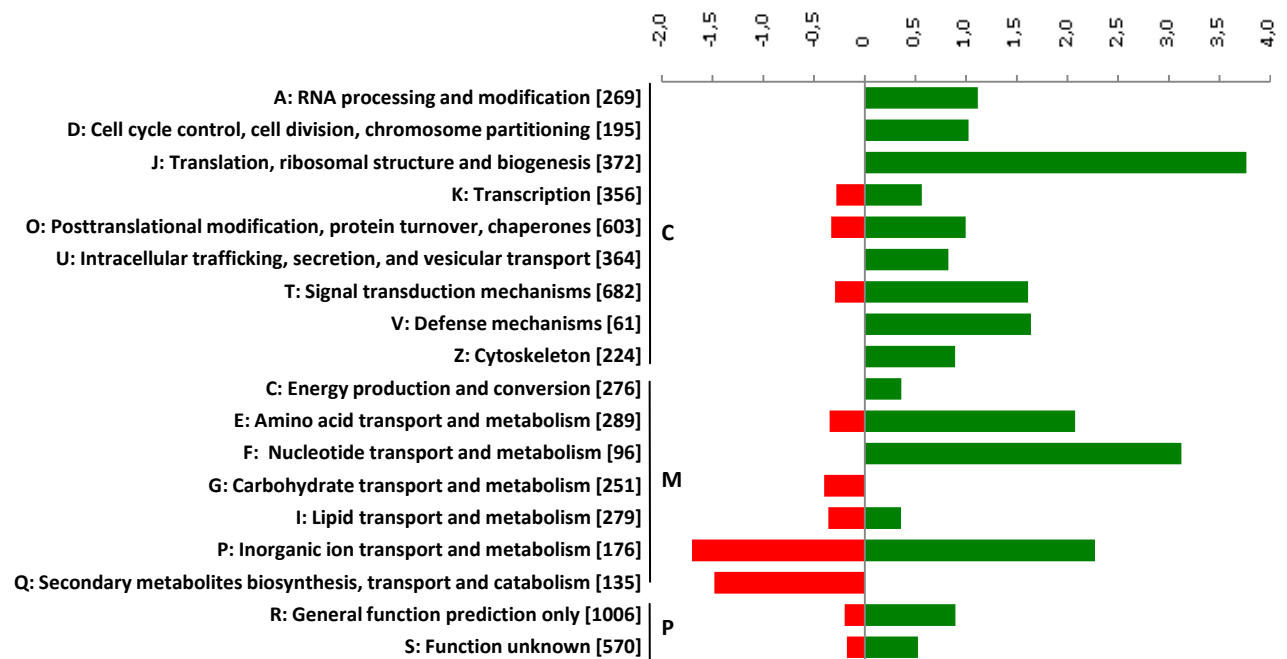


Figure 5: Square brackets: Total number of EST's within the *S. crassipalpis* EST database. On the left, KOG functional category labels are listed. C: cellular processes and signalling. M: metabolism. P: poorly characterized. Red bars: down-regulation. Green bars: up-regulation.

One challenge for transcriptomic studies like the current study that use either whole bodies or complex tissues (e.g. hemocytes, brain, or fat body) is that patterns of gene expression represent the sum of transcriptional profiles across many specialized cell types. Thus, a transcriptomics study showing some components of energy metabolism by glycolysis that are up-regulated and some pathway members that are down-regulated may reflect the fact that glycolysis is up-regulated in some cells and down-regulated in others, rather than indicating that some components of the pathway are up-regulated and others down-regulated within a single cell type. Furthermore, a single gene that may affect multiple downstream pathways and functional categories (i.e., GO or COG) can also reflect a diversity of biochemical cellular events that indicate multiple different physiological effects on organismal phenotypes. The responses of *Sarcophaga* fly hosts to envenomation by *N. vitripennis* have been studied from organismal and physiological perspectives for two decades (Rivers & Denlinger, 1994a; Rivers & Denlinger, 1994b; Rivers & Denlinger, 1995; Rivers *et al.*, 2002b; Rivers *et al.*, 2010) revealing that even without the developmental influence of the parasitoid larva, envenomation alters fly hosts in three major ways: by suppressing host immunity, arresting host development, and altering host metabolism to

favour parasitoid development. Below, we interpret current transcriptomic results in the context of these three host manipulations. Many patterns of transcript abundance are products of host manipulation by the parasitoid (e.g., changes in many downstream players in genetic information processing), but here we consciously focus the discussion on regulatory processes that we believe may be important in modulating host immunity, development, and metabolism to benefit parasitoid development. We acknowledge that other, more-subtle interactions between parasitoid venom and host physiology, beyond those observed in current data or discussed here, are undoubtedly occurring. For example, some of the transcriptional responses we observe may be the product of host responses attempting to deter parasitoid success. However, we cannot currently disentangle host-defensive responses from parasitoid manipulation of hosts. Future work comparing responses to envenomation across populations of strains of hosts that vary in their susceptibility to parasitism by *N. vitripennis* is needed. Because successful envenomation by *N. vitripennis* always leads to developmental arrest and eventual host death in the strains used here, we focus the discussion from the perspective of host molecular responses to parasite manipulation. We hope that current data, the first to our knowledge on genome wide transcriptomic responses to ectoparasitoid envenomation, will motivate additional hypothesis building that leads to careful biochemical and cellular studies of venom modes of action in the *N. vitripennis-Sarcophaga* interaction and other host-parasitoid systems.

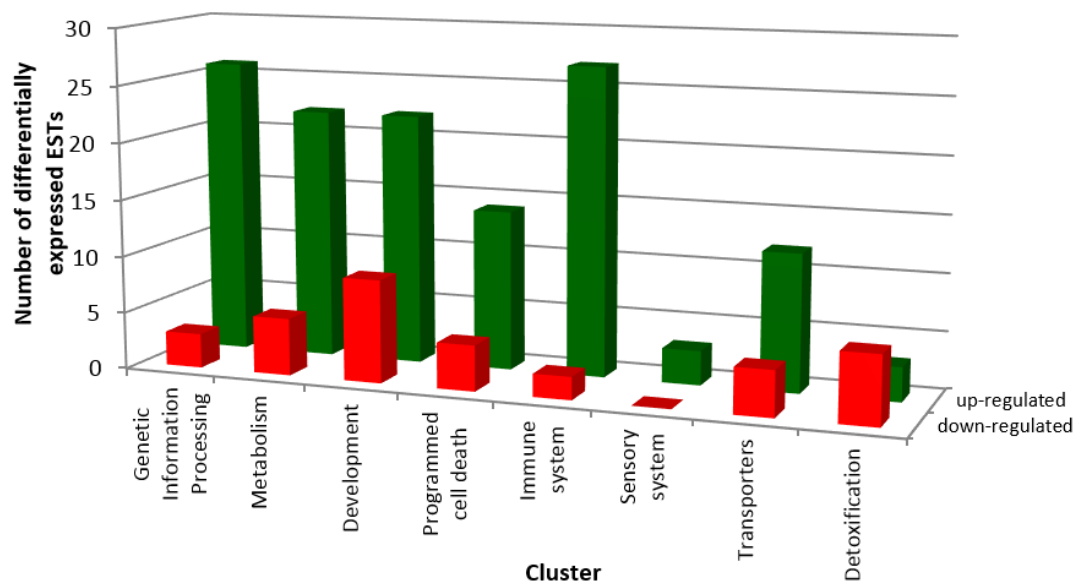


Figure 6: Differentially expressed EST's for comparisons Contr_25h vs Paras_25h, manually clustered into 8 different classes. Schematically presented.

4.5. Immunity

From the perspective of host exploitation, venoms should inhibit components of the host immune response that target parasitoid larvae while either maintaining or even enhancing components of the host immune system that target other invaders, like bacteria or fungi, that may promote degrading host quality, compete for parasitoid larvae for host resources, or infect the parasitoids themselves (Asgari & Rivers, 2011). Hosts will react to the invasion of foreign agents by producing antimicrobial peptides and reactive oxygen species by contact epithelia, fat body and hemocytes and more directly by phagocytosis, encapsulation and nodule formation in which specialized hemocytes interplay (Danneels *et al.*, 2010). Studies of hemocyte dynamics showed that envenomation of *Sarcophaga bullata* pupae by *N. vitripennis* resulted in the rapid death of plasmatocytes, inhibited proliferation and differentiation of pro-hemocytes into plasmatocytes, and diminished granulocyte spreading (Rivers & Denlinger, 1995; Rivers *et al.*, 2002b). The transcripts that are described in the following sub-sections on immunity, are marked with green asterisk (*) in tables S2 and S3.

4.5.1. Programmed cell death in hemocytes and other tissues

In vivo studies in *Sarcophaga* hosts and *in vitro* work on lepidopteran cells suggests that venom-induced hemocyte cell death occurs by apoptosis signaled by phospholipase A₂ (PLA₂) that alters cellular Ca²⁺ signalling and Na⁺ homeostasis leading to characteristic changes in cell shape, including blebbing caused by membrane separation from the cytoskeleton, cellular swelling, DNA fragmentation, phosphatidylserine externalization and activation of caspase activity (Rivers & Denlinger, 1994a, 1994b; Rivers *et al.*, 2002a, 2010; Formesyn *et al.*, 2013b). We show differential regulation of apoptotic transcripts, including up-regulation (2.15-fold) of a secretory PLA₂ transcript that may activate caspase activity. Consistent with the previous observations that PLA₂ activity is associated with changes in cellular Na⁺ and Ca²⁺ homeostasis that may induce apoptosis, we found a putative small mitochondrial calcium-binding protein transcriptionally up-regulated 25 hours after envenomation (1.83-fold), as well as a putative calcyphosine that was also up-regulated (1.44-fold). Up-regulation of a putative cytochrome oxidase III subunit (3.81-fold) may also be related to mitochondrial dysfunction induced by PLA₂ signalling. Overexpression of cytochrome III may help to induce

apoptosis by affecting the redox state of cytochrome C, a known effector of caspase activity and apoptosis (Brown & Borutaite, 2008; Wu *et al.*, 2009).

Three transcripts annotated to the Rho family of GTP-binding proteins and a guanine nucleotide exchange factor were highly up-regulated 25 hours after envenomation (13.2, 9.7, 1.38, and 1.3-fold enrichment respectively, table S2). Rho GTPases belong to the Ras superfamily and play diverse roles in intracellular signalling (Rossman *et al.*, 2005; Cox & Der, 2003). We propose that Rho GTP-binding proteins and the observed guanine nucleotide exchange factor modulate Rho signalling and promote apoptosis in hemocytes and other tissues by activating the RASSF1/Nore1/Mst1 signalling pathway that leads eventually to caspase activation. Rho signalling pathway members were also found to be differentially regulated in a study of the evolution of resistance of *D. melanogaster* hosts to the parasitoid wasp *A. tabida* (Wertheim *et al.*, 2011). Thus, further interrogation of the potential roles of Rho signalling is needed to understand how *N. vitripennis* venom affects *Sarcophaga* hosts from the perspectives of both immune modulation by hemocyte cell death and selective programmed cell death in other tissues (see 4.6.1. Developmental signalling pathways).

Rho GTPases are also known for their involvement in cytoskeletal actin reorganization (Coleman & Olson, 2002; Fiorentini *et al.*, 2003). Blebbing, where the cell membrane separates from the cytoskeleton, is one of the phenotypic hallmarks of cell death when cultured lepidopteran cells are exposed to *N. vitripennis* venom *in vitro* (Rivers *et al.*, 2002a, 2002b, 2010), and Rho signalling has been shown to promote blebbing by actin cytoskeleton reorganization in cultured mammalian cells (Aznar & Lacal, 2001). Consistent with this view, several cytoskeleton-related transcripts were up-regulated by envenomation in current study (table S2: kinesin-like protein kif1a, actin-related protein 5, map kinase-activated protein kinase, annexin b11 isoform a). Cytoskeletal disorganization could also reduce the ability of hemocytes to produce pseudopodial projections critical for endocytosis or spreading responses, and venom of several endoparasitoids has been shown to reduce the efficacy of spreading by disrupting hemocyte cytoskeletal structure (Gillespie *et al.*, 1997; Strand, 2008; Asgari & Rivers, 2011; Richards *et al.*, 2013). Differential regulation of cytoskeletal transcripts has been observed in other transcriptional studies of parasitism

(Wertheim *et al.*, 2005, 2011), suggesting conservation of this mode of action between endoparasitoid and ectoparasitoid venoms.

4.5.2. Cellular and humoral immune responses

Although *N. vitripennis* venom dramatically suppresses *Sarcophaga* immunity by rapidly killing hemocytes, hosts still do mount an immune response characterized by both cellular and humoral mechanisms (Rivers *et al.*, 2002b). The p38K and JNK cascades of the multifunctional mitogen-activated protein kinase (MAPK) pathway are promising candidates for regulating both the cellular immune response to envenomation and hemocyte apoptosis (Concannon *et al.*, 2003;Rane *et al.*, 2003). MAPKs are cytosolic proteins that translocate into the nucleus to regulate transcription when activated. The p38K and JNK signalling cascades are associated with cellular stress responses including apoptosis, immunity, and cell cycle arrest. After envenomation, a putative MAPK 3 or MAPK 4 transcript possibly belonging to either the p38 or JNK cascade was down-regulated (-1.46-fold) and transcript abundance of a putative MAPK 3 or MAPK 4-phosphatase that suppresses MAPK activity was up-regulated (1.86-fold). We currently cannot distinguish whether this transcript functions as MAPK 3 by activating the p38K cascade or acting as MAPK 4 by activating the JNK cascade because we did not detect other transcripts clearly assigned to either cascade. However, both signalling cascades participate in immune system function, and down-regulating this arm of MAPK signalling may be an important part of the parasitoid's strategy for host immune suppression. MAPKs are activated post-translationally by phosphorylation, thus testing whether any of the arms of MAPK signalling are important in immune modulation at envenomation will involve further biochemical work at the level of immune cells and other tissues.

Parasitoid detection and immune system activation may also be modulated by the NF- κ B pathway (Strand, 2008). A transcript for a putative tumor necrosis factor (TNF) receptor associated factor (TRAF) known to regulate NF- κ B activity in *Drosophila* (Zapata *et al.*, 2000) was up-regulated 25 hours after envenomation (1.7-fold). A protein tyrosine phosphatase involved in intracellular immune signalling pathways that had previously been implicated in the immune response of *D. melanogaster* to the wasp *A. tabida* (Wertheim *et al.*, 2005) was also up-regulated (2.17-fold).

The immune response in all organisms, including insects, has been associated with microRNA (miRNA) activity (O'Connell *et al.*, 2010;Asgari, 2013). Two different transcripts that annotated to the host argonaute-2 protein, a critical mediator of the RNAi response, were up-regulated 25 hours after envenomation (1.36 and 1.35-fold respectively). In addition to their known roles in antimicrobial immunity in insects (Fullaondo & Lee, 2012;Asgari, 2013), microRNAs have also recently been implicated in the response of a lepidopteran host, *P. xylostella*, to the parasitoid wasp *D. semiclastrum* (Etebari *et al.*, 2013), motivating further study of microRNAs in regulating envenomation responses. We also observed up-regulation of transcripts for two odorant-binding proteins (OBPs) (6.56 and 7.38-fold). Beyond their activities in chemoreception, OBPs have been implicated in diverse cellular responses including pathogen recognition and neutralization of invading microorganisms (Levy *et al.*, 2004). Of further interest, two distinct OBPs were also discovered in *N. vitripennis* venom (de Graaf *et al.*, 2010), but whether and how endogenous host OBPs and venom-derived parasitoid OBPs may interact is unknown and merits further study.

Envenomation by *N. vitripennis* has long been associated with decreased melanisation of *Sarcophaga* host hemolymph (Rivers & Denlinger, 1994a;Rivers *et al.*, 2002). The deposition of melanin around the intruding object forms a physical shield and prevents or retards the growth of the intruder. Given such a crucial role for melanisation in host immunity, alterations of this process in hosts likely represents a strategy for host manipulation. Analysis of *N. vitripennis* venom has revealed both serine protease inhibitors (serpins) and cysteine-rich protease inhibitors that may impede the activity of pro-phenoloxidase and the melanisation response (de Graaf *et al.*, 2010). We found substantial up-regulation of transcripts for two serine proteases (2.12 and 3.11-fold) that could compete with venom-derived protease inhibitors in an effort to disrupt the parasitoid's immune-suppressive strategy, similar to the enhanced expression of melanisation cascade transcripts observed when *D. melanogaster* was parasitized by the virulent parasitoid wasp *Leptopilina boulardi* (Schlenke *et al.*, 2007).

A targeted oxidative burst that is facilitated by both cellular and humoral elements of immunity is associated with melanisation (Strand, 2008). For hosts, this targeted burst of free radicals and pro-oxidants must be delivered precisely to stress the invader while

protecting host tissues. Concomitantly, parasitoids must reduce effects of oxidative damage on their own bodies and critical host tissues such as the fat body. Consistent with this dynamic interplay of host and parasitoid control of free radical damage, we found enrichment of detoxification proteins that could control redox events and may play roles in immune oxidative burst reactions, including down-regulation of a putative metallothionein family 5 protein (-2.01-fold), cytochrome p450-28a1 (-1.91-fold), copper uptake protein (-1.65-fold), and up-regulation of transcripts for putative myoinositol oxygenase (1.83-fold) and ribonucleoside-diphosphate reductase (1.46-fold). These redox proteins may be important in detoxification responses that facilitate immunity. Other detoxification transcripts were also differentially regulated, including down-regulation of the glutathione metabolism enzyme alanyl aminopeptidase (-2.93-fold), and an apparent ABC transporter was up-regulated (2.75-fold).

We detected no change in abundance of antimicrobial peptide transcripts, an important component of humoral immunity. Other studies on host-parasitoid responses also showed minimal up-regulation of antimicrobial peptides in responses to parasitoids relative to exposure to microorganisms (Ross & Dunn, 1989; Nicolas *et al.*, 1996; Masova *et al.*, 2010; Wertheim *et al.*, 2005).

4.6. Development

Delaying or arresting host development is a common life history tactic for parasitoids. In endoparasitoids, suppressive actions are begun by venom components, and sometimes symbiotic viruses, then reinforced by developing parasitoids (Strand, 2008; Asgari & Rivers, 2011). As ectoparasitoid without obvious viral symbionts, *N. vitripennis* uses venom to arrest host development until external parasitoid eggs can hatch and larvae can affect host physiology directly. Even without the influence of a developing wasp larva, *Sarcophaga* pupae envenomated by *N. vitripennis* enter an irreversible state of developmental arrest that can last more than a month before the host dies (Rivers & Denlinger, 1994a). Envenomation-induced developmental arrest in *Sarcophaga* pupae superficially resembles the developmental arrest induced at pupal diapause in this species. However, within 24 hours of envenomation the brain of a *Sarcophaga* host pupa undergoes substantial

programmed cell death and thus will never be able to develop into a functional adult (Rivers *et al.*, 2011).

Degeneration of the brain contributes to envenomation-induced developmental arrest because the release of ecdysteroids from the prothoracic glands instructed by the brain corpora cardiaca is needed to coordinate pupal-adult metamorphosis. However, unlike diapausing pupae that will resume development with exogenous ecdysteroid exposure, exogenous ecdysteroids cannot restart development in envenomated pupae (Rivers & Denlinger, 1994a). This suggests that the developmental arrest in envenomated *Sarcophaga* pupae is regulated differently than pupal diapause. Envenomation-induced developmental arrest must somehow disrupt ecdysteroid signalling and may also include coordinated cell death in other critical tissues, like partially differentiated imaginal wing or antennal discs. In contrast to the programmed cell death that occurs after envenomation in hemocytes and brain cells, cells of the host fat body remain healthy (Rivers *et al.*, 2011) and continue participating in intermediary metabolism of the pupae, including the accumulation of greater fat reserves (Rivers & Denlinger, 1995). This clear contrast in the cellular viability responses amongst the three *Sarcophaga* tissues that have been studied is consistent with venom manipulating the host environment to favour parasitoid larvae. Further examination may reveal that other host tissues benefiting larval development, like the heart and respiratory system, are also selectively maintained. An important question is, what cellular and biochemical factors promote survival and viability in some *Sarcophaga* pupal tissues, like the fat body which survives for weeks despite exposure to *N. vitripennis* venom, when other tissues undergo programmed cell death within hours of envenomation? The transcripts that are described in the following sub-sections on development, are marked with red asterisk (*) in tables S2 and S3.

4.6.1. Developmental signalling pathways

One third of the differentially expressed genes at 25 hours post-envenomation have putative roles in developmental processes, with 70% of these transcripts up-regulated and 30% down-regulated (tables S2 and S3). Many genes classified as promoting development by cellular proliferation and growth also have functions in apoptosis (discussed above). A possible regulator of developmental arrest is down-regulation (-2.87-fold) of a putative

alkyldihydroxy-acetonephosphate synthase (ADHAPS) required for normal development in humans and *C. elegans* (Motley *et al.*, 2000). Understanding the molecular basis of selective maintenance of some tissues during developmental arrest while others are destroyed will require picking apart the activity of candidate genes and signalling pathways in individual tissues.

As mentioned above (see 4.5.2. Cellular and humoral immune responses), 25 hours after envenomation we observed up-regulation in transcripts of a putative MAPK 3 or MAPK 4-phosphatase (1.86-fold) that could inhibit MAPK signalling through the p38K or JNK cascades, and down-regulation of transcripts for a putative MAPK 3 or MAPK 4 protein that would activate p38K or JNK signalling (-1.46-fold) and thus modulate development and immunity. Because envenomation initiates a developmental arrest in pupae preventing pharate adult metamorphosis, we expected that ERK-signalling, which promotes growth and morphogenesis, would be down-regulated after envenomation. Interestingly, Torso, a peptide-hormone receptor that activates ERK signalling was up-regulated 25 hours after envenomation (2.42-fold). Torso is the prothoracicotropic hormone (PTTH) receptor that signals the prothoracic glands to produce ecdysone to precipitate molting and morphogenesis via ERK signalling (Rewitz *et al.*, 2009). It may seem counterintuitive for transcripts of the PTTH receptor to be in greater abundance in developmentally arrested pupae compared to control animals already undergoing pupal-adult metamorphosis after 25 hours. However, by the time of metamorphosis developing flies have already completed PTTH signalling and released ecdysteroids so PTTH reception may not be necessary. In contrast, envenomated, developmentally arrested pupae may still express the PTTH receptor even though exogenous ecdysteroids cannot trigger the resumption of development (Rivers & Denlinger, 1994a), indicating that these pupae are stuck perpetually in molecular stasis. A putative Torso/PTTH-receptor transcript was also up-regulated in larvae of *P. xylostella* that failed to successfully pupate when parasitized by *D. semiclausm* (Etebari *et al.*, 2011), again suggesting developmental arrest may occur up-stream of ecdysone reception. A putative cytochrome p450-28a1 that is down-regulated (-1.91-fold) could also be involved in developmental arrest. This p450 is similar to *C. elegans* daf-9, an enzyme that regulates the dauer developmental arrest by producing the steroid hormone dafchronic acid that acts through a nuclear steroid hormone receptor, daf-12 (Gerisch & Antebi, 2004). Further

detailed investigations of both steroid hormone production and signalling are needed to tease apart potential regulatory roles in developmental arrest in host-parasitoid interactions. We expect that investigation of sensitivity to the action of PTTH and ecdysteroids through the ERK signalling cascade relative to p38K and JNK signalling holds promise for understanding the regulation of envenomation-induced arrest of host development.

Rho signalling, mentioned above in the context of immunity (see 4.5.1. Programmed cell death in hemocytes and other tissues), may also play a critical role in envenomation-induced developmental arrest as suggested by high levels of up-regulation in three Rho-family GTP-binding proteins and a guanine nucleotide exchange factor (13.2, 9.7, 1.38 and 1.3-fold enrichment respectively, table S2). Rho signalling affects cytoskeletal structure in embryonic and pupal morphogenesis in *Drosophila* (Chen *et al.*, 2004). Rho signalling is GTP-mediated and mutations or transgenes that enhance Rho signalling increase cellular proliferation and yield tissue overgrowth (Clark *et al.*, 2000). Thus, up-regulation of Rho family GTP-binding proteins and a guanine nuclear exchange factor that regulates cyclic GMP levels may help induce developmental arrest by sequestering and decreasing GTP to inhibit the Rho signalling that would normally lead to pupal-adult metamorphosis (Chang *et al.*, 1998). Rho signalling can also interact with signalling in another pathway affecting cellular proliferation and morphogenesis, the SH2 domain ankyrin repeat kinase (Src) pathway (Chan *et al.*, 1994; Pedraza *et al.*, 2004). Transcripts for a putative inhibitor protein of Src signalling, a Prl protein-tyrosine phosphatase (Pagarigan *et al.*, 2013), are up-regulated 25 hours after envenomation (2.17-fold), suggesting inhibition of Src signalling may contribute to the envenomation-induced developmental arrest.

4.6.2. Other regulators of development

The only transcript to be detectably differentially expressed 3 hours after envenomation was a down-regulated leucine rich-repeat protein (-1.67-fold). Putative homologues of this transcript are highly expressed during the pupal-adult transition in *D. melanogaster* where they regulate programmed cell death of larval-pupal structures as the animal undergoes adult morphogenesis (Berry & Baehrecke, 2007). Down-regulation of this protein may contribute to an early developmental halt upon envenomation, preventing the pupal host

from further morphogenesis that may make it less suitable for parasitoid larvae (Rivers & Denlinger, 1995). Besides their possible involvement in immunity (see 4.5.2. Cellular and humoral immune responses), miRNAs can be important for regulating development in insects because they can modulate major transcriptional programs and RNA processing (Asgari, 2013). Up-regulation of transcripts for two putative argonaute-2 proteins (1.35 and 1.16-fold respectively) in current data combined with similar observations in the interaction between the lepidopteran host *P. xylostella* and the wasp *D. semiclasum* (Etebari *et al.*, 2013) suggests that miRNAs may play important roles in regulating host developmental arrest.

4.7. Metabolism

Many parasitoids manipulate host intermediary metabolism to improve the nutritional milieu for larval development through venom and symbiotic viruses, followed by the developing parasitoid and teratocytes (Dahlman *et al.*, 2003; Nakamatsu & Tanaka, 2004, 2003; Nurullahoglu *et al.*, 2004; Formesyn *et al.*, 2011). Over the 25 hours timeframe of current study the egg is external to the host body and would not have yet hatched (Whiting, 1967). Thus the effects we observe are due to envenomation. Envenomation causes a precipitous drop in the metabolic rates of hosts (decreased O₂ consumption) (Rivers & Denlinger, 1994b, 1995). Although decreased host metabolism upon envenomation may be partly due to arresting host development, *N. vitripennis* venom clearly manipulates host intermediary metabolism because just after envenomation hosts increase levels of alanine and pyruvate, but decrease oxaloacetate (Rivers & Denlinger, 1994b). Perhaps most striking from a metabolic perspective is that envenomation by *N. vitripennis* causes flesh fly pupae to increase the lipid content of their fat bodies while decreasing circulating blood lipids (Rivers & Denlinger, 1995, 1994b). We construct hypotheses for regulation of increased lipid storage and metabolic depression based on transcript abundance. Because current data are from whole bodies, we interpret these data in the context of intermediary metabolism being increased in some tissues and decreased in others. The transcripts that are described in the following sub-sections on metabolism, are marked with blue asterisk (*) in tables S2 and S3.

4.7.1. Reorganizing metabolism to increase fat body lipids

Because many insect parasitoids, including *N. vitripennis*, show limited or no capacity to synthesize lipids themselves, they must rely on host lipids for fatty acids necessary for juvenile growth and adult reproduction (Visser *et al.*, 2010, 2012). Previous work on several systems has shown parasitoid manipulation of host lipid metabolism from physiological (Rivers & Denlinger, 1995, 1994b; Dahlman *et al.*, 2003; Nakamatsu & Tanaka, 2004, 2003; Nurullahoglu *et al.*, 2004), proteomic (Song *et al.*, 2008), and transcriptomic perspectives (Fang *et al.*, 2010; Etebari *et al.*, 2011; Provost *et al.*, 2011).

Considering that *Sarcophaga* host pupae are a nutritionally-sealed system that cannot feed, *N. vitripennis* venom must trigger a tissue-specific starvation response so nutrients are mobilized from peripheral tissues destined to degenerate, like the brain and thoracic muscles, while maintaining metabolic and synthetic function in the fat body. Autophagy is a controlled process wherein cells selectively degrade sub-cellular components, recruiting components (e.g., mitochondria) to intracellular lysosomes to break them down into trafficable units like amino acids that can be reused elsewhere (Cooper & Mitchell-Foster, 2011; Kroemer & Levine, 2008). Autophagy is a critical component of both starvation responses and responses to mild cellular damage. Mild starvation or cellular damage that lead to autophagy will induce cell cycle arrest by p53-dependent pathways to stop growth and will trigger lysosomal clearance of some sub-cellular structures, but cells can typically rebound function and resume growth when nutrients become available again or the stress abates (Lee, 2012). Prolonged starvation or chronic stress will induce a shift from the milder autophagy response to trigger apoptotic pathways. Both autophagy and apoptosis allow organisms to control the breakdown of cellular components into trafficable units that could be recycled and used to produce fat body lipid stores. Because autophagy has been associated with both starvation/nutrient recycling and cell cycle arrest, regulation of autophagy pathways provides an opportunity for parasitoids to manipulate both host development and intermediary metabolism to favor offspring production. We observe enrichment in several GO categories that are consistent with the expectation that envenomation promotes autophagy and nutrient mobilization in peripheral host tissues to support lipid synthesis in the fat body, including: cell cycle control, intracellular trafficking/vesicular transport, energy production and conversion, amino acid transport and

metabolism, nucleotide transport and metabolism, and lipid transport and metabolism (figure 5).

When assessing the gene-by-gene analysis (table S3) several autophagy-related transcripts were differentially expressed. Damage-related autophagy modulator (DRAM), shown to control autophagy in *D. melanogaster* (O'Prey *et al.*, 2009), was up-regulated 25 hours after envenomation (1.47-fold). Mitochondria play an important role in promoting or inhibiting autophagy (Cooper & Mitchell-Foster, 2011; Kroemer & Levine, 2008). Specifically, damaged or energy-stressed alterations in the mitochondrial trans-membrane potential regulated by mitochondrial Ca^{2+} signalling can trigger recruitment of mitochondria to autolysosomes (e.g., mitophagy). Two possible regulators of mitochondrial Ca^{2+} homeostasis associated with autophagy were up-regulated 25 hours after envenomation (Cardenas & Foskett, 2012; Lin *et al.*, 2012), a putative calcyphosine (1.44-fold) and a putative small mitochondrial calcium-binding protein (1.83-fold). A putative DGP-1, an elongation factor that regulates taking damaged cells out of the cell cycle and inducing autophagic repair (Gruenewald *et al.*, 2009; Blanco *et al.*, 2010) was highly up-regulated after envenomation (13.2-fold). A putative lysosomal aspartic protease was also up-regulated (1.74-fold) 25 hours after envenomation. This transcript has similarities to cathepsin D, a lysosomal protease activated in cells undergoing autophagy, apoptosis, and even necrotic cell death (Benes *et al.*, 2008; Guicciardi *et al.*, 2004). Cathepsins specifically, and other pro-autophagic genes more generally have been implicated in host responses to parasitism in other transcriptomic (Etebari *et al.*, 2011; Fang *et al.*, 2010) and proteomic studies (Song *et al.*, 2008). Although we have couched most of the autophagy-related responses as important to nutritional manipulation of host tissues, there is substantial overlap between autophagic and apoptotic genes such that it is difficult to determine from simple snapshots of transcripts or proteins what specific pathways are being triggered across studies. Clearly pro-autophagy pathways could be playing roles in the apoptotic response of host tissues after envenomation in the *Sarcophaga-Nasonia* interaction (see 4.6. Development) (Rivers *et al.*, 2002b) and other parasitoid-host manipulation systems (Song *et al.*, 2008; Fang *et al.*, 2010; Etebari *et al.*, 2011). We expect apoptotic pathways will be rapidly initiated for parasitoid suppression of host immunity, then autophagic responses will be important for longer-term manipulation of host development and nutritional state. Careful investigation of time-course patterns of

autophagy and apoptotic responses are needed across multiple tissue types to test these hypotheses about the relative contributions of autophagy and apoptosis to the three major axes of host manipulation: immunity, development, and nutrition.

Amino acids are a major product of cellular autophagy responses, and we expect that these amino acids liberated from the peripheral host tissues by envenomation are being deaminated to provide carbon skeletons for metabolic fuel as substrates for anabolic lipid synthesis. Gene Ontology categories including energy production and conversion, amino acid transport and metabolism, nucleotide transport and metabolism, and lipid transport and metabolism were all differentially expressed in hosts 25 hours after envenomation (figure 5). When considering lipid synthesis from amino acids produced by autophagy, some transporters were up-regulated including a cationic amino acid transporter (2.13-fold) and an oligopeptide transporter (YIN) (2.87-fold) associated with transport of small peptides (especially alanylalanine) in *D. melanogaster* (Charriere *et al.*, 2010). 2-amino-3-ketobutyrate coenzyme A ligase (Edgar and Polak, 2000) plays a critical role in the metabolism of serine, threonine, and glycine into acetyl-CoA for use in fatty acid synthesis and was up-regulated (1.63-fold). Up-regulation (2.05-fold) of a putative sodium-associated monocarboxylate transporter 25 hours after envenomation also suggests amino acid catabolism because monocarboxylate transporters may be moving metabolic intermediates of catabolism of peripheral tissue protein, like pyruvate, acetate, or propionate, into the fat body for lipid synthesis. A putative hydroxy-acid oxidase was also up-regulated (7.43-fold). Hydroxy-acid oxidase plays a critical role in glyoxylate and dicarboxylate metabolism following serine, threonine, and glycine metabolism. Envenomation could also trigger glyoxylate and dicarboxylate metabolism in host peripheral tissues, where two-carbon precursors could be used for gluconeogenesis; simple carbohydrates or Krebs-cycle intermediates could be trafficked to the host fat body for fatty-acid synthesis (Voet & Voet, 2011). Current data suggest that envenomation of *Sarcophaga* host pupae by *N. vitripennis* causes a substantial shift in protein and amino-acid metabolism to mobilize nutrients from peripheral tissues to the host fat body to support larval parasitoid growth, ensuring that parasitoids can acquire enough lipids during larval feeding to compensate for their inability to synthesize lipids *de novo* in adulthood (Visser *et al.*, 2010, 2012). Although several candidate metabolic pathways emerge above, particularly serine, threonine, and glycine metabolism coupled to

the glyoxylate/dicarboxylate cycle, future work should include testing which pathways of intermediary metabolism are most affected by envenomation, using techniques to carefully track labeled amino acid substrates through their metabolic intermediates (Zera, 2011; Visser *et al.*, 2012).

4.7.2. Metabolic depression

Upon envenomation by *N. vitripennis* metabolic rates of *Sarcophaga* pupae decline precipitously (Rivers & Denlinger, 1994b) and within 24 hours pupae increase alanine and pyruvate levels, but not the Krebs-cycle intermediate oxaloacetate (Rivers & Denlinger, 1994b). Although pyruvate levels increase initially they later drop as lipid is accumulated in the host fat body, suggesting that early high levels of pyruvate may support envenomation-induced alterations in host lipid metabolism (Rivers & Denlinger, 1994b). As in other parasitoid-host interactions that have been studied from a transcriptomic or proteomic perspective (Etebari *et al.*, 2011; Wertheim *et al.*, 2011; Zhu *et al.*, 2009; Song *et al.*, 2008; Nguyen *et al.*, 2008), genes associated with energy metabolism were differentially expressed in the current study (figure 5, table S2). Across a wide range of taxa from turtles to insects, metabolic depression is associated with inducing hypoxic-like states that induce greater anaerobic metabolism through glycolytic/gluconeogenic pathways and decreased reliance on the Krebs cycle (Guppy & Withers, 1999). A shift away from aerobic metabolism towards increased glycolysis and gluconeogenesis appears to occur with metabolic depression during pupal diapause in *Sarcophaga* flies despite the fact that diapausing pupae remain normoxic (Michaud & Denlinger, 2007; Ragland *et al.*, 2010), and several observations suggest envenomation may encourage a shift towards increased anaerobic metabolism. Lactate dehydrogenase regenerates NAD^+ by converting pyruvate to lactate under anaerobic conditions (Voet & Voet, 2011), and a putative lactate dehydrogenase was up-regulated 25 hours after envenomation (3.83-fold). Elevated pyruvate levels in *Sarcophaga* pupae just after envenomation (Rivers & Denlinger, 1994b) are consistent with envenomation causing increased glycolysis relative to aerobic Krebs-cycle activity, and lactate dehydrogenase may help maintain NAD^+ levels to facilitate glycolysis. Up-regulation of a putative myoinositol oxygenase (1.83-fold) also suggests greater glycolysis in envenomated hosts. Myoinositol can be used for glycolysis or to produce the lipid-precursor inositol, and myoinositol oxygenases

have been implicated in sugar balance and diabetes (Ganapathy *et al.*, 2008;Nayak *et al.*, 2011).

Envenomated pupae were kept in normal-oxygen atmospheres, so a shift towards anaerobic metabolism favouring glycolysis and reducing the activity of the Krebs cycle could be caused by components of *N. vitripennis* venom altering pathways that regulate metabolic responses to hypoxia. A putative hypoxia-inducible domain family 1 protein was up-regulated 25 hours after envenomation (2.22-fold). This protein is a downstream effector of the HIF signalling pathway and may be playing a role in directly reducing mitochondrial activity (Gorr *et al.*, 2004;Hayashi *et al.*, 2012). Future physiological and biochemical studies will be needed to test the hypothesis that envenomated host pupae are in a hypoxia-like state despite being normoxic. Although most studies of parasitoid manipulation of hosts focus on modulation of the immune response, the mechanisms that parasitoids use to manipulate host metabolism may provide new perspectives into states of metabolic depression.

5. Conclusions

These results support earlier studies demonstrating that *N. vitripennis* venom influences diverse physiological processes in one of its preferred host organism *S. crassipalpis*. Current molecular assay to confirm parasitization *post hoc* is unique because it keeps the samples intact, avoiding adverse effects caused by manipulation. Furthermore, to our knowledge this is the first transcriptomic study of the response of a host insect to attack by an ectoparasitoid, complementing a substantial body of physiological literature on the *N. vitripennis-Sarcophaga* interaction. Overall, fewer genes were found to be differentially expressed after parasitization with *N. vitripennis* than have been observed in studies with endoparasitoids. This observation is at least partly the result of venom injection alone rather than feeding by the wasp larvae because current samples were taken prior to hatching of the wasp eggs. The patterns of differential expression we observed suggest several clear candidate pathways for the molecular regulation of immune suppression, host developmental arrest, and alteration of host metabolism in response to ectoparasitoid envenomation. Many of these same pathways have also been implicated in endoparasitoid

attack of hosts, suggesting some fundamental, conserved aspects of host-parasitoid interactions to be further investigated.

6. Acknowledgements

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7. Supplementary materials

Table S1: Concentration and quality of the RNA samples used for microarray and validation experiment.

Hybridization-ID	Sample ID	260/280 ^a	260/230 ^b	Conc (ng/μl)	RIN ^c
12802	A1-Contr_3h_1	2.24	2.64	1924.11	ok
12803	A101-Paras_3h_1	2.17	2.69	5983.9	ok
12804	A164-Paras_25h_1	2.16	2.59	6271.9	ok
12805	A50-Contr_25h_1	2.17	2.75	4101.4	ok
12806	A3-Contr_3h_2	2.19	2.62	4779.1	ok
12807	A51-Contr_25h_2	2.16	2.74	3580.9	ok
12808	A165-Paras_25h_2	2.14	2.64	5480.5	ok
12809	A103-Paras_3h_2	2.20	2.73	8566.4	ok
12810	A4-Contr_3h_3	2.18	2.45	2968.11	ok
12811	A114-Paras_3h_3	2.17	2.75	5027.4	ok
12812	A167-Paras_25h_3	2.18	2.35	4349.7	ok
12813	A52-Contr_25h_3	2.16	2.72	5295.7	ok
12814	A5-Contr_3h_4	2.21	2.76	2788.5	ok
12815	A53-Contr_25h_4	2.16	2.63	3081.3	ok
12816	A168-Paras_25h_4	2.15	2.39	5053.7	ok
12817	A115-Paras_3h_4	2.17	2.50	5464.3	ok

^aRNA ratio 260/280

^bRNA ratio 260/230

^cRNA integrity number

Table S2: Differentially expressed EST's for comparisons Contr_3h vs Paras_3h and Contr_25h vs Paras_25h.

EST-ID	Name	Fold change
Control_3h vs Paras_3h		
<i>Down-regulated genes</i>		
EUA37Q302I4V57	leucine rich repeat protein	-1.67
Control_25h vs Paras_25h		
<i>Down-regulated genes</i>		
gi 296337397 gb EZ600582.1	alkaline phosphatase	-4.84
gi 296344996 gb EZ608181.1	acyl- reductase	-3.76
gi 296345268 gb EZ608453.1	af521649_1aminopeptidase 1 *	-2.93
EUA37Q301EWMAR	alkyldihydroxyacetonephosphate synthase *	-2.87
gi 296344527 gb EZ607712.1	GJ21452 [Drosophila virilis]	-2.36
EUA37Q301A5D33	metallothionein family 5 *	-2.01
EUA37Q301BGZ4G	cytochrome p450-28a1 * *	-1.91
gi 296334792 gb EZ597977.1	kda salivary protein	-1.88
gi 296347786 gb EZ610971.1	vitelline membrane protein 26aa	-1.75
EUA37Q301BDW2J	high-affinity copper uptake protein *	-1.65
EUA37Q302HYMJL	abdominal a	-1.58
EUA37Q301BG3HQ	GM12685 [Drosophila sechellia]	-1.49
gi 296336476 gb EZ599661.1	mitogen-activated protein kinase * *	-1.46
EUA37Q301DQW90	peptidyl-prolyl cis-trans isomerase 10	-1.38
gi 296339670 gb EZ602855.1	CG15096, isoform b	-1.35
EUA37Q302J1OWE	lethal nc136	-1.31
<i>Up-regulated genes</i>		
gi 296334764 gb EZ597949.1	rna polymerase ii second largest subunit	1.27
gi 296341632 gb EZ604817.1	isoleucyl trna synthetase	1.29
EUA37Q302FL5NT	guanine nucleotide exchange factor * *	1.30
gi 296354272 gb EZ617457.1	tyrosine-protein phosphatase non-receptor type 23	1.33
gi 296334548 gb EZ597733.1	glutaminyl-trna synthetase	1.34
EUA37Q302HO4B9	sin3a-associated protein sap130	1.34
gi 296335934 gb EZ599119.1	tubulin-specific chaperone b (tubulin folding cofactor b)	1.34
gi 296338738 gb EZ601923.1	argonaute-2 * *	1.35
EUA37Q301D0M2N	argonaute-2 * *	1.36
gi 296344550 gb EZ607735.1	GH15944 [Drosophila grimshawi]	1.38
gi 296344797 gb EZ607982.1	nucleolar gtp-binding protein * *	1.38
EUA37Q301BS4VV	isoleucyl trna synthetase	1.41
EUA37Q302HSGOB	CG2658, isoform b	1.41
gi 296351762 gb EZ614947.1	maleless	1.42
gi 296347793 gb EZ610978.1	CG10126, isoform a * *	1.44
gi 296339430 gb EZ602615.1	CG13365, isoform a	1.44
gi 296346174 gb EZ609359.1	ribonucleoside-diphosphate reductase large subunit *	1.46
gi 296337498 gb EZ600683.1	GJ13945 [Drosophila virilis] *	1.47
gi 296347148 gb EZ610333.1	transmembrane protein 77	1.47

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EUA37Q302FJ8LE	GJ21111 [Drosophila virilis]	1.47
EUA37Q301CP166	adenosine isoform a	1.50
gi 296335801 gb EZ598986.1	glycyl-trna synthetase	1.51
EUA37Q301B50E4	cg32479 cg32479-pa	1.61
gi 296338685 gb EZ601870.1	atp-binding cassette sub-family f member 1	1.63
gi 296347140 gb EZ610325.1	2-amino-3-ketobutyrate coenzyme a ligase *	1.63
gi 296346691 gb EZ609876.1	protease inhibitor-like protein	1.64
gi 296345742 gb EZ608927.1	ribosomal protein l7ae	1.65
gi 296350384 gb EZ613569.1	tnf receptor associated factor *	1.70
gi 296341534 gb EZ604719.1	tetraspanin 42ed	1.72
gi 296342414 gb EZ605599.1	CG13315, hypothetical conserved protein	1.72
gi 296347908 gb EZ611093.1	lysosomal aspartic protease *	1.74
gi 296336063 gb EZ599248.1	CG1572, isoform a	1.78
gi 296341133 gb EZ604318.1	CG10721, cDNA sequence	1.81
gi 296334521 gb EZ597706.1	nonsense-mediated mRNA 3	1.81
gi 296341333 gb EZ604518.1	small calcium-binding mitochondrial * *	1.83
EUA37Q301DBPNS	myoinositol oxygenase * *	1.83
gi 296344999 gb EZ608184.1	CG31086, isoform b	1.85
gi 296336730 gb EZ599915.1	map kinase phosphatase * *	1.86
gi 296343686 gb EZ606871.1	transport and golgi organization 14	1.86
EUA37Q302H71B7	CG32103, unknown	1.88
gi 296339539 gb EZ602724.1	G18822 [Drosophila mojavensis]	1.89
gi 296346814 gb EZ609999.1	tyrosyl-trna synthetase	1.90
EUA37Q302GBM2S	tyrosyl-trna synthetase	1.91
gi 296340589 gb EZ603774.1	CG6171, isoform b	1.93
gi 296338258 gb EZ601443.1	rna 3'-terminal phosphate cyclase	1.93
gi 296347532 gb EZ610717.1	annexin b11 isoform a	1.96
gi 296341392 gb EZ604577.1	lethal isoform g	1.99
gi 296336711 gb EZ599896.1	CG17549, isoform b	1.99
gi 296343254 gb EZ606439.1	CG10864, secreted peptide	2.01
gi 296338517 gb EZ601702.1	CG10444, isoform a *	2.05
EUA37Q302JKFDV	karl isoform a	2.07
EUA37Q301AOS36	serine protease *	2.12
EUA37Q302GXY23	CG5535, isoform b *	2.13
EUA37Q301DRADV	secretory phospholipase a2 *	2.15
gi 296340914 gb EZ604099.1	GG21026 [Drosophila erecta]	2.16
gi 296336709 gb EZ599894.1	CG17549, isoform b	2.16
gi 296343420 gb EZ606605.1	protein tyrosine phosphatase prl * *	2.17
gi 296334090 gb EZ597275.1	CG17734, isoform a *	2.22
gi 296336076 gb EZ599261.1	CG11050, isoform a	2.24
gi 296338864 gb EZ602049.1	sodium sialic acid	2.29
gi 296343363 gb EZ606548.1	CG4452, uncharacterized conserved protein	2.36
gi 296352558 gb EZ615743.1	receptor tyrosine kinase torso-like protein *	2.42
gi 296339089 gb EZ602274.1	CG3568	2.45
gi 296343036 gb EZ606221.1	map kinase-activated protein kinase	2.46
EUA37Q301E3IFP	actin-related protein 5	2.63

gi 296351374 gb EZ614559.1	kinesin-like protein kif1a	2.66
EUA37Q301D7PIT	osiris 7	2.68
gi 296349459 gb EZ612644.1	abc transporter *	2.75
EUA37Q301CDAR9	oligopeptide transporter *	2.87
EUA37Q301AOJ10	serine protease *	3.11
gi 296349810 gb EZ612995.1	GA13886 [Drosophila pseudoobscura pseudoobscura]	3.30
EUA37Q301BY6H3	GI23044 [Drosophila mojavensis]	3.37
gi 296346947 gb EZ610132.1	cytochrome oxidase subunit iii *	3.81
gi 296336117 gb EZ599302.1	l-lactate dehydrogenase *	3.83
gi 296339954 gb EZ603139.1	dolichyl pyrophosphate glc1man9 c2 alpha- -glucosyltransferase	4.34
EUA37Q301DOITT	sulfate transporter	5.47
gi 296347980 gb EZ611165.1	CG15650	5.55
EUA37Q301EV8JS	protein archease-like	5.90
gi 296351277 gb EZ614462.1	protein archease-like	5.95
gi 296346767 gb EZ609952.1	beta-site app-cleaving enzyme	6.11
gi 296333743 gb EZ596928.1	odorant-binding protein 56a *	6.56
EUA37Q302JMW6F	odorant-binding protein 56a *	7.38
EUA37Q301AHLSE	GK15652 [Drosophila willistoni]	7.12
gi 296346813 gb EZ609998.1	CG18003, isoform b *	7.43
gi 296344784 gb EZ607969.1	cuticular protein 76bc	8.39
gi 296334791 gb EZ597976.1	gtp-binding protein 1 * *	9.72
EOHUN8206DRP1R	dgp isoform b * * *	13.20

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Table S3: Differentially expressed EST's for comparisons Contr_25h vs Paras_25h, manually clustered into 8 different classes. Several EST's are present in more than one cluster, since they can perform multiple functionalities. * discussed in 4.5, * discussed in 4.6, * discussed in 4.7.

Classification	EST ID	Fold change	Name	#GOs	KEGG code	Enzyme Codes	Ref function
<i>Genetic Information Processing</i>							
Transcription							
	gi 296341392 gb EZ604577.1	1.99	lethal isoform g	5	K01104		P:regulation of transcription, DNA-dependent
	gi 296338258 gb EZ601443.1	1.93	rna 3 -terminal phosphate cyclase	2	K01974	EC:6.5.1.4	K01974
	gi 296345742 gb EZ608927.1	1.65	ribosomal protein l7ae	8	K12845		K12845
	gi 296351762 gb EZ614947.1	1.42	maleless	11	K13184		(Kotlikova <i>et al.</i> , 2006)
	gi 296344550 gb EZ607735.1	1.38	GH15944 [Drosophila grimshawi]	2			F:nucleic acid binding
	gi 296334764 gb EZ597949.1	1.27	rna polymerase ii second largest subunit	5	K03010	EC:2.7.7.6	P:transcription from RNA polymerase II promoter
	EUA37Q302J1OWE	-1.31	lethal nc136	7			P:transcription, DNA-dependent
	EUA37Q302HYMJL	-1.58	abdominal a	22	K09311		P:regulation of transcription, DNA-dependent
Translation							
	EUA37Q302GBMZS	1.91	tyrosyl-trna synthetase	5	K01866	EC:6.1.1.1	K01866
	gi 296346814 gb EZ609999.1	1.90	tyrosyl-trna synthetase	5	K01866	EC:6.1.1.1	K01866
	gi 296334521 gb EZ597706.1	1.81	nonsense-mediated mrna 3	2	K07562		K07562
	gi 296338685 gb EZ601870.1	1.63	atp-binding cassette sub-family f member 1	4	K06184		K06184
	gi 296335801 gb EZ598986.1	1.51	glycyl-trna synthetase	8	K01880	EC:6.1.1.14	K01880
	EUA37Q302FJ8LE	1.47	GJ21111 [Drosophila virilis]	2	K14573		K14573
	EUA37Q301BS4VV	1.41	isoleucyl trna synthetase	5	K01870	EC:6.1.1.5	K01870
	gi 296344797 gb EZ607982.1	1.38	nucleolar gtp-binding protein * *	2	K06943		K06943
	gi 296338738 gb EZ601923.1	1.35	argonaute-2 * *	21		EC:4.6.1.1	P:translational initiation
	gi 296341632 gb EZ604817.1	1.29	isoleucyl trna synthetase	9	K01870	EC:6.1.1.5	K01870
Replication and repair							
	EUA37Q301E3IFP	2.63	actin-related protein 5	7	K11672		P:double-strand break repair
	EUA37Q302GXY23	2.13	isoform b *	6			P:DNA repair

gi 296347532 gb EZ610717.1		1.96	annexin b11 isoform a	10			P:regulation of transcription from RNA polymerase II promoter
gi 296346174 gb EZ609359.1		1.46	ribonucleoside-diphosphate reductase large subunit *	11	K10807	EC:1.17.4.1	P:DNA replication
EUA37Q302HO4B9		1.34	sin3a-associated protein sap130	2			(Silverstein & Ekwall, 2005)
EUA37Q301DQW90		-1.38	peptidyl-prolyl cis-trans isomerase 10	7	K12734	EC:5.2.1.8	K12734
Folding, sorting and degradation							
EUA37Q301B50E4		1.61	cg32479 cg32479-pa	3	K11841	EC:3.1.2.15	K11841
gi 296339430 gb EZ602615.1		1.44	isoform a	10	K03798	EC:3.4.24.0; EC:3.6.1.3	K03798
EUA37Q302HSGOB		1.41	isoform b	10	K03798	EC:3.4.24.0; EC:3.6.1.3	K03798
gi 296335934 gb EZ599119.1		1.34	tubulin-specific chaperone b (tubulin folding cofactor b)	1			(Lytle <i>et al.</i> , 2004)
Metabolism							
Energy metabolism							
gi 296336117 gb EZ599302.1		3.83	L-lactate dehydrogenase *	5	K00016	EC:1.1.1.27	(Xia <i>et al.</i> , 2011)
gi 296346947 gb EZ610132.1		3.81	cytochrome oxidase subunit iii *	4	K02262	EC:1.9.3.1	(Voet <i>et al.</i> , 2011)
EUA37Q301DBPNS		1.83	myo-inositol oxygenase *	7	K00469	EC:1.13.99.1	(Vesala <i>et al.</i> , 2012)
Lipid metabolism							
EUA37Q301DRADV		2.15	secretory phospholipase a2 *	4	K01047	EC:3.1.1.4	(Stanley, 2006)
EUA37Q301EWMAR		-2.87	alkyldihydroxyacetonephosphate synthase *	7	K00803	EC:2.5.1.26	(Hajra & Bishop, 1982)
Amino acid/nucleotide metabolism							
gi 296346767 gb EZ609952.1		6.11	beta-site app-cleaving enzyme	2		EC:3.4.23.0	(Kotani <i>et al.</i> , 2005)
gi 296339954 gb EZ603139.1		4.34	dolichyl pyrophosphate glc1man9 c2 alpha- - glucosyltransferase	5	K03849	EC:2.4.1.265	(Sorte <i>et al.</i> , 2012)
gi 296347908 gb EZ611093.1		1.74	lysosomal aspartic protease *	6	K01379	EC:3.4.23.0	(Benes <i>et al.</i> , 2008)
gi 296350384 gb EZ613569.1		1.70	tnf receptor associated factor *	18	K09848	EC:6.3.2.19	(Deng <i>et al.</i> , 2000)
gi 296346691 gb EZ609876.1		1.64	protease inhibitor-like protein	3			(Niimi <i>et al.</i> , 1999)
gi 296347140 gb EZ610325.1		1.63	2-amino-3-ketobutyrate coenzyme a ligase *	8	K00639	EC:2.3.1.29	
EUA37Q301B50E4		1.61	cg32479 cg32479-pa (ubiquitin thiolesterase)	3	K11841	EC:3.1.2.15	(Osaka <i>et al.</i> , 2003)

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EUA37Q301CP166	1.50	adenosine isoform a	2	K01952	EC:6.3.5.3	(Tiong & Nash, 1993)
gi 296346174 gb EZ609359.1	1.46	ribonucleoside-diphosphate reductase large subunit *	11	K10807	EC:1.17.4.1	(Nordlund & Reichard, 2006)
gi 296339430 gb EZ602615.1	1.44	isoform a	10	K03798	EC:3.4.24.0; EC:3.6.1.3	(Juhola <i>et al.</i> , 2000)
EUA37Q302HSGOB	1.41	isoform b	10	K03798	EC:3.4.24.0; EC:3.6.1.3	(Juhola <i>et al.</i> , 2000)
EUA37Q301D0M2N	1.36	argonaute-2 * *	1			(Linder & Schultz, 2003)
gi 296338738 gb EZ601923.1	1.35	argonaute-2 * *	21		EC:4.6.1.1	(Linder & Schultz, 2003)
gi 296334764 gb EZ597949.1	1.27	rna polymerase ii second largest subunit	5	K03010	E.C.2.7.7.6	(Cramer <i>et al.</i> , 2001)
EUA37Q301DQW90	-1.38	peptidyl-prolyl cis-trans isomerase 10	7	K12734	EC:5.2.1.8	(Schiene-Fischer & Yu, 2001)
gi 296345268 gb EZ608453.1	-2.93	af521649_1aminopeptidase 1 (alanyl aminopeptidase) *	4		EC 3.4.11.2	(Nakanishi <i>et al.</i> , 2002)
Others						
gi 296346813 gb EZ609998.1	7.43	isoform b *	3	K11517	EC:1.1.3.15	
gi 296336076 gb EZ599261.1	2.24	isoform a	3	K07023	EC:3.1.4.0	
gi 296337498 gb EZ600683.1	1.47	GJ13945 [Drosophila virilis] (4-nitrophenyl phosphatase) *	1	K01101	EC:3.1.3.41	(Versaw <i>et al.</i> , 1991)
EUA37Q302HO4B9	1.34	sin3a-associated protein sap130	2		E.C:3.5	
gi 296334792 gb EZ597977.1	-1.88	kda salivary protein	0			
gi 296337397 gb EZ600582.1	-4.84	alkaline phosphatase	5	K01077	EC:3.1.3.1	(Funk, 2001)
Development						
gi 296346947 gb EZ610132.1	3.81	cytochrome oxidase subunit iii *	4	K02262	EC:1.9.3.1	(Singtripop <i>et al.</i> , 2007)
EUA37Q301AOJ10	3.11	serine protease *	1			(Robich & Denlinger, 2005)
gi 296349459 gb EZ612644.1	2.75	abc transporter *	6			(Yabe <i>et al.</i> , 2005)
gi 296351374 gb EZ614559.1	2.66	kinesin-like protein kif1a	11	K10392		P:neuromuscular junction development
EUA37Q301E3IFP	2.63	actin-related protein 5	7	K11672		(Meagher <i>et al.</i> , 2005)
gi 296352558 gb EZ615743.1	2.42	receptor tyrosine kinase torso-like protein *	4		EC:2.7.10.0	(Furriols <i>et al.</i> , 1998)
gi 296343420 gb EZ606605.1	2.17	protein tyrosine phosphatase prl * *	3	K01104		(Diamond <i>et al.</i> , 1994)
EUA37Q301AOS36	2.12	serine protease *	3		EC:3.4.21.0	(Robich & Denlinger, 2005)
gi 296343686 gb EZ606871.1	1.86	transport and golgi organization 14	2			P:wing disc development
gi 296336730 gb EZ599915.1	1.86	map kinase phosphatase * *	3	K04459	EC:3.1.3.48	(Kim <i>et al.</i> , 2004)
gi 296334521 gb EZ597706.1	1.81	nonsense-mediated mrna 3	2	K07562		(Metzstein & Krasnow, 2006)
gi 296347908 gb EZ611093.1	1.74	lysosomal aspartic protease *	6	K01379	EC:3.4.23.0	(Gui <i>et al.</i> , 2006)

gi 296350384 gb EZ613569.1	1.70	tnf receptor associated factor *	18	K09848	EC:6.3.2.19	P:instar larval development
gi 296345742 gb EZ608927.1	1.65	ribosomal protein l7ae	8	K12845		P:peripheral nervous system development
gi 296346691 gb EZ609876.1	1.64	protease inhibitor-like protein	3			(Oppert <i>et al.</i> , 2003)Oppert et al., 2003
gi 296335801 gb EZ598986.1	1.51	glycyl-trna synthetase	8	K01880	EC:6.1.1.14	(Uwer <i>et al.</i> , 1998)
gi 296344797 gb EZ607982.1	1.38	nucleolar gtp-binding protein * *	2	K06943		(Paridaen <i>et al.</i> , 2011)
EUA37Q301D0M2N	1.36	argonaute-2 * *	1			(Deshpande <i>et al.</i> , 2005)
gi 296338738 gb EZ601923.1	1.35	argonaute-2 * *	21		EC:4.6.1.1	(Deshpande <i>et al.</i> , 2005)
gi 296334548 gb EZ597733.1	1.34	glutaminyl-trna synthetase	5		EC:6.1.1.18	(Yebra <i>et al.</i> , 2006)
EUA37Q302FL5NT	1.30	guanine nucleotide exchange factor * *	6			(Van & D'Souza-Schorey, 1997)
gi 296334764 gb EZ597949.1	1.27	rna polymerase ii second largest subunit	5	K03010	EC:2.7.7.6	(Nowock <i>et al.</i> , 1978)
EUA37Q301DQW90	-1.38	peptidyl-prolyl cis-trans isomerase 10	7	K12734	EC:5.2.1.8	(Pemberton & Kay, 2005)
gi 296336476 gb EZ599661.1	-1.46	mitogen-activated protein kinase * *	8	K04432		(Widmann <i>et al.</i> , 1999)
EUA37Q302HYMJL	-1.58	abdominal a	22	K09311		P:oenocyte development; P:genitalia development;
						P:midgut development; P:gonadal mesoderm development; P:cardiac muscle tissue development;
						P:peripheral nervous system development;
						P:neuroblast development; P:positive regulation of muscle organ development;
EUA37Q301BDW2J	-1.65	high-affinity copper uptake protein *	3	K14686		name: high-affinity copper uptake protein
gi 296334792 gb EZ597977.1	-1.88	kda salivary protein	0			name: kda salivary protein
EUA37Q301BGZ4G	-1.91	cytochrome p450-28a1 * *	1	K15005		(Gerisch & Antebi, 2004)
EUA37Q301EWMAR	-2.87	alkyldihydroxyacetonephosphate synthase *	7	K00803	EC:2.5.1.26	(Motley <i>et al.</i> , 2000)
gi 296337397 gb EZ600582.1	-4.84	alkaline phosphatase	5	K01077	EC:3.1.3.1	(Yi & Adams, 2001)
<i>Programmed cell death</i>						
EOHUN8206DRP1R	13.20	dgp- isoform b * * *	3			(Takai <i>et al.</i> , 2001)
gi 296334791 gb EZ597976.1	9.72	gtp-binding protein 1 * *	3			(Bustelo <i>et al.</i> , 2007)
gi 296346947 gb EZ610132.1	3.81	cytochrome oxidase subunit iii *	4	K02262	EC:1.9.3.1	(Wu <i>et al.</i> , 2009)
gi 296343036 gb EZ606221.1	2.46	map kinase-activated protein kinase	5	K04443	EC:2.7.11.17	(Concannon <i>et al.</i> , 2003)

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EUA37Q301DRADV	2.15	secretory phospholipase a2 *	4	K01047	EC:3.1.1.4	(Rivers <i>et al.</i> , 2002a)
gi 296336730 gb EZ599915.1	1.86	map kinase phosphatase * *	3	K04459	EC:3.1.3.48	(Dickinson & Keyse, 2006)
gi 296347908 gb EZ611093.1	1.74	lysosomal aspartic protease *	6	K01379	EC:3.4.23.0	(Benes <i>et al.</i> , 2008)
gi 296350384 gb EZ613569.1	1.70	tnf receptor associated factor *	18	K09848	EC:6.3.2.19	(Kuranaga <i>et al.</i> , 2002)
gi 296347148 gb EZ610333.1	1.47	transmembrane protein 77	1			(O'Prey <i>et al.</i> , 2009)
gi 296346174 gb EZ609359.1	1.46	ribonucleoside-diphosphate reductase large subunit *	11	K10807	EC:1.17.4.1	(Akdemir <i>et al.</i> , 2007)
gi 296344797 gb EZ607982.1	1.38	nucleolar gtp-binding protein *	2	K06943		(Paridaen <i>et al.</i> , 2011)
EUA37Q301D0M2N	1.36	argonaute-2 * *	1			(Parisi <i>et al.</i> , 2011)
gi 296338738 gb EZ601923.1	1.35	argonaute-2 * *	21		EC:4.6.1.1	(Parisi <i>et al.</i> , 2011)
EUA37Q302HO4B9	1.34	sin3a-associated protein sap130	2		E.C:3.5	(Zilfou <i>et al.</i> , 2001)
gi 296335934 gb EZ599119.1	1.34	tubulin-specific chaperone b (tubulin folding cofactor b)	1			(Rossman <i>et al.</i> , 2005)
gi 296336476 gb EZ599661.1	-1.46	mitogen-activated protein kinase * *	8	K04432		(Cai <i>et al.</i> , 2006)
EUA37Q302HYMJL	-1.58	abdominal a	22	K09311		(Bello <i>et al.</i> , 2003)
EUA37Q301BGZ4G	-1.91	cytochrome p450-28a1 * *	1	K15005		(Scott & Wen, 2001)
EUA37Q301A5D33	-2.01	metallothionein family 5 *	2			(Shimoda <i>et al.</i> , 2003)

Detoxification

gi 296349459 gb EZ612644.1	2.75	abc transporter *	6			(Glavinas <i>et al.</i> , 2004)
EUA37Q301DBPNS	1.83	myoinositol oxygenase * *	7	K00469	EC:1.13.99.1	(Duan <i>et al.</i> , 2012)
gi 296338685 gb EZ601870.1	1.63	atp-binding cassette sub-family f member 1	4	K06184		(Sipos & Kuchler, 2006)
gi 296346174 gb EZ609359.1	1.46	ribonucleoside-diphosphate reductase large subunit *	11	K10807	EC:1.17.4.1	
EUA37Q301BDW2J	-1.65	high-affinity copper uptake protein *	3	K14686		(Balamurugan & Schaffner, 2006)
EUA37Q301BGZ4G	-1.91	cytochrome p450-28a1 * *	1	K15005		(Scott & Wen, 2001)
EUA37Q301A5D33	-2.01	metallothionein family 5 *	2			(Coyle <i>et al.</i> , 2002)
gi 296345268 gb EZ608453.1	-2.93	af521649_1aminopeptidase 1 (alanyl aminopeptidase) *			EC 3.4.11.2	(Cappiello <i>et al.</i> , 2004)
gi 296337397 gb EZ600582.1	-4.84	alkaline phosphatase	5	K01077	EC:3.1.3.1	(Arenas <i>et al.</i> , 2010)

Immune system

gi 296334791 gb EZ597976.1	9.72	gtp-binding protein 1 * *	3			(Traver <i>et al.</i> , 2011)
EUA37Q302JMW6F	7.38	odorant-binding protein 56a *	6			(Levy <i>et al.</i> , 2004)
gi 296333743 gb EZ596928.1	6.56	odorant-binding protein 56a *	5			(Levy <i>et al.</i> , 2004)

EUA37Q301AOJ10	3.11	serine protease *	1			(Tang <i>et al.</i> , 2011)
EUA37Q301CDAR9	2.87	oligopeptide transporter *	3	K14206		(Charrier & Merlin, 2006)
gi 296349459 gb EZ612644.1	2.75	abc transporter *	6			(van de Ven <i>et al.</i> , 2009)
gi 296351374 gb EZ614559.1	2.66	kinesin-like protein kif1a	11	K10392		(Hanada <i>et al.</i> , 2000)
EUA37Q301E3IFP	2.63	actin-related protein 5	7	K11672		(Yutin <i>et al.</i> , 2009)
gi 296343036 gb EZ606221.1	2.46	map kinase-activated protein kinase	5	K04443	EC:2.7.11.17	(Cargnello & Roux, 2011)
gi 296343420 gb EZ606605.1	2.17	protein tyrosine phosphatase prl * *	3	K01104		(Pruissers & Strand, 2007)
EUA37Q301DRADV	2.15	secretory phospholipase a2 *	4	K01047	EC:3.1.1.4	(Shrestha <i>et al.</i> , 2010)
EUA37Q301AOS36	2.12	serine protease *	3		EC:3.4.21.0	(Tang <i>et al.</i> , 2011)
gi 296341392 gb EZ604577.1	1.99	lethal isoform g	5	K01104		P:phagocytosis, engulfment
gi 296347532 gb EZ610717.1	1.96	annexin b11 isoform a	10			(Yutin <i>et al.</i> , 2009)
gi 296336730 gb EZ599915.1	1.86	map kinase phosphatase * *	3	K04459	EC:3.1.3.48	
gi 296334521 gb EZ597706.1	1.81	nonsense-mediated mrna 3	2	K07562		(El-Bchiri <i>et al.</i> , 2008)
gi 296347908 gb EZ611093.1	1.74	lysosomal aspartic protease *	6	K01379	EC:3.4.23.0	(Colbert <i>et al.</i> , 2009)
gi 296341534 gb EZ604719.1	1.72	tetraspanin 42ed	1			(Wright <i>et al.</i> , 2004)
gi 296350384 gb EZ613569.1	1.70	tnf receptor associated factor *	18	K09848	EC:6.3.2.19	(Lund & Delotto, 2011)
gi 296346691 gb EZ609876.1	1.64	protease inhibitor-like protein	3			(Clermont <i>et al.</i> , 2004)
gi 296347793 gb EZ610978.1	1.44	isoform a *	3			P:phagocytosis, engulfment
EUA37Q301BS4VV	1.41	isoleucyl trna synthetase	5	K01870	EC:6.1.1.5	(Park <i>et al.</i> , 2008b)
EUA37Q301D0M2N	1.36	argonaute-2 * *	1			(van Rij <i>et al.</i> , 2006)
gi 296338738 gb EZ601923.1	1.35	argonaute-2 * *	21		EC:4.6.1.1	(van Rij <i>et al.</i> , 2006)
EUA37Q302HO4B9	1.34	sin3a-associated protein sap130	2			(Cambi & Figdor, 2009)
EUA37Q302FL5NT	1.30	guanine nucleotide exchange factor * *	6			(Hall <i>et al.</i> , 2006)
gi 296341632 gb EZ604817.1	1.29	isoleucyl trna synthetase	9	K01870	EC:6.1.1.5	(Park <i>et al.</i> , 2008b)
gi 296336476 gb EZ599661.1	-1.46	mitogen-activated protein kinase * *	8	K04432		(Chen <i>et al.</i> , 2010)
EUA37Q301BGZ4G	-1.91	cytochrome p450-28a1 * *	1	K15005		(Morfin, 2002)
Sensory system						
EUA37Q302JMW6F	7.38	odorant-binding protein 56a *	6			(Wertheim <i>et al.</i> , 2005)
gi 296333743 gb EZ596928.1	6.56	odorant-binding protein 56a *	5			(Wertheim <i>et al.</i> , 2005)
gi 296351762 gb EZ614947.1	1.42	maleless	11	K13184		(Peixoto & Hall, 1998)

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Transporters

EUA37Q302JMW6F	7.38	odorant-binding protein 56a *	6			(Gong <i>et al.</i> , 2009)
EUA37Q301DOITT	5.47	sulfate transporter	4	K14708		(Mount & Romero, 2004)
EUA37Q301CDAR9	2.87	oligopeptide transporter (YIN) *	3	K14206		(Charriere <i>et al.</i> , 2010)
gi 296349459 gb EZ612644.1	2.75	abc transporter *	6			(Glavinas <i>et al.</i> , 2004)
gi 296338864 gb EZ602049.1	2.29	sodium sialic acid	4	K12303		(Havelaar <i>et al.</i> , 1998)
EUA37Q302GXY23	2.13	isoform b *	6			GO:0015326
gi 296338517 gb EZ601702.1	2.05	isoform a *	4			GO:0006814
gi 296339539 gb EZ602724.1	1.89	GI18822 [<i>Drosophila mojavensis</i>]	3			GO:0006810
EUA37Q302H71B7	1.88	Unknown	0			GO:0006810
gi 296343686 gb EZ606871.1	1.86	transport and golgi organization 14	2			
gi 296341333 gb EZ604518.1	1.83	small calcium-binding mitochondrial * *	6	K14684		(Satrustegui <i>et al.</i> , 2007)
gi 296338685 gb EZ601870.1	1.63	atp-binding cassette sub-family f member 1	4	K06184		(Sipos & Kuchler, 2006)
gi 296337498 gb EZ600683.1	1.47	GJ13945 [<i>Drosophila virilis</i>] (4-nitrophenyl phosphatase) *	1	K01101	EC:3.1.3.41	(Bramkamp <i>et al.</i> , 2004)
gi 296339670 gb EZ602855.1	-1.35	isoform b	3			GO:0005316
EUA37Q301DQW90	-1.38	peptidyl-prolyl cis-trans isomerase 10	7	K12734	EC:5.2.1.8	GO:0006810
EUA37Q301BDW2J	-1.65	high-affinity copper uptake protein *	3	K14686		(Balamurugan & Schaffner, 2006)
gi 296337397 gb EZ600582.1	-4.84	alkaline phosphatase	5	K01077	EC:3.1.3.1	GO:0042045

Table S4: Randomly selected genes used in the RT-qPCR experiment to validate the microarray results.

Sequence Name	Sequence Description	Symbol	Primer sequence (5' to 3')	Amlicon length (bp)	E (%) ^a	R ^{2,b}	log ratio microarray	log ratio RT-qPCR
EUA37Q302I4V57	leucine rich repeat protein	lrr-pr	GTGACGATAGTGGGCAGGAT GTTTTCGGGTGCCTATACCA	83	106.5	0.992	-0.744	-0.505
gi 296336476 gb EZ599661.1	mitogen-activated protein kinase	mapk	TCATAAATCGCAACGGTGAA CGGGTGCCATATAGGGTTTA	111	107.9	0.910	-0.515	-0.845
gi 296334792 gb EZ597977.1	kda salivary protein	sal-pr	TGGCCTCTTTGGCTATTTTG TGCTTTGGTTCTTGTGTCTG	94	97.8	0.996	-0.914	-1.664
EUA37Q301BDW2J	high-affinity copper uptake protein	cup	TTTGGGTGCTGGTATTGGTT TTGCACATTGCTAGTGACAGTG	89	100.1	0.998	-0.721	-1.239
EUA37Q301BGZ4G	cytochrome p450-28a1	cytochr	GCAGGCGTATTGTACACAC GCATCCAAATAGGGCAATTC	143	106.5	0.988	-0.901	-0.886
gi 296344784 gb EZ607969.1	cuticular protein 76bc	cut-pr	TGCCAGAATACGTCAAGTGC AGGCTGGAAATCCTCCTGTT	137	97.9	0.999	3.068	2.661
gi 296334791 gb EZ597976.1	gtp-binding protein 1	gtp-bpr	GTAGTGTGCGGCACCTGTCT CGCCTCTTACCTCTTTCACG	142	94.1	0.998	3.280	2.948
EOHUN8206DRP1R	dgp- isoform b	dgp-isoformb	TTCCGCATGAGTTAAACACC GGCAACGTAAAGTTGAAAAAGG	149	92.7	0.999	3.722	2.946
gi 296350384 gb EZ613569.1	tnf receptor associated factor	traf	GGAATTGGTCTACCACCAT AGGACCATTGCCATTCAAAA	82	88.4	0.995	0.763	0.593
gi 296343254 gb EZ606439.1	secreted peptide	sec-pep	TCTAAGGACATTCCTCATCG AAGCCCATTTGTGGAACAGA	127	85.8	0.992	1.006	0.703
gi 296335934 gb EZ599119.1	tubulin-specific chaperone b (tubulin folding cofactor b)	tub-cha	CAATTGATCCTGGCATGAGA ACCCATGCGATTCTTTTCA	149	87.3	0.998	0.426	-0.027

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gi 296343036 gb EZ606221.1	map kinase-activated protein kinase	mapk-apk	TCCACCGTTTTACAGCAACA TCCATTCAGGATTGGGAAAA	100	86.0	0.998	1.296	0.527
gi 296347908 gb EZ611093.1	lysosomal aspartic protease	la-prot	CTACAGCAAAGCCAACACGA CCTTTGTGGATTTTGGGAGA	85	91.5	0.999	0.801	-0.129
gi 296341632 gb EZ604817.1	isoleucyl trna synthetase	isol-synt	AGTCTTTGGGCCAACGTTTA ATACGCTGGCCTTCACTTC	125	107.4	0.967	0.372	0.118
gi 296336117 gb EZ599302.1	l-lactate dehydrogenase	l-lact-dehydro	CAAACGTGATTACCCCTCCT GCAACACGGTTCCAATTTCT	127	97.4	0.999	1.937	1.952
EUA37Q302GBMZS	tyrosyl-trna synthetase	tyr-synth	TGTACTTCCAATGCGGATCA CTTCGAAGGCTAAACGGCTA	85	90.9	0.997	0.934	0.570
gi 296346813 gb EZ609998.1	isoform b	isoformb	ACGGGATGTTTCTCAAGTGG CACCAGCAGCTTTTGCATTA	137	92	0.984	2.894	2.753
EUA37Q302JMW6F	odorant-binding protein 56a	odorant	CCTTGAAGGATGGCCAAGTA AGCCAGTATCACAGCGTCCT	132	93.9	0.999	1.528	2.409
EUA37Q301DOITT	sulfate transporter	sulfatetr	CGCTTTAAGTCGTTGGTGCT TAATAGCCGCCAATGTAGCC	147	94.4	0.996	1.293	2.002
EUA37Q301AHLSE	GK15652 [<i>Drosophila willistoni</i>]	GK15652	TGACAACGATATTGCCATTG ATCCGGTAACAGTGCCAGTC	122	97.8	0.98	2.832	2.790

^aMeasure of the real-time PCR reaction efficiency (calculated by the standard curve method)

^bReproducibility of the real-time PCR reaction

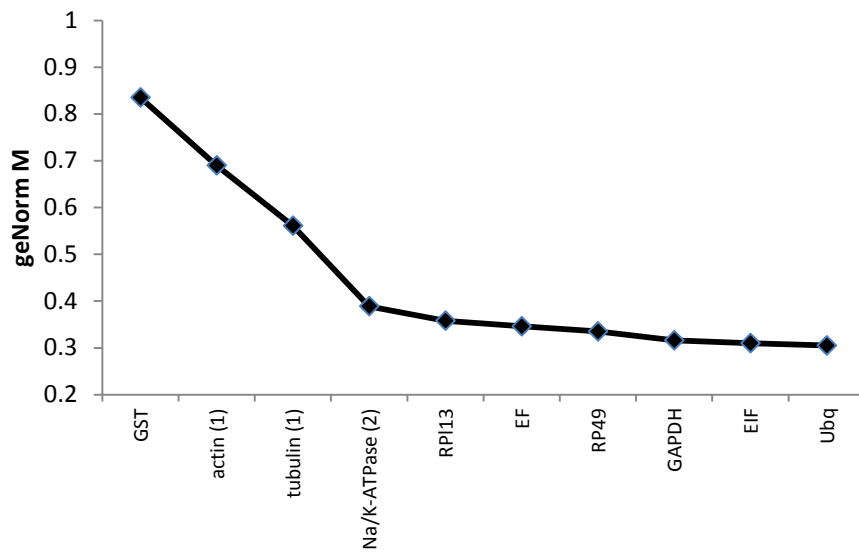
Table S5: Candidate reference genes for *S. crassipalpis*, of which the stability was analyzed with the geNorm^{PLUS} algorithm.

Seq. Name	Seq. Description	Symbol	Primer sequence (5' to 3')	Amlicon length (bp)	E (%) ^a	R ^{2b}
gi 296333647 gb EZ596832.1	actin	actin(1)	GGTATGTGCAAGGCTGGTTT TTTTGACCCATACCGACCAT	107	102.6	0.998
gi 296336747 gb EZ599932.1	tubulin alpha chain	tubulin (1)	CAATTATGCTCGAGGCCATT ACCACTTCCAGTTCCACCAC	142	102.9	0.992
gi 296336001 gb EZ599186.1	glutathione-s-transferase gst	GST	TTGAGAAAACGAGCCGAAGT GGAGCAATCAGTGGGAACCAT	114	110.4	0.990
gi 296334900 gb EZ598085.1	glyceraldehyde 3 phosphate dehydrogenase 2	GAPDH	AGCAGGAGCCTGAGCATTTA CCATGAGGTGGATTTGCTT	112	107.4	0.995
gi 296334383 gb EZ597568.1	ribosomal protein 49	RP49	CGCACAAATGGAGAAAACCT GGTACGCTTGTGGAACCAT	104	112.4	0.987
gi 296347152 gb EZ610337.1	ribosomal protein l13	RPI13	GGAATTGAAGGGTGCTGGTA GATATTGCGTTGACGGGATT	106	102.1	0.993
»» gi 296337299 gb EZ600484.1	ubiquitin-conjugating enzyme	Ubq	AAAATGGCGAATCAATCCAC TGTCGTCGTCATTTCAAGG	127	104.0	0.993
gi 296339815 gb EZ603000.1	elongation factor isoform a	EF	CTCAAGCTGATTGTGCCGTA TGTTTGACACCCAAGGTGAA	121	94.7	0.993
»» gi 296334587 gb EZ597772.1	eukaryotic translation initiation factor 1a	EIF	CAAGATTCAAAGGCCGATGT AACCGTCTTCCACAAAGGTG	124	99.9	0.984
gi 296340585 gb EZ603770.1	sodium potassium-dependent atpase beta-2 subunit	NaK-ATPase(2)	GTTTTCGCCCATAGTGAA TTGCCACTGTGAGTGCTTTC	139	103.5	0.995

^aMeasure of the real-time PCR reaction efficiency (calculated by the standard curve method)^bReproducibility of the real-time PCR reaction

»»Reference genes used to normalize the results of the RT-qPCR experiment for microarray data validation.

A



B

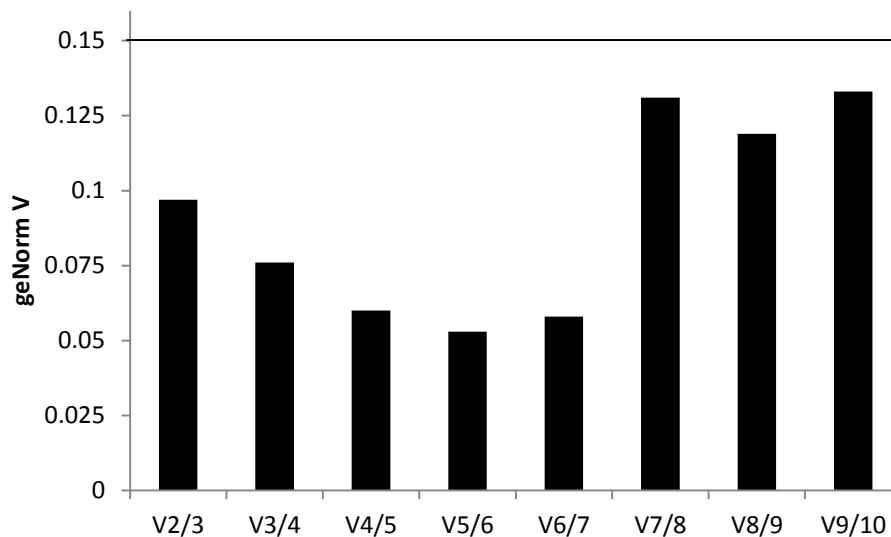


Figure S1: A. Average expression stability of remaining reference targets (geNorm M-value < 0.5). The M-value calculates the average for all pairwise variation values, so a lower value indicates increased gene stability across all tested genes. B. Determination of the optimal number of reference targets with geNorm^{PLUS} (geNorm V-value < 0.15). The V-value refers to the pairwise variation between reference targets that is reduced by the inclusion of additional reference genes and thus indicates the number of genes required to achieve an arbitrarily selected threshold of reference gene stability. Since every amount of reference genes that keep high stability stay below the threshold of 0.15, the minimum amount of reference genes can be selected (2 reference genes were chosen).

ADDENDUM TO CHAPTER 3

Exploring functionalities of novel venom compounds

Personal contribution:

- Insect cultures
- Sample preparation and PCR
- Recombinant expression and host injections
- RT-qPCR
- Parasitization experiments
- dsRNA production and *Nasonia* pupal injections
- Functional analysis of the dataset
- Writing the manuscript

1. Introduction

Once the complexity of animal venoms gained interest among scientists, venomomics or the analysis of venom proteomes eventually received its proper attention. First studies on parasitoid venoms consisted out of bioassays trying to understand what parasitization was triggering in a host organism. Alterations in development, the immune reaction, the nutrient levels, in short the complete biochemical and physiological profile was explored. The logic next step was to find out what exactly in this venomous mixture was causing these effects, bringing research activities to a completely different level. Originally by HPLC fractionation, the venom compounds were separated from each other and bioassays were conducted (Moore *et al.*, 2006; Rivers *et al.*, 2006; Dani *et al.*, 2003). The production of cDNA libraries made it possible to start identifying the structure of these proteins and looking for typical functional domains (Asgari *et al.*, 2003; Crawford *et al.*, 2008; Baek & Lee, 2010; Zhu *et al.*, 2010; Falabella *et al.*, 2007; Price *et al.*, 2009). But it took until mass spectrometry and genome characterizations were brought to life, before high-throughput venom compound screening really started to kick in.

When conducting functional studies on parasitoid venom compounds, producing this compound by recombinant expression is one approach. Since HPLC fractionation mostly involve time-consuming dissections, the availability of the recombinant venom protein enormously broadens the possibilities in functional research. The mode of action of many venom proteins was revealed in this way, partly because they showed homology with other venom proteins or because they contained certain structural domains that hint at their possible function. If for instance, the venom compound shows sequence similarity with a known enzyme, specific assays can be performed with the recombinant protein to assess this potential enzyme activity (Falabella *et al.*, 2007; Colinet *et al.*, 2011; Zhu *et al.*, 2008). If the venom protein contains similarities with proteins from the serpin family for instance (serine protease inhibitors), melanisation assays can be performed using the recombinant compound since these proteins are known to tightly regulate the phenoloxidase cascade (Zhang *et al.*, 2004; Colinet *et al.*, 2009). When the venom components show sequence similarity to known proteins, like the examples above, the proposed function can rather

quickly be verified when performing recombinant expression combined with focussed bio-assays.

Next to the approach of recombinant expression, a method to assess venom protein functionalities that is gaining interest the last few years, is RNA interference (RNAi) (Box 1) (Scott *et al.*, 2013). This relatively new technique in insects makes use of the RNAi machinery that contributes to immunity against viruses with a double stranded RNA (dsRNA) genome or dsRNA replicative intermediates in the cytoplasm of infected cells (Blair, 2011). The accumulating body of literature on insect RNAi has revealed that the efficiency of RNAi varies between different species, the mode of RNAi delivery and the genes being targeted. Crucial in effective silencing, is the cellular uptake of the RNAi molecule and the transfer to other cells. If RNAi-mediated silencing is transmitted widely throughout the treated organism, RNAi is described as systemic, which seemed to be the case for dsRNA microinjection into several parasitoid wasps (Colinet *et al.*, 2014; Burke *et al.*, 2013; Bonvin *et al.*, 2005). The most widely used routes for administering RNAi to insects are injection into the hemolymph and feeding.

BOX 1: RNA interference

RNAi is a post-transcriptional gene silencing mechanism conserved from plants to humans that relies on exogenous dsRNA delivery. When dsRNA enters the cell, it is cleaved by RNase III Dicer into 20-25 bp fragments. These fragments are incorporated into the multi-protein RNA-induced silencing complex (RISC), where one strand (the “passenger” strand) is eliminated and the other “guide” strand is retained. The catalytic component of RISC is an Argonaute protein, which cleaves single-stranded RNA molecules having sequence complementary to the guide RNA. Endogenous gene transcripts can be cut by Dicer into 20-25 bp RNAs called microRNAs (miRNAs), which function in the regulation of gene expression. dsRNA molecules can also be cleaved by Dicer into small interfering RNAs (siRNAs), providing defense against viruses and transposable elements (Sidahmed *et al.*, 2014). This siRNA pathway in which cells degrade a single-stranded RNA (ssRNA) (including mRNA) with sequence identity to the administered dsRNA molecules is exploited by the experimental RNA interference technique (Scott *et al.*, 2013).

Microinjection has been applied to all life stages in hemi- and holometabolous insects. In *N. vitripennis*, RNAi has been proven to be successful via microinjection into the larval and the pupal stage (Lynch & Desplan, 2006; Werren *et al.*, 2009). Injecting *N. vitripennis* pupae with

dsRNA and examining their progeny for developmental defects, called parental RNAi (pRNAi), was proven to be efficient in aiding the study of gene function during embryonic patterning (Zwier *et al.*, 2012). However, when dsRNA is injected in a developmental stage of *N. vitripennis* before the third larval stage, developmental arrest was induced, possibly through induction of diapause by the stress of injection (Werren *et al.*, 2009). Silencing the abundant venom compound LbGAP in the endoparasitoid wasp *L. boulardi* remained at least for 15 days after female emergence, suggesting that the knock-down of gene expression lasts the entire lifetime of the injected parasitoid females (Colinet *et al.*, 2014). In that specific study, microinjection of dsRNA into the hemolymph of wasp larvae or pupae was shown to produce a long term and highly efficient silencing of gene expression in very different tissues. In addition, 2 bracovirus genes could be effectively knocked down by RNAi in *Chelonus inanitus* and *M. demolitor* (Burke *et al.*, 2013). Coupling the RNAi method with functional bio-assays, gave insight into the role of these genes in viral replication. Using RNAi in *N. vitripennis* to elucidate the function of its venom compounds, would imply the knock down of this specific component in female wasps followed by specific assays on the host after parasitization by this wasp.

When in 2010 the venom composition of *N. vitripennis* became available (de Graaf *et al.*, 2010), the major task remained to link these 79 proteins to their respective function. Interestingly, 23 of these venom proteins showed no similarities to any known protein, constituting an intriguing group of newly discovered venom compounds. Therefore, we started the quest of functionality with this challenging group of venom proteins. The two separate lines of approaches explained above were explored using the *N. vitripennis* - *S. crassipalpis* parasitoid – host system. Design of the two experimental procedures is outlined in figure 1. The possible employment of recombinant expression of *N. vitripennis* venom proteins in *E. coli* was first analysed by injecting the bacterial background (still present after His-tag purification) into the host. This was done to investigate possible host immune effects against these impurities and is described below. Additionally, before applying the second approach that makes use of injecting dsRNA into the host, the exact timing of dsRNA injection into *N. vitripennis* pupae and the verification process of successful silencing was investigated, which is described below.

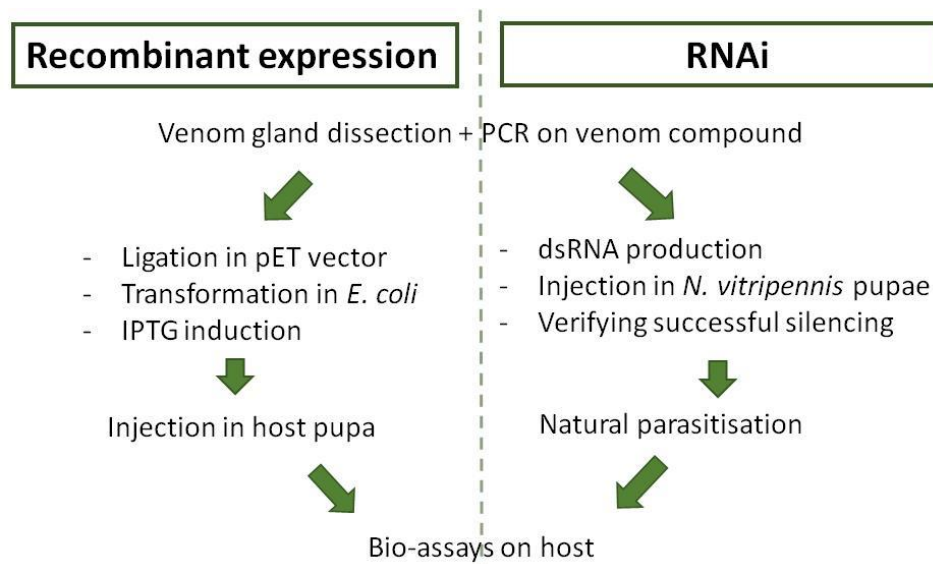


Figure 1: Overview of two approaches to investigate functionalities of venom proteins from *N. vitripennis*.

2. Materials and methods

2.1. Biological material

N. vitripennis AsymC wasps were reared on pupae of the flesh fly, *Sarcophaga crassipalpis*, and maintained at 25°C with a daily 16:8 light:dark cycle (van den Assem & Jachmann, 1999). For the collection of 8 different developmental stages of *N. vitripennis*, 20 specimens per life stage were pooled, except for the eggs, where 50 specimens were harvested from the host. The *N. vitripennis* venom gland tissue preparation was done by dissecting the venom reservoirs and associated venom gland in insect saline solution (ISB) (150 mM NaCl, 10 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 10 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid, pH7) and separating secretions from the tissues by rapid spinning. After removal of the supernatant, the pellet was resuspended in RNALater® (Ambion) for temporal storage. *S. crassipalpis* Macquart was maintained in the laboratory and cultured as described by Denlinger (Denlinger, 1972). Larvae were fed on beef liver at 25°C and exposed to a 16:8 light:dark cycle.

2.2. RNA extraction and cDNA conversion

From the developmental stages of *N. vitripennis*, total RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's protocol. Isolated RNA was subsequently

reverse transcribed with the RevertAid™ H Minus First Strand cDNA Synthesis Kit using the oligo(dT)18 primer. In order to investigate possible splice variants of venom compounds, RNA from adult female *N. vitripennis* wasps, was also converted into cDNA using the 3'RACE Adapter primer (5'-GCGAGCACAGAATTAATACGACTCACTATAGGT12VN-3') from the FirstChoice® RLM Race Kit (Invitrogen, Ambion), synthesizing poly(A)-tailed RNA and adding an adapter sequence as anchor for primers used in PCR amplification.

From venom gland tissues, RNA was extracted from the pellet (see 2.1. Biological material) using the RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas) using the oligo(dT)18 primer.

From the single female wasp abdomens, total RNA was isolated with TRIzol. Due to the hard exoskeleton, the Precellys®24 Homogenizer (Bertin Technologies, Montigny le Bretonneux, France) was used after adding one stainless steel bead (2,3 mm mean diameter) and ¼ of a PCR tube of zirconia/silica beads (0,1 mm mean diameter) to an individual abdomen. Further extraction was performed according to the manufacturer's protocol. First-strand cDNA was synthesized with the RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas) using a mix of one part oligo(dT)18 primer and six parts random hexamer primer.

RNA extraction from flesh fly pupae was performed using the RNeasy Mini Kit (Qiagen). Tissues were disrupted and homogenized by adding one stainless steel bead, ¼ of a PCR tube of zirconia/silica beads and 1 ml Qiazol Lysis Reagent and using the Precellys®24 Homogenizer (Bertin Technologies, Montigny le Bretonneux, France). An on-column DNase I treatment with the RNase-free DNase set (Qiagen) was performed. RNA was eluted twice, first with 30 µl RNase free water, then with 20 µl RNase free water, and stored at -80 °C. Five µg of total RNA from each sample was converted to cDNA using oligo(dT)18 primer and was carried out according to the RevertAid™ H Minus First strand cDNA Synthesis kit protocol.

2.3. Primer design and PCR

For amplification of transcripts that correspond with the complete coding sequence (CDS) of predicted genes from *N. vitripennis*, primers were developed at the extreme ends with a melting temperature of approximately 60°C. The different primer sets are listed in Table S1. For amplification of transcripts from the cDNA with the adapter sequence at the poly(A)tail, the 3'RACE outer primer (5'-GCGAGCACAGAATTAATACGACT-3') was used in combination

with the CDS forward primer. The 3'RACE outer primer is complementary to the anchored adapter and ensures, together with the CDS forward primer, amplification of the possible splice variants that have been spliced at the 3' end.

PCR reactions were carried out in the Eppendorf Mastercycler®. Following an initial step at 95°C for 15 minutes, the reaction mixtures were subjected to 35 cycles consisting of denaturation at 93°C for 1 minute, primer annealing at 55°C for 30 seconds, and DNA extension of 2 minutes at 72°C. The reaction was completed by a final extension step at 72°C for 10 minutes.

2.4. Recombinant expression

Plasmids containing the pET100/D-TOPO vector (without insert) were transformed in *E. coli* BL21 star (DE3). Bacteria were grown in 100 ml Luria Bertani (LB) broth medium at 37°C to an optical density of 0.5 at 600 nm. Subsequently, isopropyl-1-thio- β -D-galactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued for 4 hours. Cells were harvested and resuspended in 8 ml lysis buffer (6 M guanidine HCl, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8). After 10 minutes incubation at room temperature, the cell lysate was sonicated on ice with three five-second pulses at high intensity and centrifuged. The supernatant was loaded on Profinity IMAC Ni-Charged Resin (Bio-Rad) that was equilibrated with binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8). The column was washed with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8) and eluted in elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8) in eight steps of 1 ml each. Fractions with recombinant protein are pooled and buffer exchange was performed from elution buffer to ISB by using the SnakeSkin™ Pleated Dialysis Tubing (Pierce).

2.5. Injection of *S. crassipalpis* pupae

The puparium was removed from the posterior end of the pupa. Several minutes before injection, the cuticle was pricked with a sterile insect pin and a small droplet of hemolymph was removed with a piece of absorbent filter paper. Otherwise, the turgor of the pupae would prevent the injection of more fluid into the body. Injections were performed into the

last abdominal segments by using a 10 ml Hamilton microsyringe. The injected volume was 1.5 µl solution per pupa.

2.6. Reference gene selection, primer design and RT-qPCR

Candidate reference genes for *N. vitripennis* were chosen due to their stable expression in other studies (Cheng *et al.*, 2013a; Sun *et al.*, 2010). Primer sets for the novel venom proteins (table S2) and the candidate reference genes (table S3) were developed to contain at least one exon-exon spanning primer and to produce products with size-ranges of 80-120 base pairs using PerlPrimer (Marshall, 2004). Similarly, primer sets were also developed for 6 immune genes in *S. crassipalpis* (table S4). RT-qPCR was executed as described in chapter 3. Each sample was run in triplicate.

2.7. Computational selection of reference and test genes

Primer efficiencies, R^2 values and melt curves were calculated with CFX Manager Software (Bio-Rad). Reference gene stability was analyzed using the geNormPLUS algorithm within the qBasePLUS environment (Biogazelle NV). Default settings were kept, except that target specific amplification efficiencies were used. RT-qPCR data was evaluated using analysis of variance, ANOVA followed by Dunn's or Bonferroni posthoc test. P-value 0.05 was considered statistically significant. All statistical analyses were performed with Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

3. Results and Discussion

3.1. Injection of recombinant venom compounds into the host

The first approach to investigate the function of *N. vitripennis* venom proteins was the injection of recombinant expressed protein into the host. Since the large amount of 23 novel venom proteins caught our interest, we needed an expression system that is high-throughput. The bacterial expression in *E. coli* meets these requirements, but bacteria have the disadvantage to lack the enzymatic machinery to perform complex post-translational modifications like glycosylations. First characterization of *N. vitripennis* venom with infrared

spectroscopy revealed that proteins are unglycosylated (Rivers *et al.*, 2006). This is a quite uncommon feature for parasitoid venoms, but a feature shared with *P. turionella* (Uckan *et al.*, 2004) and many social hymenopterans (Leluk *et al.*, 1989). Next to this, the presence of several carbohydrates, including polysaccharides, mucopolysaccharides, glycoproteins and glycolipids, was also investigated in the Laboratory of Molecular Entomology and Bee Pathology. Periodic acid Schiff (PAS) staining of dissected *N. vitripennis* venom resulted in confirmative evidence that *N. vitripennis* venom lacks carbohydrates (data not presented).

Once the venom proteins are overexpressed in *E. coli*, they can be purified over a column with Ni-Charged Resin due to the N-terminal His-tag. This purification procedure does not completely eliminate all bacterial fragments present in the protein elution solution. Since it is our goal to investigate the effect of the (recombinant) venom protein on the developmental and immune systems of the host, the effects caused by these background fragments must be kept to a minimum. To investigate possible side effects, plasmids cloned with the pET100/D-TOPO vector without an inserted fragment were transformed into *E. coli* BL21 bacteria. After induction with IPTG, cell lysates were sonicated and transferred to a Ni-Charged Resin column. The buffer of the purified *E. coli* lysates was exchanged to ISB via overnight dialysis. *S. crassipalpis* pupae, 5 days post-pupation, were either injected with ISB or with the purified *E. coli* lysate. Not injected pupae served as control group. Viability was measured on 40 pupae per group which resulted in 100% for the control group, 93% for the ISB injected group and 75% for the purified *E. coli* lysate injected group. This decline in survival rate already indicated that the pupae suffered more from the injection with the purified *E. coli* lysate than with the ISB solution. RNA was extracted and converted into cDNA from 3 individual pupae per group. RT-qPCR was performed on 6 immune genes of *Sarcophaga* that are known to participate in immune processes: sapecin (AMP defensin homologue from *D. melanogaster*), sarcotoxin I, (AMP cecropin homologue), sarcotoxin IIA (AMP attacinB homologue), dopa decarboxylase (melanisation pathway), dorsal (Toll pathway) and relish (Imd pathway) (Natori, 2010). Ten candidate reference genes (see chapter 3) were tested for their stable expression levels across all conditions (not injected, ISB injected and purified *E. coli* lysate injected pupae) with geNorm, resulting in two selected reference genes; EIF or eukaryotic translation initiation factor 1 and Ubq or ubiquitin

conjugating enzyme. Ct-values from the 6 tested immune genes of ISB injected pupae and not injected pupae were normalized and fold changes were calculated (figure 2A).

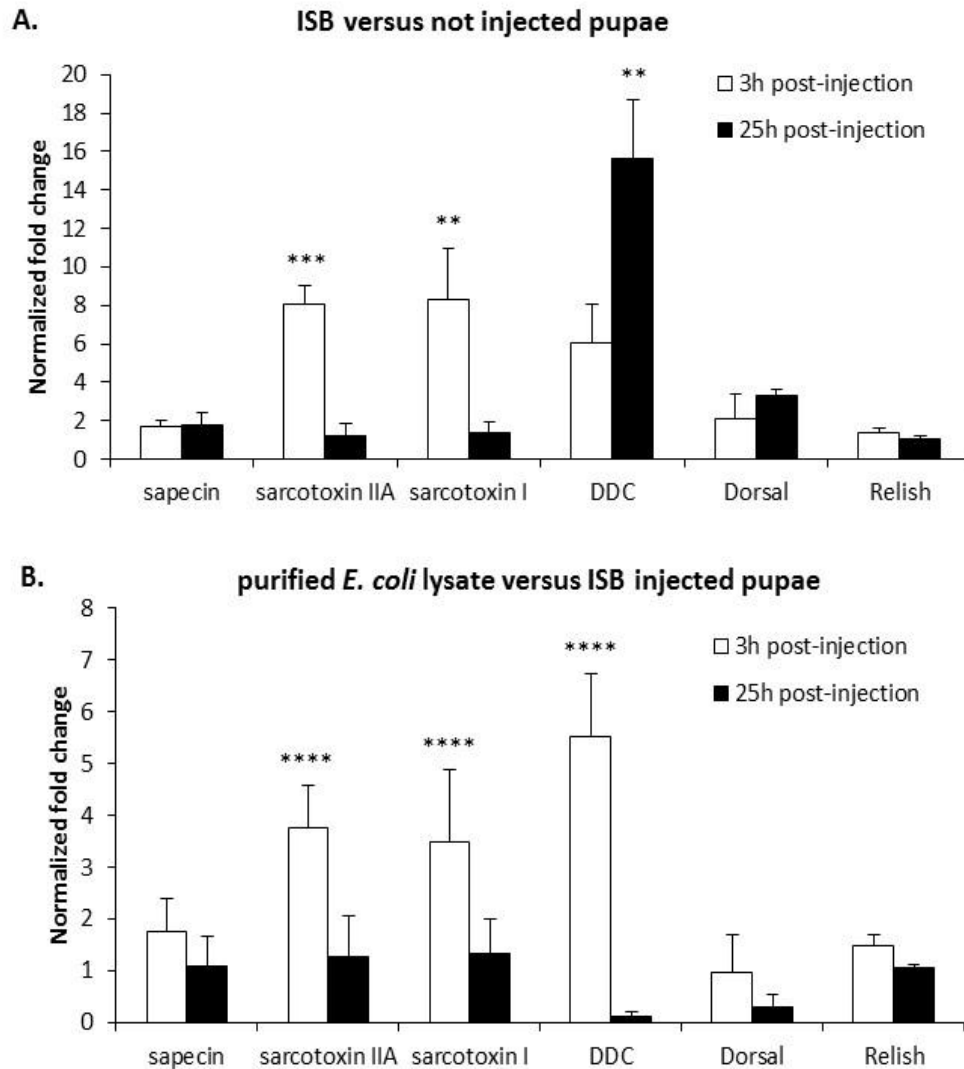


Figure 2: Normalized fold change of 6 immune genes in *S. crassipalpis* pupae. A. ISB injected pupae versus not injected pupae. B. Purified *E. coli* lysate injected pupae versus ISB injected pupae. White bars represent relative mRNA expression levels 3 hours post-injection, black bars 25 hours post-injection. Ubq and EIF were used to normalize the data. The data are expressed as the mean $\pm$ SD of three biological replicates. Statistical analysis on ISB injected compared to not injected (A), and on purified *E. coli* lysate injected compared to ISB injected (B), ** p 0.01, *** p 0.001, **** p 0.0001, ANOVA with Bonferroni posthoc test.

The injection of the saline solution showed an 8-fold increase in mRNA expression of the antimicrobial peptides sarcotoxin IIA and sarcotoxin I, 3 hours after the injection. These two

AMPs target Gram-negative bacteria and play a major role in the innate immunity (Natori, 2010). Their homologues in *D. melanogaster*, cecropin and attacin respectively, are very effective against Gram-negative bacteria, where attacin also targets fungi and Gram-positive bacteria (Lemaitre & Hoffmann, 2007). Sterile wounding and the injection of a saline solution in *Drosophila* cause melanotic spots at the site of the lesion shortly after the injection and trigger lamellocyte development as part of the cellular immunity (Markus *et al.*, 2005). Next to this, the mRNA level of the antimicrobial peptide drosomycin is up regulated after sterile wounding of *Drosophila* embryos (Stramer *et al.*, 2008).

The wounding of the *S. crassipalpis* pupae in the current experiment by a needle and the injection of ISB, without the presence of bacteria, already induces the expression of antimicrobial peptides. Twenty-five hours after the ISB injection, dopa decarboxylase (DDC) transcripts showed a 16-fold increase compared to not injected pupae. DDC converts dopa into dopamine, which is further metabolized to produce melanin and sclerotin in epidermal tissues. Needle injury to *Drosophila* larvae or adults induces a melanisation reaction at the wounding site, caused by de novo synthesis of melanin by phenoloxidase (PO) (Binggeli *et al.*, 2014). This enzyme catalyses the tyrosine to dopa conversion, that in turn, can be converted to dopamine by DDC, eventually leading to melanin production. The elevation of DDC in ISB injected *S. crassipalpis* can therefore be expected as sclerotisation spots were detected at the injection site of these pupae. These results indicate that the ISB injected pupae must be used as negative control for recombinant protein injections, since wounding and the injection of a 'harmless' saline solution already is a minimal and sufficient trigger for a humoral immune response in *S. crassipalpis* pupae.

When comparing expression levels of the same 6 genes from pupae injected with the purified *E. coli* lysate with pupae injected with ISB, a stimulation of the immune system can be noted 3 hours after injection (figure 2B). These changes cannot be attributed to wounding by needle insertion since the ISB injected pupae served as control group. Two AMPs, sarcotoxin I and sarcotoxin IIA, were triggered by the purified *E. coli* lysate. Since both AMPs are known to play a role in immunity against Gram-negative bacteria, we can conclude that probably still fragments of *E. coli* are present in the purified sample, resulting in the production of these AMPs in the injected pupae. DDC was also strongly up-regulated 3 hours

after injection. Because in *Drosophila* the melanisation cascade cooperates with the Toll pathway in promoting host defence, there is a possibility that the DDC expression is linked to the up-regulation of sarcotoxin I and IIA, explaining its induction 3 hours post-injection.

Only by looking at 6 important immune related genes, a significant effect of injecting the *E. coli* background solution into the *S. crassipalpis* pupae could be noted on the immune system. From chapter 3, we know that alterations in host immunity can be quite subtle and do not always involve large up or down regulations in mRNA expression levels. This means that when the recombinant protein still contains traces of bacterial fragments and in this way already causes a boost of immune processes in the flesh fly pupa, the potential changes caused by the recombinant protein will be overshadowed. To proceed with this approach, other purification steps need to be applied, suggested in the section 'Conclusions'. Since recombinant expression was already successfully performed for 10 out of the 23 novel venom proteins (data not presented), extra purification would be the logical next step. Next to this method to investigate functionalities of the venom proteins, a different approach was explored, described below.

3.2. Silencing of venom compound in *N. vitripennis* pupae

Instead of directly looking for effects in the host caused by an injected venom protein, the effect of normal parasitization but by a parasitoid lacking one (or more) compounds can also be a way to tackle functionality studies. This approach, by silencing a venom component in *N. vitripennis* and letting it parasitize its host, has the advantage that no large needles need to be inserted into the host, already causing an immune boost. In this way, the parasitization process can be kept as natural as possible, which was also the intention in the microarray experiment (see chapter 3). For this reason, silencing a venom protein in *N. vitripennis* is another way to explore its functionality. Of the 80 venom proteins discovered, no less than 23 venom proteins were found to have no similarity with any other protein. The complete lack of knowledge on the functionalities of this group of venom compounds had drawn our attention. But investigating the functions of 23 venom proteins seems an enormous task, therefore we started by optimizing the experiment with 2 of them. Instead of randomly pinning the tail on the donkey, we pinpointed 2 venom compounds purely by practical considerations, explained below.

3.2.1. Selection of venom compound

Since the protocol for pupal injection of dsRNA in *N. vitripennis* is well-documented, we decided to perform the silencing of venom compounds in the pupal life stage. In order to be successful, the mRNA expression of this specific venom compound may only be initiated after this developmental stage, more specifically the yellow pupa-stage. Otherwise, the mRNA that is already present before the silencing could give rise to the corresponding venom proteins through translation, making the following silencing ineffective. The expression profile of the 23 novel venom compounds was determined in 8 different *N. vitripennis* developmental stages via RT-qPCR, more specifically egg, white larva, brown larva, white pupa, yellow pupa, red-eyed pupa, black & white pupa, black pupa and adult. Ten candidate reference genes were tested for their stable expression level across all developmental stages with geNorm, resulting in the mean of 2 stable reference genes, 60S ribosomal protein L13a-like and elongation factor 1 α , as optimal normalization factor. mRNA expression levels were extrapolated into percentages, presenting 12 out of the 23 venom compounds that showed nearly complete mRNA expression (> 90%) during the adult stage (table 1).

The mRNA sequences of the 12 remaining venom compounds were individually blasted against the nucleotide database of *N. vitripennis* to check for sequence specificity. If the sequence of one gene shows overlap with sequences of other genes, the specificity of all produced siRNAs cannot be guaranteed. Sequences of venom proteins P, V and Z showed coverage with sequences from other genes, making them less suited for RNAi (table 2).

If several splice variants of a venom compound would be present in the venom reservoir, possibility exists that not all variants are successfully silenced by RNAi, still giving rise to functional venom compounds. Therefore, potential splice variants of the remaining 9 venom proteins in *N. vitripennis* female wasps was investigated. Therefore, extracted RNA from adult *N. vitripennis* females was converted into cDNA using a 3'RACE adapter that anneals to poly(A)tails. When using a gene-specific forward primer in combination with the 3'RACE adapter reverse primer, possible splice variants that mostly occur at the 3' region are picked up by PCR (figure 3). Next to this, PCR was also performed on cDNA from venom glands with CDS primers constructed on the coding sequence of the predicted sequences.

Exploring functionalities of novel venom compounds

Table 1: Percentages of relative mRNA expression levels of 23 novel venom compounds throughout *N. vitripennis* developmental stages (for egg stadium n=50, for other life stages n=20) (sum of percentages from all life stages equals 100%). The venom compounds that show more than 90% expression level in the adult stage are marked in grey.

venom compound	Relative mRNA expression level (%)								
	egg	white larva	brown larva	white pupa	yellow pupa	red-eyed pupa	salt & pepper pupa	black pupa	adult
VPD	1,10	0,73	1,38	9,78	13,94	4,76	6,97	5,53	55,81
VPE	6,91	9,13	27,63	0,12	0,11	0,12	0,24	0,43	55,31
VPF	1,84	1,86	3,67	16,36	14,69	14,28	29,39	14,35	3,56
VPG	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	99,98
VPH	0,00	0,00	0,00	0,01	0,01	0,01	0,02	0,02	99,93
VPI	0,04	0,32	0,31	0,01	0,04	0,01	0,04	0,01	99,22
VPJ	1,75	0,16	0,31	3,47	6,23	4,38	3,12	1,39	79,20
VPK	0,01	0,00	0,02	1,55	1,41	1,27	0,74	0,15	94,84
VPL	0,01	0,00	0,00	0,11	0,02	0,01	0,01	0,02	99,82
VPM	0,07	5,55	84,73	2,95	2,65	0,72	0,66	0,26	2,41
VPN	2,45	0,30	0,49	4,33	3,89	3,34	4,90	1,82	78,47
VPO	2,68	2,97	8,76	5,01	4,50	2,46	9,00	28,58	36,04
VPP	0,21	0,43	0,10	0,23	0,20	0,22	0,42	1,64	96,55
VPQ	0,02	0,07	0,39	0,00	0,00	0,00	0,00	0,01	99,52
VPR	1,17	7,64	46,23	8,11	2,89	1,56	8,26	18,35	5,78
VPS	0,68	9,87	86,49	0,07	0,07	0,05	0,04	0,03	2,70
VPT	0,01	0,01	1,30	0,72	0,41	0,42	0,65	13,11	83,36
VPU	0,47	0,20	0,38	1,67	0,47	0,41	0,13	0,09	96,18
VPV	0,00	0,00	0,04	0,00	0,03	0,00	0,00	0,01	99,91
VPW	2,70	1,25	0,95	8,16	12,02	16,41	7,57	2,80	48,13
VPX	0,04	0,02	0,04	0,43	0,16	0,17	0,19	0,31	98,64
VPY	0,01	0,00	0,00	0,01	0,00	0,00	0,00	0,01	99,95
VPZ	0,00	0,00	0,04	0,01	0,00	0,00	0,00	0,00	99,95

Table 2: Venom compounds that show sequence coverage with other *N. vitripennis* genes. Percentage over sequence coverage is presented, together with the percentage of similarity of the covering sequence (Identity). Last column presents the accession number of the gene that shows sequence coverage with the venom protein.

Venom compound	Sequence coverage	Identity	Description of covering gene	Accession Number
VPP	14%	100%	tubulin alpha-1 chain-like	XM_001606820
VPV	93%	82%	venom protein Z	NM_001161697.1
VPV	77%	79%	polyamine-modulated factor 1-binding protein 1-like	XM_003425717.2
VPZ	98%	82%	venom protein V	NM_001161569.1

Venom proteins H and U did not show any band when CDS primers were used, meaning that the 3' sequence anchoring the CDS reverse primer is different than what was predicted. Therefore, 3'RACE would need to be performed in order to gain their full sequence. Venom proteins G, I, L, Q and Y showed several splice variants. Venom protein K also shows one band around 700 base pairs, which is exactly double the size of the band around 350 base pairs. Sequencing data confirmed that this band represents a dimer of the VPK gene, and therefore will not pose a problem for silencing. This drop out race finally resulted in 2 venom proteins, VPK and VPX, that present good candidates for functionality research by gene silencing.

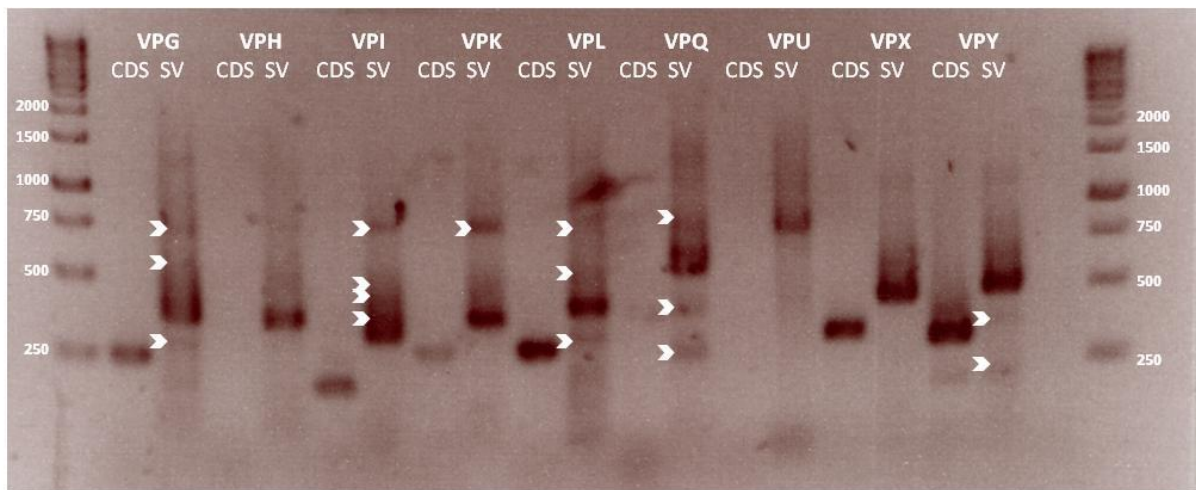


Figure 3: Splice variants of 9 novel venom proteins. 'CDS' represents transcripts of PCR performed with coding sequence primers of the predicted sequences, 'SV' represents transcripts of PCR performed with the coding sequence forward primer combined with the 3'-RACE primer on cDNA with the adapter attached to the poly(A)tail. White arrows show splice variants for the respective venom protein.

3.2.2. Experimental setting

N. vitripennis adults, immediately after host emergence, were placed individually in an egg-laying chamber (figure 4A). In this system, only the posterior region of the host pupa is accessible for the *N. vitripennis* female to parasitize, since venom injection in this side causes the best survival rate for the parasitoid offspring (Rivers & Denlinger, 1995). At specific time points (figure 4B) a flesh fly host, 5 days after pupation, is placed in the chamber so that the female wasp can inject venom and eggs into the host. In order to correlate the results of this experiment with the differentially expressed EST data from the microarray (see chapter 3)

the same experimental conditions were chosen. This means that only a 2 hour time frame was allowed for parasitization (except the first host, since it had solely feeding purposes). Twenty-four hours thereafter, the hosts were snap-frozen and stored in -80°C . The first hosts served as training, so that effective parasitization can be guaranteed in the final host during this short time frame. Since performing RT-qPCR on the dissected venom reservoir of a single *N. vitripennis* female is too time-consuming for the complete experiment, the abdomens of single parasitoids were snap-frozen and used for RNA extraction.

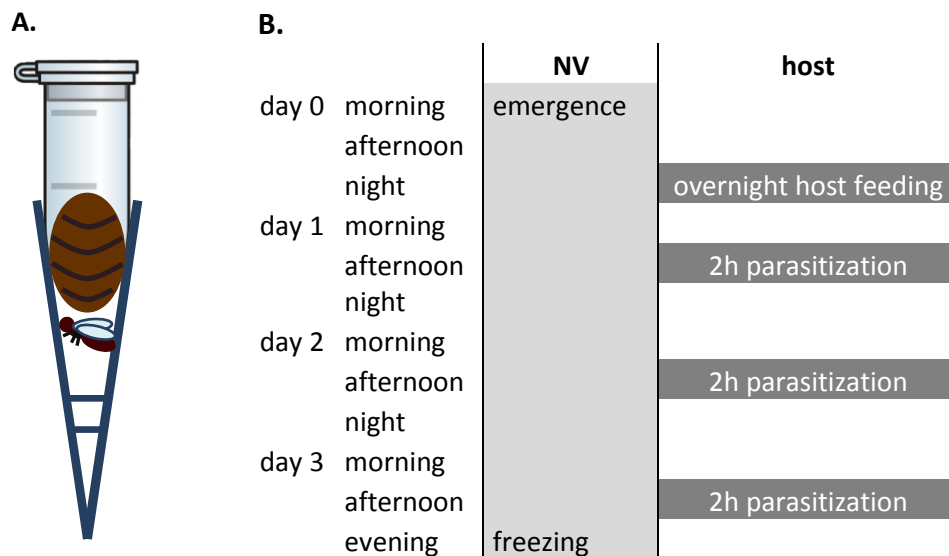


Figure 4: A. Schematic representation of an egg-laying chamber in which only the posterior part of the host is accessible for the parasitoid. A filter tip is on the bottom and an eppendorf tube with the bottom cut off is placed on top. The flesh fly pupa is placed with its posterior end downwards and a single *N. vitripennis* female is placed in the filter tip. B. Time schedule of the parasitization experiment. In light grey, the life span of the parasitoid is represented, in dark grey the placement of a host in the egg-laying chamber. 24 hours after the parasitization process, these hosts are snap-frozen and stored in -80°C .

Before starting the dsRNA injections, the practical setup was first put to the test by the control group of parasitoids that were not injected. Twenty *N. vitripennis* females, directly after emergence, were given hosts in the time-frame mentioned above (figure 4B). At the end of day 3, their abdomens were cut off and frozen. RNA was extracted and converted into cDNA. The expression profile of 5 venom compounds were determined via RT-qPCR: VPK, VPX and 3 other venom compounds that are expected to be abundantly present in the venom (aminotransferase-like venom protein 1 (aminoVP1), γ -glutamyl transpeptidase-like venom protein (glutVP1) and laccase). Again, the 10 candidate reference genes for *N.*

vitripennis (table S3) were tested for their stable expression level with geNorm. This resulted in the use of the geometrical mean of 2 reference genes, 60S ribosomal protein L13 α -like (RPL13a) and eukaryotic translation initiation factor 1 α (EF1a), as optimal normalization factor. The normalized relative mRNA expression levels of these 5 venom compounds for 18 single *N. vitripennis* females are presented in table 3.

Table 3: Normalized mRNA expression levels of 5 venom compounds for 18 not injected female *N. vitripennis* female wasps after several parasitizations on flesh fly hosts. For every venom compound, the 5 highest mRNA levels are marked in orange, the 5 lowest in green. (NV = *Nasonia vitripennis*)

NV female	VPK	VPX	aminoVP1	glutVP1	laccase
1	0.021	0.043	0.101	0.015	0.113
2	0.485	0.506	0.772	0.319	0.012
3	0.009	0.021	0.134	0.016	0.499
4	0.043	0.097	0.460	0.091	0.169
5	2.180	1.858	3.298	3.057	2.790
6	1.610	2.198	3.750	4.258	4.258
7	3.566	1.841	2.854	2.127	1.461
8	0.112	0.129	0.648	0.252	0.327
9	2.056	1.832	2.170	2.010	1.481
10	0.674	0.785	0.558	0.352	0.627
11	0.403	0.222	0.852	0.366	0.331
12	2.949	2.949	1.362	2.033	0.529
13	0.014	0.055	0.259	0.035	0.093
14	0.006	0.012	0.085	0.011	0.003
15	0.016	0.047	0.237	0.037	0.013
16	0.049	0.153	0.724	0.101	0.298
17	0.010	0.013	0.120	0.008	0.012
18	0.005	0.019	0.057	0.014	0.073

Large fluctuations in expression levels can be observed for the different samples for all 5 venom compounds. When the 5 highest (in orange in table 3) and the 5 lowest (in green in table 3) expression levels are marked, it is remarkable that a parasitoid has either low or high expression levels for all 5 tested venom compounds. Possibly, the amount of mRNA of venom compounds present in the parasitoid is linked with the amount of venom present in the venom reservoir. If we assume that a low mRNA level implies low amount of venom present in the reservoir, these females have injected their venom and have parasitized a host. Accordingly, high mRNA levels either imply that no parasitisation occurred or that new transcription of venom compounds has been performed after parasitization. Therefore, monitoring the reduction of transcript level after RNAi by RT-qPCR does not guarantee successful knock-down, since this *N. vitripennis* female can simply have an empty venom

reservoir due to successful parasitization. Snake venom revealed a clear dynamic in gene expression of newly synthesized venom compounds following venom depletion (Currier *et al.*, 2012). Since this occurs very rapidly, venomous snakes retain the ability to efficiently predate and remain defended from predators. In parasitoids, only the time-related expression of the venom protein acid phosphatase has been investigated by RT-PCR for the endoparasitoid wasp *Pteromalus puparum* (Zhu *et al.*, 2008). They showed expression of acid phosphatase immediately after emergence, but with low abundance. At 2 to 4 days, the transcript level increased and dropped after 4 days, but was still detectable with low abundance at 7 days. However, the expression level of parasitoid venom transcripts after replenishment of the venom reservoir is not known. Therefore, we investigated whether a similar dynamics of venom gene expression as seen in snakes, can be detected for venom proteins in *N. vitripennis*. Female *N. vitripennis* wasps were fed with sugar water until 6 days after emergence from the host. RNA was extracted from the abdomens of single wasps, converted into cDNA and RT-qPCR was performed (3 biological replicates and 3 technical replicates) for the same 5 venom components used in table 3. Since all 5 venom constituents showed a similar expression pattern, only the graph for aminoVP1 is presented (figure 5). Black bars show that gene expression peaks at 2 days after emergence and decrease after day 4, similarly as in *P. puparum*. A group of wasps that had been feeding on sugar water for 2 days, received a host (1 host per wasp in egg-laying chamber) at day 3 for the duration of 8 hours to parasitize. The following 3 days, they only received sugar water. The presence of wasp eggs was verified in the hosts in order to guarantee successful parasitization. The dotted line in figure 5 represents the moment of parasitization on the host. After that, the white bars show the expression of aminoVP1 in the following days (at day 3, sampling occurred immediately after the removal of the host), while the black bars represent pupae that were only fed with sugar. The instantaneous decrease in gene expression and the duration until at least 3 days after parasitization, shows that replenishment of the venom reservoir during parasitization enforces new transcription and translation of venom proteins.

Due to the relatively short parasitization periods used (figure 4), successful venom injection cannot be guaranteed at the end of this specific setup. The host that was presented to the wasps at day 1 hardly could have been successfully parasitized, since the venom compounds are only maximally transcribed 2 days after emergence. Female wasps with numbers 5, 6, 7,

9 and 12 that show high gene expression levels for at least 4 out of 5 tested venom compounds (see table 3), probably did not inject their entire venom stock into the hosts or did not parasitize the host at all and only ate on it. Wasps with the numbers 3, 14, 17 and 18 that show the lowest gene expression levels successfully parasitized at least one of the offered hosts, assuming that the low transcript levels correlate with low protein levels. The latter should, however, be verified by SDS-PAGE.

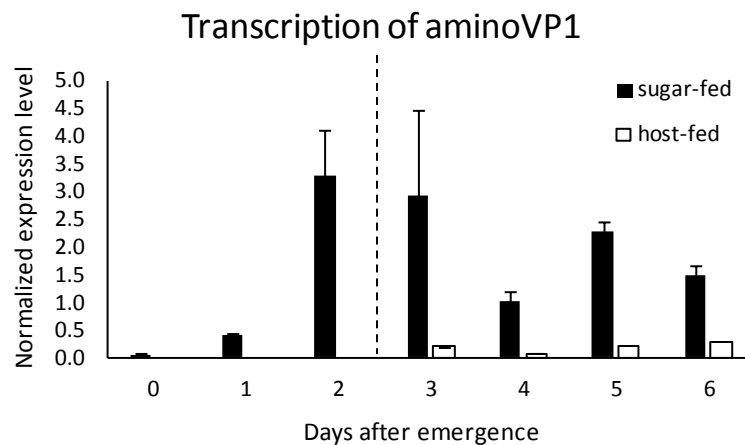


Figure 5: Expression of aminoVP1 tested by RT-qPCR, normalized with ETIF and RPL13a. The average of 3 biological replicates and 3 technical replicates is presented $\pm$ SD. Dotted line marks the moment of host feeding. This graph is representative for transcription of VPK, VPX, glutVP1 and laccase.

Large fluctuations in expression levels of venom compounds in the control group implies that the experimental setting for silencing needs to be altered. Unless gene transcription of venom compounds elevates again after parasitization (at least later than 6 days post emergence), successful silencing of venom compounds cannot be verified by RT-qPCR, since the control group will also show low expression levels due to emptying the venom reservoir during parasitization. When however, 100% successful silencing of venom compounds can be obtained, the control group of not injected wasps can be omitted. Unfortunately, due to shortness of time, the optimization of the RNAi experiment could not be performed before submitting the thesis.

4. Conclusion

The discovery of 23 completely novel venom proteins impelled us to explore the functionalities of this large group of components. Two different approaches were handled. First, recombinant expression of venom proteins in *E. coli* bacteria followed by host injection was assessed. The standard on-column purification with Profinity IMAC Ni-Charged Resin and subsequent buffer exchange was suggested. As a control, the empty vector was expressed in *E. coli* and purification was performed on the bacteria lysate. This sample was injected in *S. crassipalpis* pupae and RT-qPCR was performed on several immune genes in order to test the effect of this *E. coli* background sample on the host immunity. This resulted in significant up-regulation of half of the tested immune genes, which can overshadow possible effects of recombinant expressed venom proteins on the host immune system. Therefore, other or extra purification steps could be included to remove all bacterial rest fragments and obtain a sample with recombinant protein of extreme purity. A commonly used protocol for purification of novel protein samples is the combination of ion exchange chromatography to capture the protein, hydrophobic interaction chromatography as an intermediate purification, followed by gel filtration to polish the purified product (Recombinant Protein Purification Handbook, GE Healthcare).

Another practical adjustment to this experiment would be the use of automated injection equipment instead of Hamilton syringes. The Femtojet (Eppendorf), that is used for the injection of dsRNA in order to silence venom compounds in female *N. vitripennis* wasps, are used in combination with Femtotips that have a 0.7 µm outer diameter. The Hamilton needles, recommended for animal injections, are 0.16 mm in outer diameter, meaning that they can harm the delicate pupae much easier than the Femtotips. The use of the automated injection equipment could result in less melanisation spots appearing at the injection site, reducing the highly up-regulated expression of DDC after the injection of saline solution.

On the other hand, another expression system also belongs to the possibilities. For instance, other insect venom proteins have been successfully expressed by recombinant baculovirus infection in insect cells (e.g. Sf9 cells) (Seismann *et al.*, 2010; Van Vaerenbergh *et al.*, 2013). Even though venom proteins in *N. vitripennis* have been shown to be unglycosylated, insect

cells can perform other post-translational modifications, which is lacking in the bacterial expression system.

The second approach used to explore the functionalities of *N. vitripennis* venom proteins, consisted out of silencing the compound in the female wasp by RNAi followed by bioassays on parasitized hosts. By performing the preliminary experiments described in Results and Discussion, several focus points still need to be addressed. Apparently, the developmental stage of *N. vitripennis* for injection of the dsRNA is crucial for successful silencing of the targeted gene. When injecting dsRNA in the yellow pupal stage of *N. vitripennis* for a target component that is only expressed 2 days after emergence, the gap of 5 to 6 days may cause the injected dsRNA to be broken down until transcripts of the target gene appear. Therefore, for each venom compound individually, the exact timing of dsRNA injection needs to be defined, since their expression differs among each other. Larval RNAi in *N. vitripennis* has already been described (Werren *et al.*, 2009) and in the Centre for Ecological and Evolutionary Genetics in Groningen, embryonic injections are in trial. In an attempt to assess the amount of venom transcripts in the female wasp after parasitization, it became clear that replenishment of the venom reservoir also involves new transcription of the venom compounds next to translation in order to refill the reservoir. When 100% successful silencing can be guaranteed, there is no longer need to control the effectiveness of the injected dsRNA. Then, successful parasitization by the silenced wasp, however, needs to be verified which can be done by opening the puparium of the host and scavenging for *N. vitripennis* eggs. Preliminary experiments for silencing venom compounds have now been performed and the adjusted practical setting can be put to the test. The technique of injecting dsRNA into the pupal stage of *N. vitripennis* was acquired during a 4-week internship at the Centre for Ecological and Evolutionary Genetics in Groningen and can therefore, readily be applied at the Laboratory of Molecular Entomology and Bee Pathology in Ghent.

Both approaches had some road blocks along the way and due to shortness of time, they could not be solved by the end of this dissertation. Several solutions have been suggested and both recombinant expression as well as silencing of *N. vitripennis* venom compounds still belong to the possibilities of investigating their functionalities.

5. Supplementary materials

Table S1: Primer sets developed to amplify transcripts by PCR that contain the coding sequence of the corresponding venom proteins in *N. vitripennis*. (VP = venom protein)

Gene	Primer sequence	Amplicon length
VPG_CDS	5'-GAATTGACCGAGGAAGACGG-3' 5'-TTAATTCTTCTGGTTCTCGGC-3'	243
VPH_CDS	5'-GAACCTTCGCTTGAATCGCA-3' 5'-TTAAGTTAATTTAACTCCTTGAGT-3'	252
VPI_CDS	5'-ATGTACCTAACTCCTCTGTGT-3' 5'-TTAGCCAACAGCATTGTAAAAAG-3'	189
VPK_CDS	5'-CAAGAGATTAAATTTACAGATGAG-3' 5'-TTATTGATCATCGTTTCAATCG-3'	282
VPL_CDS	5'-CAAGACATAGAAGATGACGAC-3' 5'-TTAAGCTGCGGAACCGTGT-3'	288
VPQ_CDS	5'-AAACCAACGGCAGATGTTGAG-3' 5'-TCACAGCTGCTTCTGTTGTTT-3'	441
VPU_CDS	5'-ATGAACTAATATTCACAACCTA-3' 5'-TTAAGCTTCCAAACAAGCCTTT-3'	621
VPX_CDS	5'-CAAGTTTATAATTTTTTTCG-3' 5'-TTAATTCTCGGAGGACTTGATG-3'	334
VPY_CDS	5'-ATGAAAGCCTTTACCGTGCTA-3' 5'-TTACTGGCTGTGATATATATTCC-3'	312

Table S2: Primer sets developed to amplify transcripts by RT-qPCR (80 to 120 base pairs long) of venom proteins in *N. vitripennis*. (VP = venom protein)

Primer name	Primer sequence	Accession	Gene name
VPD_qPCR	5'-GATGTCGATATTGCATTGTCAG-3' 5'-GTTCTTGTCCTGACTTCAG-3'	NM_001161699.1	venom protein D
VPE_qPCR	5'-AGTAACACCAATCGACTTCTACAG-3' 5'-CGTTCAGTTCAATTCTTCCGT-3'	XM_001603388.2	venom protein E
VPF_qPCR	5'-GGCAATGATGGACAGAAGGA-3' 5'-AGGTATCTCAGTAACTAACGACAG-3'	NM_001161688.1	venom protein F
VPG_qPCR	5'-AATTGACCGAGGAAGACGGA-3' 5'-CGCTGTTAACGTTGTACAC-3'	XM_008205495.1	venom protein G
VPH_qPCR	5'-CAACACAGCAAACATGAAGG-3' 5'-TATGCGATTCAAGCGAAGGT-3'	NM_001161555.1	venom protein H
VPI_qPCR	5'-CCTGCTCTATAGTTTGATTGAAGC-3' 5'-TGTCTGCTCTTGATTGTAACG-3'	NM_001170875.1	venom protein I
VPJ_qPCR	5'-CTCATTTCTTGTTTCAGTCGCC-3' 5'-CACTGGATTGGCTTCTTCGT-3'	NM_001170876.1	venom protein J
VPK_qPCR	5'-GAAATCGACAAATTAGTCGCTGAG-3' 5'-GAAGATCATCTCGCAAAGTTGC-3'	NM_001161556.1	venom protein K
VPL_qPCR	5'-GAAGAACAAGTCCAGAAGAACC-3' 5'-TCATAATTAACAGGCAGACGG-3'	NM_001161557.1	venom protein L
VPM_qPCR	5'-GTACTCATCGTCTGCCTCCT-3' 5'-GCCATCCTTGCAAGTAGTAACC-3'	NM_001161558.1	venom protein M
VPN_qPCR	5'-ATCAACGATGCCCTCAAGGA-3' 5'-GTACAATTGGTTCTCACAGGTGG-3'	NM_001170878.1	venom protein N
VPO_qPCR	5'-CTCATCAGCAAGAGAATCCAG-3' 5'-CTAAGGTCTAGTCCAGTTGGTC-3'	NM_001161559.1	venom protein O
VPP_qPCR	5'-TGGTTCACGATATCTTAAAGGCAG-3' 5'-TAGTTTCCCGTCATCATCTGGT-3'	XM_001606115.2	venom protein P
VPQ_qPCR	5'-GTACTCTGATACTGGCCAAACC-3' 5'-GAGCTCCACCAGTTCATTACC-3'	NM_001161689.1	venom protein Q

VPR_qPCR	5'-CTACCTCATCACTCAGATCCGT-3' 5'-GACATTGCTGTAGCCCACTC-3'	NM_001161692.1	venom protein R
VPS_qPCR	5'-TGCTCGAGAATTAGACGACTG-3' 5'-CGAGAAAAGCTTCCTCACAAAGAC-3'	XM_001606782.2	venom protein S
VPT_qPCR	5'-CGAATACAAGACCCACATCG-3' 5'-TTTCGACCAGATCTTGACAGG-3'	NM_001161694.1	venom protein T
VPU_qPCR	5'-GCCTAGGAATGTCTAATTTGGA-3' 5'-TATTCCACATCTCGTTGAACAC-3'	NM_001161698.1	venom protein U
VPV_qPCR	5'-GAAATTCAACGAGTTCGTCGTG-3' 5'-TTTGTCTTAACGTTCTGGCC-3'	NM_001161569.1	venom protein V
VPW_qPCR	5'-AGAATCCAGTGACGAAGTAGTCC-3' 5'-TGGTGATTGCGCCGATTCTG-3'	XM_001602674.2	venom protein W
VPX_qPCR	5'-CGGTGAAGCTTTTCGATCAAT-3' 5'-TTGATGGGTGGTTCATCAGA-3'	NM_001161695.1	venom protein X
VPY_qPCR	5'-TTATGTTCAATGGCGGTTCA-3' 5'-CATAAAATGGCGGCAACTCT-3'	NM_001161696.1	venom protein Y
VPZ_qPCR	5'-CAATCCAAATTACGGGTTTCGC-3' 5'-GGATACTTTGTCTTCAAGTTGTG-3'	NM_001161697.1	venom protein Z
aminoVP1_qPCR	5'-CTATGAAATTGACGAAGACTAGCG-3' 5'-TCTTGTGTTAAAGAGGCTCCT-3'	XP_001607226	aminotransferase-like VP1
glutVP1_qPCR	5'-ATTAACCATATTCTTGGCGCAC-3' 5'-CTCAGAAGGTTCAACCAGGA-3'	XP_001607488	γ -glutamyl transpeptidase-like VP1
laccase_qPCR	5'-CGAATTATATCCAGTACATCGCAC-3' 5'-TCCTTATTGTATCCGCTCTCAC-3'	XP_001600917	laccase

Table S3: Primer sets developed to amplify transcripts by RT-qPCR of the candidate reference genes for *N. vitripennis* (transcripts of 80 to 120 basepairs long).

Primer name	Primer sequence	Accession number	Gene name
actin_qPCR	5'-GCATCACACCTTCTACAACGA-3' 5'-ATCTGGGTCATCTTCTCACGG-3'	XM_001601454.3	actin-3-like
tubulin_qPCR	5'-GCTATCGCAACCATCAAGACC-3' 5'-GTAGTTGATACCGACCTTGAACC-3'	XM_001607649.3	tubulin alpha-1 chain
RPL13a_qPCR	5'-CTATCATCGCCAAGACCATCC-3' 5'-AGCTTGTTCTGAAGAAGTTACC-3'	XM_008215505.1	60S ribosomal protein L13a
nanos_qPCR	5'-ATTTGCGACTGATGACGTCTG-3' 5'-CCGGTACGATCACTTCATCTG-3'	NM_001134922.1	nanos
RPS18l_qPCR	5'-AAATACTCACAGCTTACGAGTTCC-3' 5'-TAGTGTCGGAGACCTCTGTG-3'	XM_008215790.1	40S ribosomal protein S18
GAPDH_qPCR	5'-GCTTATATCGCCGCTTTGTG-3' 5'-GTTTGTGTTAGACGGAGACGA-3'	XM_001607424.3	glyceraldehyde 3-phosphate
SDHA_qPCR	5'-TGCTGGATCTGGTTGTATTTGG-3' 5'-CTTCTCCAGCGTTAGTTCCG-3'	NM_001172330	succinate dehydrogenase
EF1a_qPCR	5'-CACTTGATCTACAAATGCGG-3' 5'-GAAGTCTCGAATTCCACAG-3'	NM_001172756	elongation factor 1-alpha
ETIF1a_qPCR	5'-CGAAGAATAAGGGAAAGGGAG-3' 5'-GCCATCCTCTTGAAGACCA-3'	XM_008206136.1	eukaryotic translation
Med8_qPCR	5'-CATCTCATAAACAGGTAGCACAG-3' 5'-TTTCACTTTCCCACTCTTCAC-3'	XM_003427117	mediator of RNA polymerase

Exploring functionalities of novel venom compounds

Table S4: Primer sets developed to amplify transcripts by RT-qPCR of immune proteins in *S. crassipalpis*.

Gene name <i>S. crassipalpis</i>	Accession number	Gene name <i>D. melanogaster</i>	Accession number	% identity	e-value	Primer sequences
sapecin	EZ598683.1	defensin	NP_523672.1	67.39	1.00E-15	5'-CCTGTTGAAGCTGCTGAGGA-3' 5'-TACGCAGACACCTTTGCCAT-3'
sarcotoxin IIA	EZ599257.1	attacin B	NP_523746.1	65.71	8.00E-66	5'-CAGCAGCAAAAACCTTGTTCCA-3' 5'-TCCTCCACCCACTAGACCAC-3'
sarcotoxin I	EZ603663.1	cecropin A1	NP_524588.1	78.00	2.00E-18	5'-CAACCAACCAGCCTGACTTT-3' 5'-GCGGGGAGCATCATTTACT-3'
DDC	EZ600971.1	DDC	AAC67582.1	85.57	4.00E-143	5'-ATCCATTTGTGGGGATTGAA-3' 5'-GTCAGTGCCTGCTGATGAAA-3'
Dorsal	EZ615368.1	Dorsal	P15330.2	83.87	2.00E-37	5'-CCACCTCTTCGGGTGTTAAA-3' 5'-ATGTTGATTTGACCGAAGCC-3'
Relish	EZ604192.1	Relish	NP_996187.1	48.39	2.00E-35	5'-GCTCACAACAGCATTGCAT-3' 5'-ATATGTAGCGCGGTATTGCC-3'

PART III

BIOMEDICAL APPLICATION OF *NASONIA* *VITRIPENNIS* VENOM

CHAPTER 4

How the venom from the ectoparasitoid wasp *Nasonia vitripennis* exhibits anti-inflammatory properties on mammalian cell lines

Personal contribution:

- Cell cultures
- Venom dissections
- Sample preparation
- RT-qPCR
- Reporter gene analysis and ELISA
- Western blot analysis
- Immunofluorescence staining
- Functional analysis of the dataset
- Writing the manuscript

Published in PLoS ONE in 2014 as:

How the venom from the ectoparasitoid wasp *Nasonia vitripennis* exhibits anti-inflammatory properties on mammalian cell lines.

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1. Abstract

With more than 150,000 species, parasitoids are a large group of hymenopteran insects that inject venom into and then lay their eggs in or on other insects, eventually killing the hosts. Their venoms have evolved into different mechanisms for manipulating host immunity, physiology and behavior in such a way that enhance development of the parasitoid young. The venom from the ectoparasitoid *N. vitripennis* inhibits the immune system in its host organism in order to protect their offspring from elimination. Since the major innate immune pathways in insects, the Toll and Imd pathways, are homologous to the NF- κ B pathway in mammals, we were interested in whether a similar immune suppression seen in insects could be elicited in a mammalian cell system. A well characterized NF- κ B reporter gene assay in fibrosarcoma cells showed a dose-dependent inhibition of NF- κ B signalling caused by the venom. In line with this NF- κ B inhibitory action, *N. vitripennis* venom dampened the expression of IL-6, a prototypical proinflammatory cytokine, from LPS-treated macrophages. The venom also inhibited the expression of two NF- κ B target genes, I κ B α and A20, that act in a negative feedback loop to prevent excessive NF- κ B activity. Surprisingly, we did not detect any effect of the venom on the early events in the canonical NF- κ B activation pathway, leading to NF- κ B nuclear translocation, which was unaltered in venom-treated cells. The MAP kinases ERK, p38 and JNK are other crucial regulators of immune responses. We observed that venom treatment did not affect p38 and ERK activation, but induced a prolonged JNK activation. In summary, current data indicate that venom from *N. vitripennis* inhibits NF- κ B signalling in mammalian cells. We identify venom-induced up regulation of the glucocorticoid receptor-regulated GILZ as a most likely molecular mediator for this inhibition.

2. Introduction

Animal venoms have long been known for their inflammatory effects, for instance stings from honeybees, snakes and scorpions can induce ongoing pain and even hyperalgesia (Chen *et al.*, 2006; Liu *et al.*, 2007; Teixeira *et al.*, 2009). But the last few decades, there has been growing interest in the anti-inflammatory effects of these venoms. Since Billingham and colleagues have discovered the anti-inflammatory effects of honeybee venom, it has been used for the treatment of various inflammatory diseases in the oriental medicine (Billingham *et al.*, 1973). With the aim of finding treatments for several chronic inflammatory diseases (as rheumatoid arthritis and atherosclerosis) and cancer, venom components in diverse animal groups have been screened for possible anti-inflammatory characteristics (Park *et al.*, 2008a; Tsai *et al.*, 2007; Dkhil *et al.*, 2010).

In the search for animal venom-derived immune suppressive agents, the parasitoid-host interaction is a highly intriguing system. When parasitoid wasps lay their eggs in or on a host organism (endo- or ecto-parasitoid respectively), they also inject a mixture of virulence factors that consist of ovarian and venom fluids (Asgari & Rivers, 2011). These parasitoid fluids comprise of different active substances, exerting a large range of activities in diversified biological functions (Moreau & Guillot, 2005). This complexity could allow for adaptation for new or widened host ranges and could increase the difficulty for a resistance to emerge considering that a multimodal tolerance might be set up by the host to ensure its survival. The diverse mode of action of venom proteins from parasitoid wasps is dependent largely on their life strategy (Asgari & Rivers, 2011). In ectoparasitoids, like for instance *N. vitripennis*, the host generally undergoes a developmental arrest and immune suppression in order to present a good environment for the development of the parasitoid offspring (Danneels *et al.*, 2010; Small *et al.*, 2012). The precise working mechanism of this immune suppression is not yet exactly characterized. But the polydnviruses (PDVs) present in the venom of several endoparasitoid wasp species interfere with the Toll signalling pathway, which is crucial for maintaining the host immune response (Gueguen *et al.*, 2013).

Toll/Imd receptors are essential for embryonic development and immunity of insects (Valanne *et al.*, 2011). The induction of the Toll/Imd pathway by Gram-positive bacteria and

fungi or Gram-negative bacteria, respectively, leads to the activation of cellular immunity as well as the systemic release of certain antimicrobial peptides. Studies in fruit flies and in mammals have revealed that the defensive strategies of invertebrates and vertebrates are highly conserved at the molecular level (Kimbrell & Beutler, 2001). Infectious microorganisms trigger activation of Toll/Imd receptors in insects or Toll-like receptors in mammals, which induces a signalling cascade culminating in the activation of NF- κ B transcription factors (Silverman & Maniatis, 2001). NF- κ B is the generic term for members of a family of transcription factors, that act as homo- or heterodimers and regulate transcription of multiple genes involved in immunity and inflammation. The NF- κ B family in insects contains three members (Dif, Dorsal and Relish) and has expanded in mammals, which have seven NF- κ B family members (p50, p105, p52, p100, p65/RelA, RelB and c-Rel). Different insults trigger the activation of different NF- κ B dimers, which allows fine-tuning of the immune response (Hayden & Ghosh, 2012). Importantly, the NF- κ B pathway is subject to tight feedback regulation, which ensures termination of NF- κ B responses once the infectious danger has been eliminated (Ruland, 2011). NF- κ B is kept inactive in resting cells by the inhibitor protein Cactus in insects and by I κ B family members in mammals. When cells sense infectious danger, kinase cascades are activated that promote phosphorylation and degradation of the NF- κ B inhibitor proteins, leading to NF- κ B release and translocation to the nucleus, eventually activating the transcription of target genes involved in inflammation (Brasier, 2006; Hayden & Ghosh, 2011).

Whole genome microarrays on parasitized *D. melanogaster* by *Leptopilina* species revealed gene activation of Toll/NF- κ B and JAK/STAT pathway components, involved in regulating immune responses toward microbes and macroparasites. The up-regulation of genes involved in these particular immune pathways suggests these hosts are better protected against microorganisms at parasitoid oviposition (Schlenke *et al.*, 2007). In endoparasitoid venoms, the PDVs encode proteins with ankyrin repeats that are also found in Cactus, the inhibitor protein of NF- κ B signalling in *Drosophila*, and in mammalian I κ B family members. These viral ankyrin repeats (vankyrins) however, lack the amino acid sequences necessary for their degradation/turnover. Therefore, they act as antagonists of NF- κ B nuclear translocation, hence supporting parasite success. During the last decade, several PDVs in endoparasitoids have been found to express I κ B-related vankyrin genes that suppress NF- κ B

activity during immune responses in parasitized hosts (Kroemer & Webb, 2005; Bae & Kim, 2009).

In mammalian organisms, the nuclear factor kappa B (NF- κ B) transcription factors regulate important physiological processes, including inflammation and immune responses (Yamamoto & Gaynor, 2001). Maintenance of appropriate levels of NF- κ B activity is a critical factor in immune system development and normal cell proliferation. Aberrant activation of the NF- κ B pathway is involved in the pathogenesis of a number of human diseases including those related to inflammation, enhanced cellular proliferation, viral infection and genetic diseases (DiDonato *et al.*, 2012; Kumar *et al.*, 2004). Asthma for instance is a chronic inflammation of the bronchial tubes, of which the activation of NF- κ B is stimulated by agents such as allergens, ozone and viral infections (Christman *et al.*, 2000). NF- κ B also participates in many aspects of oncogenesis, since it can suppress apoptosis and induce expression of proto-oncogenes. The constitutive NF- κ B activity has been observed in a number of human cancers and the inhibition of NF- κ B abrogates tumour cell proliferation (Bharti & Aggarwal, 2002). Regulation and control of NF- κ B activation can be a powerful therapeutic strategy for inhibiting tumour growth and viral infections and for reducing the tissue damage that follows the release of inflammatory mediators.

Various stimuli give rise to the phosphorylation and degradation of the inhibitor of NF- κ B (I κ B) via the I κ B kinase (IKK) complex, leading to NF- κ B release and translocation to the nucleus, eventually activating the transcription of target genes involved in inflammation (Brasier, 2006). On the other hand, activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK), is another major signal transduction pathway involved in inflammation and is closely linked to the activation of the NF- κ B pathway (Guha & Mackman, 2001). It is clear that deregulation of the transcription factor NF- κ B can mediate several inflammatory diseases. Therefore several proteins tightly control its activation (Wong & Tergaonkar, 2009). Among these proteins are the NF- κ B inhibitory proteins, I κ B α and A20, the expression of which leads to a negative-feedback response that terminates activation of NF- κ B (Lee *et al.*, 2000). Moreover, the NF- κ B inflammatory response is also modulated by endogenous glucocorticoids (GCs) that are released following a challenge with various cytokines, with the

aim to restore homeostasis. These GCs are steroid hormones that can diffuse freely across the plasma membrane and bind to their intracellular receptor, the glucocorticoid receptor (GR). Because of their strong anti-inflammatory characteristics, GCs have been used since the late 1940s to treat inflammatory and autoimmune disease conditions (Schacke *et al.*, 2007).

N. vitripennis is an ectoparasitoid wasp that prefers *Sarcophaga* flesh flies as host organism. Bioassays uncovered that the venom of this wasp causes developmental arrest, increase of lipid levels, induction of apoptosis in certain tissues and suppression of the host immune system (Rivers & Denlinger, 1994a; Rivers *et al.*, 2002b). In contrast with several endoparasitoid wasps, *N. vitripennis* only injects venom and no PDVs into the host and therefore cannot express I κ B-related vankyrin genes. Interestingly, microarray analysis on parasitized *S. crassipalpis* pupae by *N. vitripennis* suggested that the venom also targets the NF- κ B and MAPK pathways in the host in order to regulate the immune response (Danneels *et al.*, 2013). Since conserved parallels have been noted between the inflammatory Toll/I κ m pathways of *Drosophila*, and immune signalling pathways that activate NF- κ B in mammals, we have investigated whether venom from *N. vitripennis* modulates NF- κ B activation in mammalian cells.

Using a well characterized NF- κ B reporter gene assay in fibrosarcoma cells (Plaisance *et al.*, 1997), we found that *N. vitripennis* venom, at subcytotoxic doses, inhibits NF- κ B activity. In addition, we found that *N. vitripennis* venom, in murine macrophage-like Raw264.7 cells, inhibited LPS-induced expression of the pro-inflammatory NF- κ B target Interleukin-6 (IL-6). The findings suggest that the venom-induced up-regulation of GILZ, a GR-regulated gene, is most likely the molecular mediator for this inhibition. One has to keep in mind, however, that this venom presents a highly complex mixture that, besides the 79 proteins identified (de Graaf *et al.*, 2010), consists of organic molecules, amines, salts, minerals and alkaloids (Wong & Belov, 2012). The unique find that the venom of an ectoparasitic wasp is able to inhibit one of the most important immune pathways in mammals, focused further research activities in unravelling the effect of the venom on this pathway in depth. This innovative study therefore, explored not only the effect of *N. vitripennis* venom on the canonical NF- κ B

pathway, but also the effect on the MAPK signalling pathways and negative regulatory mechanisms of NF- κ B activation.

3. Materials and methods

3.1. Isolation of crude wasp venom

N. vitripennis wasps were reared on pupae of the flesh fly, *S. crassipalpis*, and maintained at 25°C with a daily 16:8 light : dark cycle. Female wasps were allowed to host feed on flesh fly pupae for 24 hours. Venom gland reservoirs were dissected into insect saline buffer (ISB) [150 mM NaCl, 10 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 10 mM Hepes] (Formesyn *et al.*, 2013a) and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant containing the venom was transferred to a clean microcentrifuge tube and stored frozen at -70°C. Total protein in crude venom was determined colorimetrically at 595 nm using a Coomassie Protein Assay Reagent (no 23200, Thermo Scientific).

3.2. Chemicals

The origin and activity of TNF, as well as the preparation of luciferase (luc) reagent, were described previously (Vanden Berghe *et al.*, 1998). Dexamethasone (DEX), LPS from *Escherichia coli*, mouse anti-tubulin- α and the specific GR antagonist RU38486 were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies to rabbit I κ B α (C-21), mouse A20 (A-12), rabbit PARP-1/2 (H-250 and rabbit NF- κ B p65 (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to rabbit phospho-IKK α (ser180)/(ser181) β , rabbit phospho-NF- κ B p65(ser536), rabbit phospho-ERK MAPK and rabbit phospho-p38 MAPK were purchased from Cell Signalling Technology (Danvers, MA, USA). Corresponding fluorescent-coupled secondary antibodies were purchased from Rockland, Gilbertsville, PA.

3.3. Cell culture

Raw264.7 cells, a mouse macrophage-like cell line, were maintained in RPMI 1640 medium, murine fibrosarcoma L929sA cells and human embryonic kidney (HEK) 293T cells were maintained in DMEM at 37°C in 5% CO₂ humidified air. Both media were supplemented with

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10% fetal bovine serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Twenty-four hours before induction, cells were seeded in multiwell dishes such that they were confluent at the time of the experiment.

3.4. Plasmids and transfection procedure

pGal4, pGal4-p65 and p(Gal)₂-50hu.IL6P-luc+ were previously described (Schmitz & Baeuerle, 1991). pPGKβgeobpA was a gift from P. Soriano (Fred Hutchinson Cancer Research Center, Seattle). p(IL6κB)₃50hu.IL6P-luc+ (Plaisance *et al.*, 1997) and p(GRE)₂-50-luc (De Bosscher *et al.*, 2005) were described previously. Transient transfections were performed using polyethylenimine (PEI) (Longo *et al.*, 2013). Stable transfection was performed by the calcium phosphate precipitation procedure according to standard protocols (Vanden Berghe *et al.*, 1998).

3.5. Reporter gene analysis

Luciferase (luc) and galactosidase reporter assays were carried out according to the manufacturer's instructions (Promega) and have been described previously (Vanden Berghe *et al.*, 1998). Luc activity, expressed in arbitrary light units, was corrected for differences in protein concentration and transfection efficiency in the sample by normalization to constitutive β-gal levels. B-gal levels were quantified with a chemiluminescent reporter assay Galacto-Light kit (TROPIX).

3.6. Enzyme-linked immunosorbent assay of IL-6

Murine IL-6 ELISA was performed using the Mouse IL-6 CytoSet from Invitrogen.

3.7. Western blot analysis

After inductions (indicated in the figure's legends) or solvent controls where appropriate, cells were washed with ice-cold phosphate-buffered saline (PBS), harvested with a rubber policeman and collected in 1 ml of PBS by centrifugation for 10 minutes at 500 g (4°C). Preparation of cytoplasmic or nuclear cell extracts have been described previously (Plaisance

et al., 1997). If total cell lysates were used, cells were lysed in SDS sample buffer [62.5 mM Tris-HCl, pH 7.5, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol (DTT) and 0.01% (w/v) bromophenol blue]. To shear DNA and reduce sample viscosity, lysates were sonicated for 1 minute in a water bath sonicator and then heated to 95°C for 5 minutes after which they were immediately cooled in ice and microcentrifuged for 5 minutes. The lysates were separated by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Blots were probed using the appropriate antibodies (1:1,000), and their corresponding immunoreactive protein (1:10,000) was detected on an Odyssey imaging system (LI-COR Biosciences, Lincoln, USA).

3.8. RNA analysis

RNA was isolated by using TRIzol Reagent (Invitrogen) as described previously (De Bosscher *et al.*, 2005). Total RNA was reverse-transcribed into cDNA using the Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas) following the manufacturer's instructions. The expression of the genes was determined by RT-qPCR in a CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA, USA) using the SYBR Green mastermix (Invitrogen). The mouse-specific primers that were used, are mentioned in table S1, together with the primers of the household genes, used for normalization. The thermal cycling conditions were as follows: 5 minutes at 50°C, 2 minutes at 95°C and then 40 cycles of 20 seconds at 95°C and 40 seconds at 60°C. Melting curve analysis was used to determine primer specificity.

3.9. Immunofluorescence staining

Cells were seeded on coverslips and grown for 24 hours. Fixation, permeabilization, and staining were performed as described previously (De Bosscher *et al.*, 2005). Presence of p65 was visualized with a 1:200 dilution of anti-p65 antibody, followed by probing with a 1:800 dilution of Alexa Fluor 488-conjugated goat anti-rabbit IgG. Staining with 4,6-diamidino-2-phenylindole (DAPI) was used for visualization of the cell nuclei. A Zeiss Axiovert 200M microscope was used for visualization of the images, and data were analyzed using Carl Zeiss Axiovision software (Carl Zeiss Instruments, Jena, Germany).

3.10. Statistical analysis

All assays were carried out in triplicate from three biological repeats and results were expressed as the mean $\pm$ SDs. Prior to statistical tests, normality of the data was confirmed by Shapiro-Wilk tests. With this assumption met, data was evaluated using analysis of variance, ANOVA followed by Dunn's or Bonferroni posthoc test. P-value < 0.05 was considered statistically significant. All statistical analyses were performed with Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

4. Results

4.1. Effect of venom on NF- κ B signalling

4.1.1. Venom inhibits NF- κ B activation in TNF-stimulated L929sA fibrosarcoma cells.

To assess the effects of *N. vitripennis* venom on NF- κ B activity, we used a well characterized NF- κ B reporter gene assay (Plaisance *et al.*, 1997). Briefly, the system makes use of L929sA cells stably transfected with a recombinant promoter with an NF- κ B-responsive element derived from the IL-6 gene promoter (p(IL6 κ B)₃50hu.IL6P-luc+), and a constitutively expressed reporter gene construct (pPGKbGeobpA), expressing β -galactosidase (Vanden Berghe *et al.*, 1998). L929sA cells respond to TNF, followed by an induction of the NF- κ B-reporter gene. Only subcytotoxic concentrations of venom were used on the cells (see figure S1 for MTT cell viability assay). As shown in figure 1, enhanced luciferase expression levels were measured in response to TNF, whereas pre-treatment with venom was found to potently inhibit reporter gene expression in a dose-dependent manner (see figure S2 for the separate luciferase and β -galactosidase read-outs of the NF- κ B-reporter assay). Similar assays were performed on L929sA cells stably transfected with a recombinant promoter with either the CRE (cyclic AMP response element) responsive element or the AP-1 responsive element, which confirmed the specificity of the venom towards NF- κ B (see figure S3).

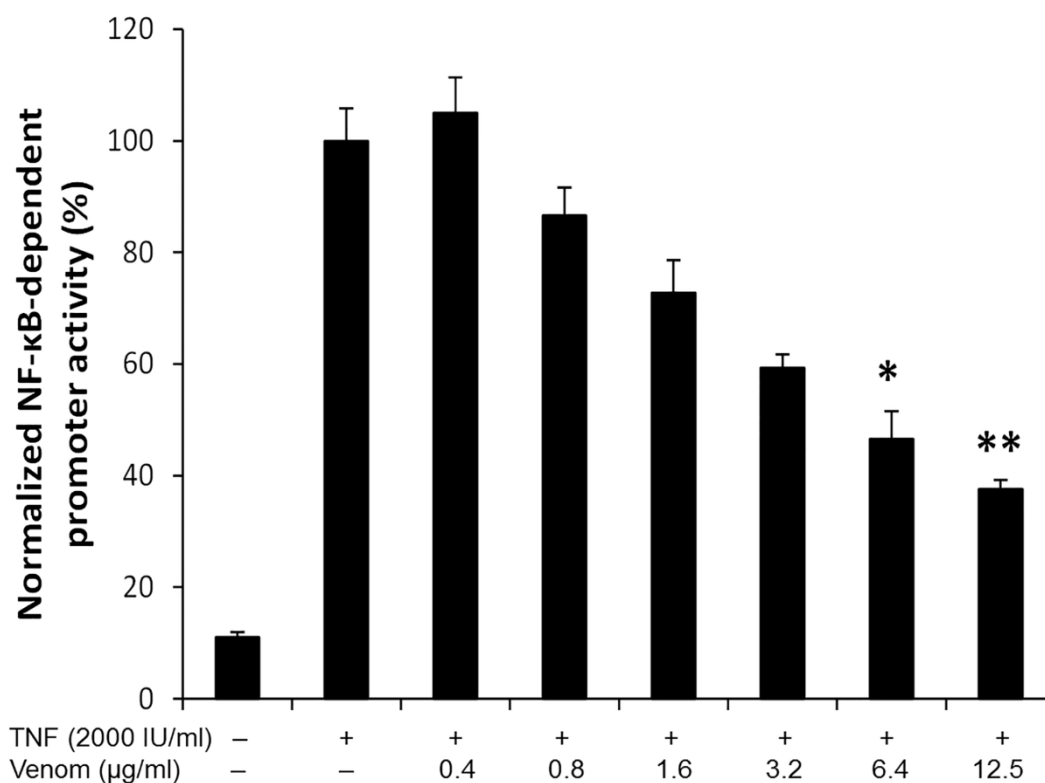


Figure 1: Venom from *N. vitripennis* inhibits TNF-induced expression of a NF-κB-dependent reporter gene. L929sA cells stably transfected with a NF-κB-dependent reporter gene were pre-treated with indicated concentrations of venom for 15 minutes followed by stimulation for 6 hours with TNF (2000 IU/ml). Luciferase activity was normalized for β-galactosidase expression. The data are expressed as the mean ± SDs of three biological replicates. Normality was confirmed by a Shapiro-Wilk test ($W=0.9689$). * $p<0.05$, ** $p<0.01$ versus TNF alone; ANOVA with Bonferroni posthoc test.

4.1.2. Venom inhibits IL-6 mRNA and protein expression in LPS-stimulated Raw264.7 macrophages.

Interleukin (IL)-6 is a pleiotropic cytokine involved in acute phase and immune reactions and inflammatory responses, the expression of which in response to inflammatory stimuli, strongly depends on NF-κB (Libermann & Baltimore, 1990). Macrophages are key players in the inflammatory response, and they secrete high levels of IL-6 upon ligation of Toll-like receptors (TLRs) by pathogen-associated molecular patterns (PAMPs). We therefore explored the effect of *N. vitripennis* venom on TLR4-dependent, LPS-induced IL-6 production in Raw264.7 mouse macrophages. As shown in figure 2A, the elevated levels of IL-6 protein detected after 6 hours LPS treatment were strongly repressed in the presence of the venom in a concentration-dependent manner.

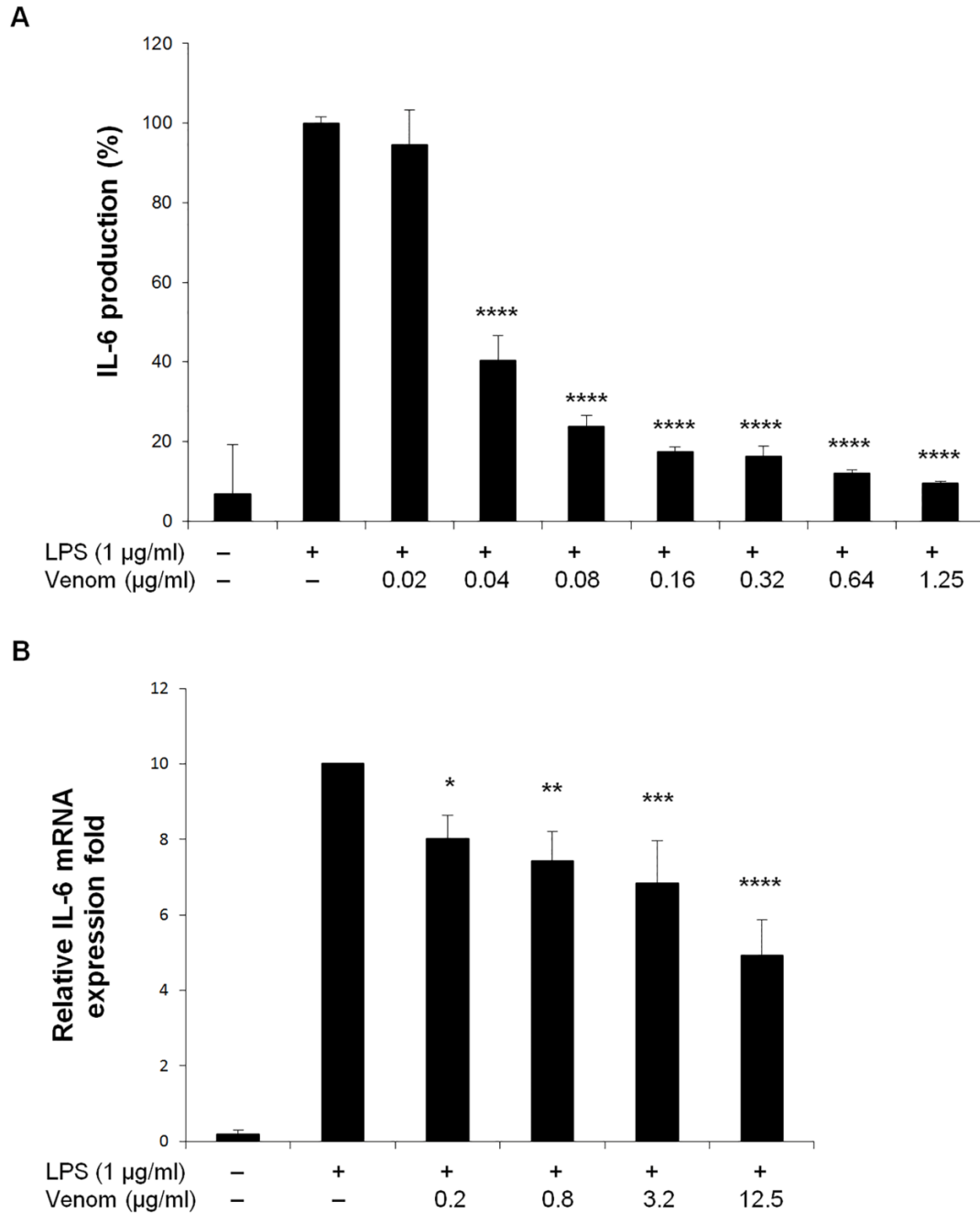


Figure 2: Effect of *N. vitripennis* venom on IL-6 production in LPS-stimulated Raw264.7 cells. A. Cells were stimulated with LPS (1 µg/ml) for 6 hours, alone or after 15 minutes pre-incubation with various concentrations of venom. IL-6 produced and released into the culture medium was assayed by ELISA. B. Cells were stimulated with LPS (1 µg/ml) alone or with indicated amount of venom for 6 hours. Total RNA was isolated and mRNA expression level of IL-6 was determined by RT-qPCR. hPRT1 and cyclophilin were used to normalize the data. The data are expressed as the mean $\pm$ S.D. of three biological replicates. Normality was confirmed by a Shapiro-Wilk test ($W=0.7617$ for A, $W=0.8673$ for B). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ venom and LPS treated versus LPS alone, ANOVA with Bonferroni posthoc test.

N. vitripennis venom pre-treatment also inhibited LPS-induced IL-6 mRNA levels, yet this effect was less prominent and required ten-fold higher doses of venom (figure 2B).

4.1.3. Venom does not interfere with canonical NF- κ B signalling in the cytoplasm.

One of the major mechanisms involved in the transcriptional activation of NF- κ B is the activation of the IKK kinase complex and the concomitant phosphorylation and degradation of the I κ B α protein, which allows the release of free NF- κ B and its subsequent translocation to the nucleus. Furthermore, post-translational modifications of p65 have been demonstrated to be critical for p65-driven gene expression of many target genes (Naumann & Scheidereit, 1994). The IKK complex is known to phosphorylate p65 at serine 536 (Sakurai *et al.*, 1999), in this way regulating p65 nuclear localization. We assessed whether *N. vitripennis* venom affects these early LPS-induced events in Raw264.7 cells (figure 3). We found that LPS promoted phosphorylation of IKK in its activation loop that was apparent after 5 minutes and lasted up to 15 minutes of LPS treatment. Venom pre-treatment did not affect IKK phosphorylation. Next, it was investigated whether the venom alters LPS-induced phosphorylation of p65. As the Western blot analysis in figure 3 shows, p65 phosphorylation in Raw264.7 cells is highly increased after 15 minutes LPS induction. However, no difference in LPS-induced p65 phosphorylation was apparent after venom co-treatment (figure S4).

4.1.4. Venom does not interfere with nuclear translocation of the p65 subunit.

LPS induction in Raw264.7 macrophage cells leads to canonical NF- κ B activation, that is followed by nuclear translocation of the NF- κ B p65 subunit prior to NF- κ B/DNA binding. Therefore, the level of p65 was measured in nuclear extracts of Raw264.7 cells either untreated or venom-treated for 10, 30 or 60 minutes LPS induction. Antibody against Poly ADP-Ribose polymerase (PARP) was used as a loading control for the nuclear fractions. When analyzed by Western blotting, LPS-induced p65 nuclear translocation was not affected by *N. vitripennis* venom (figure 4A). These results were confirmed in figure 4B by immunofluorescence staining to visualize the trafficking of the p65 subunit. LPS induction after 30 and 60 minutes caused an accumulation of green fluorescent signal in the nucleus, confirm the translocation of p65 from the cytoplasm to the nucleus. Co-incubation of these cells with venom, however, did not alter this translocation.

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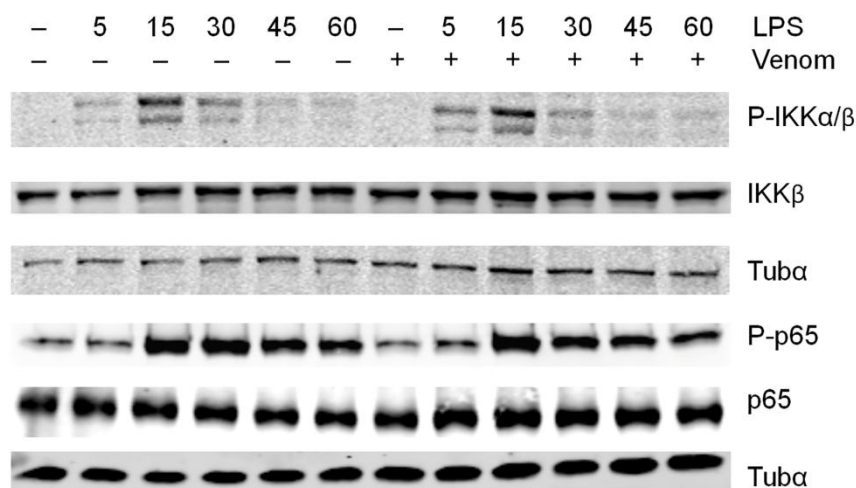
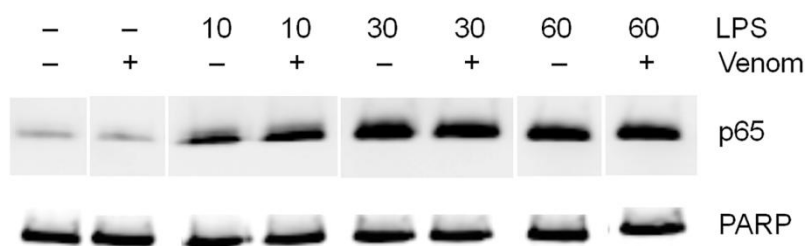


Figure 3: Effect of venom on cytosolic protein activity. Raw264.7 cells were left untreated or were pretreated with 5 $\mu\text{g}/\text{ml}$ venom for 15 minutes and then stimulated with 1 $\mu\text{g}/\text{ml}$ LPS for the indicated times. Total cell extracts were assayed by Western blot analysis using antibodies against indicated proteins. The unphosphorylated protein and Tubulin- α were used as loading controls. A representative result from three separate experiments is shown.

A



B

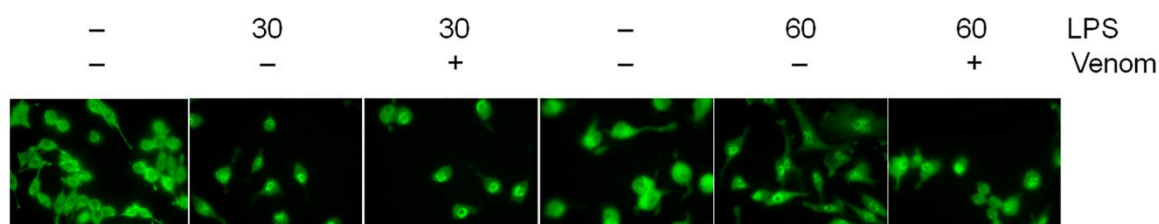


Figure 4: Effect of venom on nuclear translocation of p65. A. Western blot analysis of nuclear extracts from Raw264.7. After pre-incubation with *N. vitripennis* venom (5 $\mu\text{g}/\text{ml}$) for 15 minutes, cells were induced for the indicated times with LPS (1 $\mu\text{g}/\text{ml}$). Subsequently, nuclear extracts were subjected to Western blot analysis to determine p65 levels. Separation of nuclear and cytoplasmic fractions was verified using PARP as control for the nuclear fractions. B. After 15 minutes pre-incubation with *N. vitripennis* venom, cells were induced for the indicated times with LPS (1 $\mu\text{g}/\text{ml}$). Immunofluorescence staining was performed to visualize the trafficking of the p65 subunit. Representative results from three separate experiments are shown.

4.1.5. Venom does not interfere with NF- κ B transactivation independent from the promoter context.

Since no influence of *N. vitripennis* venom was apparent on the subcellular localization of p65 NF- κ B following inflammatory stimuli, the next step was to investigate whether the observed inhibition of NF- κ B activity (figure 1) could be explained by a nuclear phenomenon. To approach this question, it was investigated whether *N. vitripennis* venom could interfere with NF- κ B transactivation, independent from the promoter context. Therefore, its effect on the transcriptional activation of a Gal4-p65-dependent reporter gene, transactivated by the p65 chimera, was analyzed. This Gal4-p65 fusion protein, composed of the DNA-binding domain of the yeast nuclear protein Gal4 and the transactivation domain of the NF- κ B subunit p65, stimulates p(Gal)₂-50hu.IL6P-luc+, a luciferase reporter gene, preceded by two Gal4-binding DNA sequence elements and the minimal IL-6 promoter. In this particular nuclear setup, no direct influence of other responsive element-bound transcription factors, which are normally present in the IL-6 promoter context, or interference of cytoplasmic events need to be taken into account. When the glucocorticoid receptor (GR), a ligand-activated transcription factor present in the cytoplasm, binds to its agonist, e.g. the synthetic glucocorticoid dexamethasone (DEX), the receptor translocates to the nucleus and interferes with the transactivating function of NF- κ B, in a process called transrepression. Cells were incubated with DEX as a positive control for transrepression of the NF- κ B pathway.

In these experiments, HEK293T cells that are devoid of GR, were transiently transfected with the expression constructs, and treated with venom, DEX or were left untreated. Finally, GAL4-p65 driven expression of luciferase was quantified based on the measurement of its activity. Whereas Gal4 protein on its own cannot activate the reporter, overexpression of the nuclear fusion protein Gal4-p65 clearly activated reporter gene expression, as expected (figure 5). After adding DEX to the cells, the level of transactivation by the nuclear fusion protein Gal4-p65 could be specifically repressed, when sufficient exogenous GR was present. However, repression of Gal4-p65-induced transcriptional activation could not be achieved by the *N. vitripennis* venom, indicating that compounds in the venom do not interfere with the transactivation potential of the p65 subunit in the nucleus or that they act on specific components of the transcriptional machinery that is not involved in this Gal4 assay.

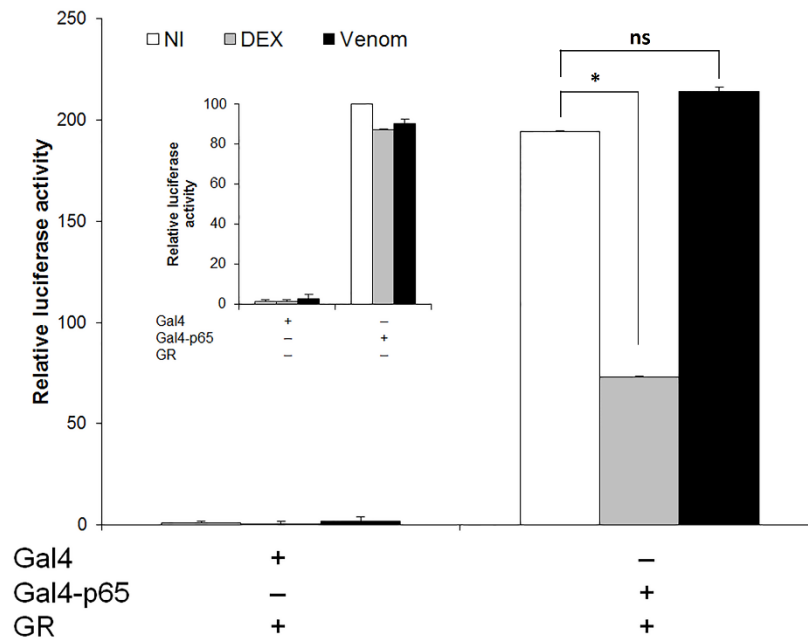


Figure 5: Effect of *N. vitripennis* venom on the Gal4-p65-mediated expression of a reporter gene in a Gal4 one-hybrid setup. HEK293T cells were transiently transfected by the PEI method with 100 ng of p(Gal)2-50.huIL6P-luc+ and with various expression plasmids, the total amount of DNA being fixed at 250 ng, i.e., pGal4 (10 ng) or pGal4-p65 (10 ng) whether or not with pGR (25 ng). 10^{-6} M DEX or 5 μ g/ml of venom was added 24 hours before analysis. Lysates were made and the relative luciferase activity was determined by using β -gal values as a basis for normalization. The data are expressed as the mean $\pm$ S.D. of three biological replicates. * $p < 0.05$, ANOVA with Bonferroni posthoc test.

4.2. Effect of venom on MAPK activation pathways: venom causes prolonged JNK-activation.

It is well established that the phosphorylation and, in turn, activation of MAPKs, i.e. p38, ERK1/2 and JNK, are crucial for LPS-induced AP-1 and NF- κ B activation and subsequent up-regulation of pro-inflammatory mediators in macrophages (Kaminska, 2005). p38 MAP kinase and/or ERK are required for NF- κ B activation in response to LPS and JNK is essential for activation of transcription factor AP-1 (Davis, 2000; Xia *et al.*, 1995; Wilkinson & Millar, 1998). To investigate whether regulation of the activation of these MAPKs is involved in the molecular mechanism of action by *N. vitripennis* venom, phosphorylation of these MAPKs was further examined. Hereto, Raw264.7 cells were pre-treated with venom followed by addition of LPS for different time points. Whole-cell lysates were prepared and the total expression of these different MAP kinases as well as levels of phosphorylated MAPKs were

determined by Western blot analysis. The results showed that LPS stimulation activates MAPKs in a time-dependent manner. Pre-treatment with venom had no effect on the LPS-induced phosphorylation of ERK1/2 and p38 (figure 6 and figure S5). However, after 60 minutes LPS induction, prolonged JNK activation could be observed when *N. vitripennis* venom was added to the cells.

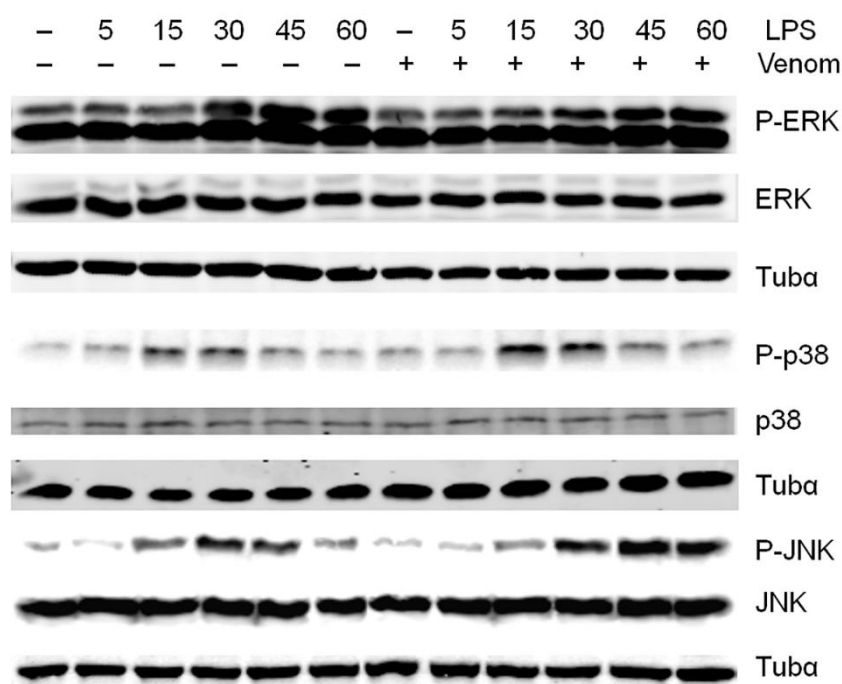


Figure 6: Effect of *N. vitripennis* venom on LPS-induced phosphorylation of MAPKs. Raw264.7 cells were either left untreated or treated with venom (5 µg/ml) for 15 minutes followed by stimulation with 1 µg/ml LPS for the indicated times. Total cell extracts were assayed by Western blot analysis using the indicated antibodies. Tubulin-α and the respective unphosphorylated proteins were used as loading controls. A representative result from three separate experiments is shown.

4.3. Effect of venom on NF-κB regulated secondary anti-inflammatory processes.

Next to the well-studied direct regulation of NF-κB signalling, the regulatory networks that control the NF-κB response are extensive. The best studied and well accepted mechanism for termination of the NF-κB response involves the resynthesis of IκBα proteins induced by activated NF-κB (Pahl, 1999). Newly synthesized IκBα can enter the nucleus, remove NF-κB from the DNA, and relocalize it to the cytosol (Hayden & Ghosh, 2004). Another important

negative regulator of the NF- κ B response is A20, which is up-regulated following NF- κ B activation. Subsequently, A20 down regulates NF- κ B through its dual function as a deubiquitinase and ubiquitin ligase (Heyninck & Beyaert, 2005). Furthermore, glucocorticoids (GCs) are also negative regulators of NF- κ B activation and are widely used as anti-inflammatory agents (Auphan *et al.*, 1995). GCs diffuse through the cell membrane and bind to their inactive cytosolic receptors (GRs), which then undergo conformational modifications that allow for their nuclear translocation. In the nucleus, activated GRs modulate transcriptional events by directly associating with DNA elements. In addition, activated GRs also act by antagonizing the activity of transcription factors, in particular NF- κ B, by direct and indirect mechanisms (Almawi & Melemedjian, 2002). For these reasons, we were interested whether the venom from *N. vitripennis* causes any differences in these secondary regulatory mechanisms of the NF- κ B response. We first tackled this question by looking at the mRNA expression of two immediate-early genes, I κ B α and A20 (figure 7A), and of three GR-regulated genes, glucocorticoid inducible leucine zipper (GILZ), mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP1), and FK506 binding protein 51 (FKBP5) (figure 7B). To study the I κ B α and A20 mRNA expression, Raw264.7 cells were either pre-treated with venom or with insect saline buffer (ISB) as a control and subsequently stimulated with LPS for 30 minutes, 1 hour, 3 or 6 hours. Then, RNA was isolated from the cells, reverse-transcribed and analyzed by real-time PCR. As shown in figure 7A, mRNA expression of I κ B α and A20 is up-regulated after 1 hour of LPS induction and remains elevated for 6 hours after LPS induction. When venom is applied to the cells, both I κ B α and A20 expression are significantly suppressed after 1 hour and after 6 hours LPS induction.

Next, the transcriptional response on several GR-regulated genes was investigated. The synthetic ligand of GR, dexamethasone (DEX) was used as a positive control and the GR antagonist RU38486 was used to monitor whether the ligand-binding domain of GR was affected by the venom. Raw264.7 cells were pre-incubated with RU38486 for 1 hour when indicated and then stimulated with DEX or with venom for 6 hours. As the results in figure 7B show, the transactivation by DEX causes an increase in all three tested genes, while the prior addition of the antagonist resulted in a decrease of their expression, as expected. Interestingly, when *N. vitripennis* venom was added to the cells, an increase in GILZ expression could also be seen, halfmaximal to the increase caused by DEX. Notably, venom-

induced MKP1 expression was even higher than the response to DEX. However, no effect of venom was apparent on FKBP5 expression. RU38486 addition prior to venom did not affect gene expression of these three GC indicator genes.

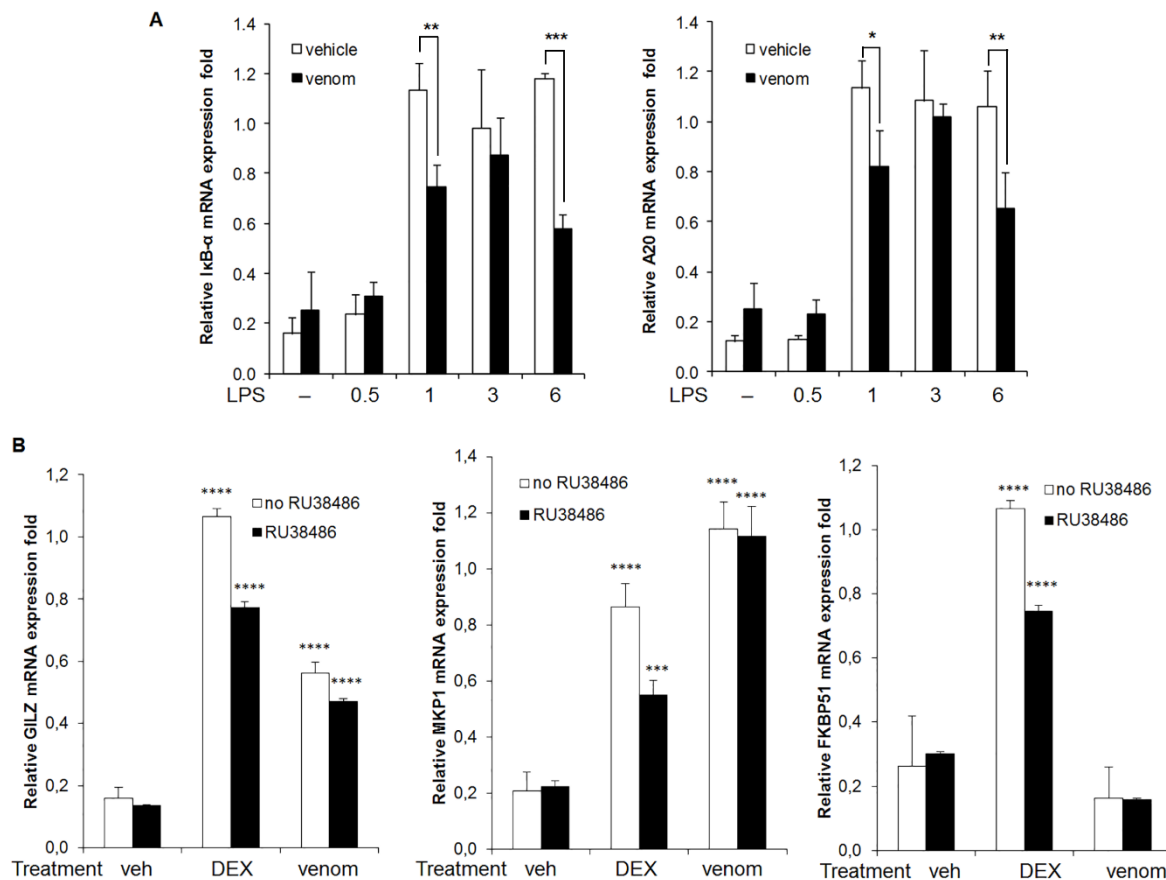


Figure 7: Effect of *N. vitripennis* venom on the mRNA expression of secondary anti-inflammatory processes. A. Raw264.7 cells were stimulated with LPS (1 μ /ml) alone or with 5 μ g/ml venom for 30 minutes, 1 hour, 3 or 6 hours. Total RNA was isolated and the mRNA expression of $\text{IkB}\alpha$ and A20 was determined by RT-qPCR. hPRT1 and cyclophilin were used to normalize the data. The data are expressed as the mean $\pm$ S.D. of three biological replicates. Normality was confirmed by a Shapiro-Wilk test ($W=0.9131$ for $\text{IkB}\alpha$, $W=0.8630$ for A20). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for the venom-treated group compared with the vehicle group, ANOVA with Bonferroni posthoc test. B. Raw264.7 cells were pre-incubated with RU38486 for 1 hour when indicated and then stimulated with 10^{-6} M DEX or with 5 μ g/ml venom for 6 hours. Total RNA was isolated and the mRNA expression of GILZ, MKP1 and FKBP5 was determined by RT-qPCR. hPRT1 and cyclophilin were used to normalize the data. The data are expressed as the mean $\pm$ S.D. of three biological replicates. Normality was confirmed by a Shapiro-Wilk test ($W=0.8952$ for GILZ, $W=0.8834$ for MKP1, $W=0.8424$ for FKBP5). *** $p<0.001$, **** $p<0.0001$ for the DEX or venom group compared with the respective vehicle group and for the DEX + RU38486 or venom + RU38486 group compared with the respective RU38486 group; ANOVA with Bonferroni posthoc test.

Since venom suppressed I κ B α and A20 mRNA expression, we were interested whether this was concomitant with a suppression at the protein level. Therefore, Raw264.7 cells either or not pre-treated with *N. vitripennis* venom, were treated with LPS for 30 minutes, 1 hour, 2, 3 or 6 hours (figure 8 and figure S6). Western blot analysis showed that I κ B α is almost completely degraded after 30 minutes and its resynthesis was detectable after 2 hours of LPS induction. When venom was applied on the cells, the resynthesis of I κ B α remains suppressed even after 6 hours of LPS induction. This probably means that the lowered amount of I κ B α mRNA caused by the venom (figure 7A) resulted in less translated I κ B α protein. When looking at the presence of A20, an elevation at the protein level was noticed after 2 and 3 hours LPS induction. Venom caused a slight elevation of A20 after 2 hours of LPS induction, but a significant suppression could be noted when LPS was applied to the cells for 3 hours. This is probably the result of the suppressed A20 mRNA expression (figure 7A).

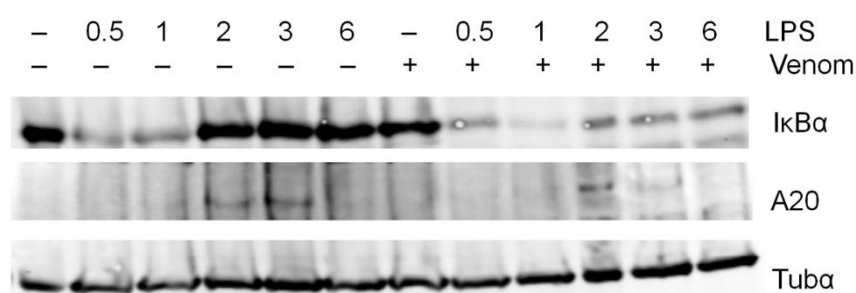


Figure 8: Effect of venom on I κ B α and A20 protein levels. Raw264.7 cells were left untreated or were pretreated with 5 μ g/ml venom for 15 minutes and then stimulated with 1 μ g/ml LPS for the indicated times. Total cell extracts were assayed by Western blot analysis using antibodies against indicated proteins. Tubulin- α was used as a loading control. A representative result from three separate experiments is shown.

5. Discussion

Studies on the immune suppressive effect of *N. vitripennis* venom have always been directed on hemocytes of their hosts (Zhang *et al.*, 2005; Qian *et al.*, 2013) or cultured insect cells (Rivers *et al.*, 2002a, 2005; Abt & Rivers, 2007). While investigating the possible function of parasitoid venom on mammalian cells may seem unlogic at first, the plethora of available research tools in these cell lines, that highly exceeds what is available in insect cell lines, convinced us to start working on mammalian cell lines. Secondly, NF- κ B transcription factors,

the MAPK signal transduction pathways and the glucocorticoid receptor signalling pathway that were studied, are evolutionary conserved in metazoan animals (Hatada *et al.*, 2000; Bolton *et al.*, 2007). Finally, there is an enormous request for anti-inflammatory compounds in medicine that represents the high amount of future applications available (D'Acquisto *et al.*, 2002). Since so many diseases are driven by chronic inflammatory responses, finding good drugs to control this excessive immune response has been the focus of much research. The last few decades, venoms have also been added to the growing list of anti-inflammatory drugs (Rajendra *et al.*, 2004). The extraordinary finding that the venom of a parasitoid wasp is able to suppress the major mammalian immune pathway, intrigued us to fully disentangle this interaction. Respectively, we are the first to investigate the inhibitory effect of *N. vitripennis* venom on the NF- κ B pathway in mammalian cells and to suggest its mode of action.

Previous experiments on natural host organisms have shown that maternally derived venom from *N. vitripennis* disrupts host immune responses almost immediately following oviposition (Rivers *et al.*, 2002b). Microarray analysis on parasitized *S. crassipalpis* pupae by *N. vitripennis* already suggested NF- κ B and MAPK pathways in the host as potential candidates for the regulation of the immune response to envenomation (Danneels *et al.*, 2013). The current study was conducted to investigate the potential anti-inflammatory and anti-NF- κ B responses of *N. vitripennis* venom on mammalian cells. A proposed model of the interactions between the venom and the NF- κ B pathway is illustrated in figure 9. Since this venom has a high cytotoxicity to certain insect cell types (Zhang *et al.*, 2005), cell viability was tested to establish the appropriate concentration ranges of venom for the analysis of ongoing experiments. When incubation periods of 6 hours were applied, 5 or 10 μ g/ml venom appeared to be non-toxic concentrations and were used in further experiments. The initial focus was to see whether *N. vitripennis* venom could inhibit the NF- κ B immune signalling pathway. This was first studied in murine fibrosarcoma cells stably transfected with a recombinant promoter that has an NF- κ B-responsive element. Interestingly, when these cells were incubated with *N. vitripennis* venom, a clear dose-related inhibition of relative luciferase activity could be noted. Venom-mediated suppression of the NF- κ B pathway could be confirmed in Raw264.7 macrophages, since the venom dose-dependently suppressed IL-6 mRNA and protein expression. This is a remarkable result, since this is the

first report that venom from an ectoparasitic wasp is able to suppress NF- κ B signalling in mammalian cell lines.

The following study focused on localizing the intrusion step by venom compounds. In 2006, Gilmore and Herscovitch reported on the identification of more than 785 inhibitors of NF- κ B signalling (Gilmore & Herscovitch, 2006). They noted that in most cases, inhibition is based on a given agent's ability to block one or more steps of NF- κ B signalling in a tissue culture-based system after stimulation of resting cells with an NF- κ B inducer, most commonly either TNF or LPS. More than half of those described NF- κ B inhibitors block a signal before the NF- κ B dimer is able to bind the DNA in the nucleus. *N. vitripennis* venom however, did not cause an effect on the cytosolic events, nor on the translocation of the NF- κ B dimer to the nucleus for binding to the DNA, nor did it disturb the transactivation function of p65. These intriguing results forced further research activities into the direction of other pathways or regulatory mechanisms that could result into this inhibition of NF- κ B activity.

The phosphorylation and activation of three major MAPKs – p38, ERK1/2 and JNK – have been shown to initiate inflammatory gene expression in LPS-induced macrophages. Therefore, the effect of venom on these pathways was also investigated. Interestingly, we found that venom prolonged LPS-induced JNK activation in Raw264.7 macrophages. Genotoxic stresses, e.g. UV or γ -irradiation, and TNF-induced reactive oxygen species (ROS) accumulation induce long-lasting or prolonged MAPK activation, which in turn contributes to cell death (Sakon *et al.*, 2003). NF- κ B on the other hand, can protect cells against TNF cytotoxicity by inhibiting the sustained phase of TNF-induced JNK activation (Wullaert *et al.*, 2006). Prolonged JNK activation as a result of venom induction could be the result of NF- κ B inhibition and eventually even lead to cell death. Indeed, *N. vitripennis* venom induces apoptosis of insect cells (Formesyn *et al.*, 2013b), while in murine macrophages, only high doses and long induction times were cytotoxic to the cells.

Since aberrant NF- κ B activity can directly lead to uncontrolled tissue damage and deleterious disease (Staudt, 2010; Lawrence, 2009), the NF- κ B driven inflammatory responses need to be down-regulated and properly terminated. Therefore, many distinct negative regulatory feedback mechanisms have evolved that operate at different molecular levels in the NF- κ B

signalling pathway (Ruland, 2011). For instance, several proteins among which I κ B α and A20, are NF- κ B target genes, constituting a negative-feedback response. *N. vitripennis* venom was, by causing suppression of inhibitors of NF- κ B signalling, indirectly found to give rise to an elimination of this negative-feedback response. The abrogation of this negative regulation can therefore be seen as the normal consequence of the venom-induced suppression of the NF- κ B activity. Another regulation of the NF- κ B activation is realized by glucocorticoids (Almawi & Melemedjian, 2002). Today, nothing is known about possible steroids or corticoid-like substances in *N. vitripennis* venom. However, besides peptides and proteins, venoms are known to contain organic molecules, amines, alkaloids, salts and minerals (Fry *et al.*, 2009) and recently, the venom from the toad *Bufo bufo gargarizans* was found to contain steroids that displayed potent inhibitory activities against cancer cells (Tian *et al.*, 2013). When we looked at genes that are regulated by GR, *N. vitripennis* venom induction caused an up-regulation of GILZ and MKP1. Intriguingly, GILZ and MKP1 are known to inhibit NF- κ B signalling; GILZ through the interaction with p65 (Riccardi *et al.*, 2001), and MKP1 via dephosphorylation and inactivation of members of MAPK family, preferably JNK and p38 MAPK (Franklin & Kraft, 1997). Studies in T lymphocytes demonstrated that GILZ reduced NF- κ B transcriptional activity (Ayroldi *et al.*, 2001). Subsequent in vivo studies showed that administration of a GILZ peptide inhibits disease development in the experimental autoimmune encephalomyelitis model of multiple sclerosis, in part via effects on T cell activation (Srinivasan & Janardhanam, 2011). Together, these findings demonstrate a suppressive role of GILZ in the regulation of immune cell activation. We speculate GILZ up-regulation as the most likely candidate for the venom-induced effect, but the precise mode of action still needs to be elucidated.

We can conclude that in analogy with inhibition of immune responses in the natural insect host, also in mammalian cells anti-inflammatory responses of the venom can be observed. However, an interesting difference concerning MAPK pathways was observed. While the microarray study on the insect host showed significant down-regulation of a MAPK gene and up-regulation of a MAPK-phosphatase, an increase in activation of the JNK pathway was seen in the mammalian system. Apparently, the venom has a different mode of action in insects than in mammalian cells, which seems interesting to investigate more into detail.

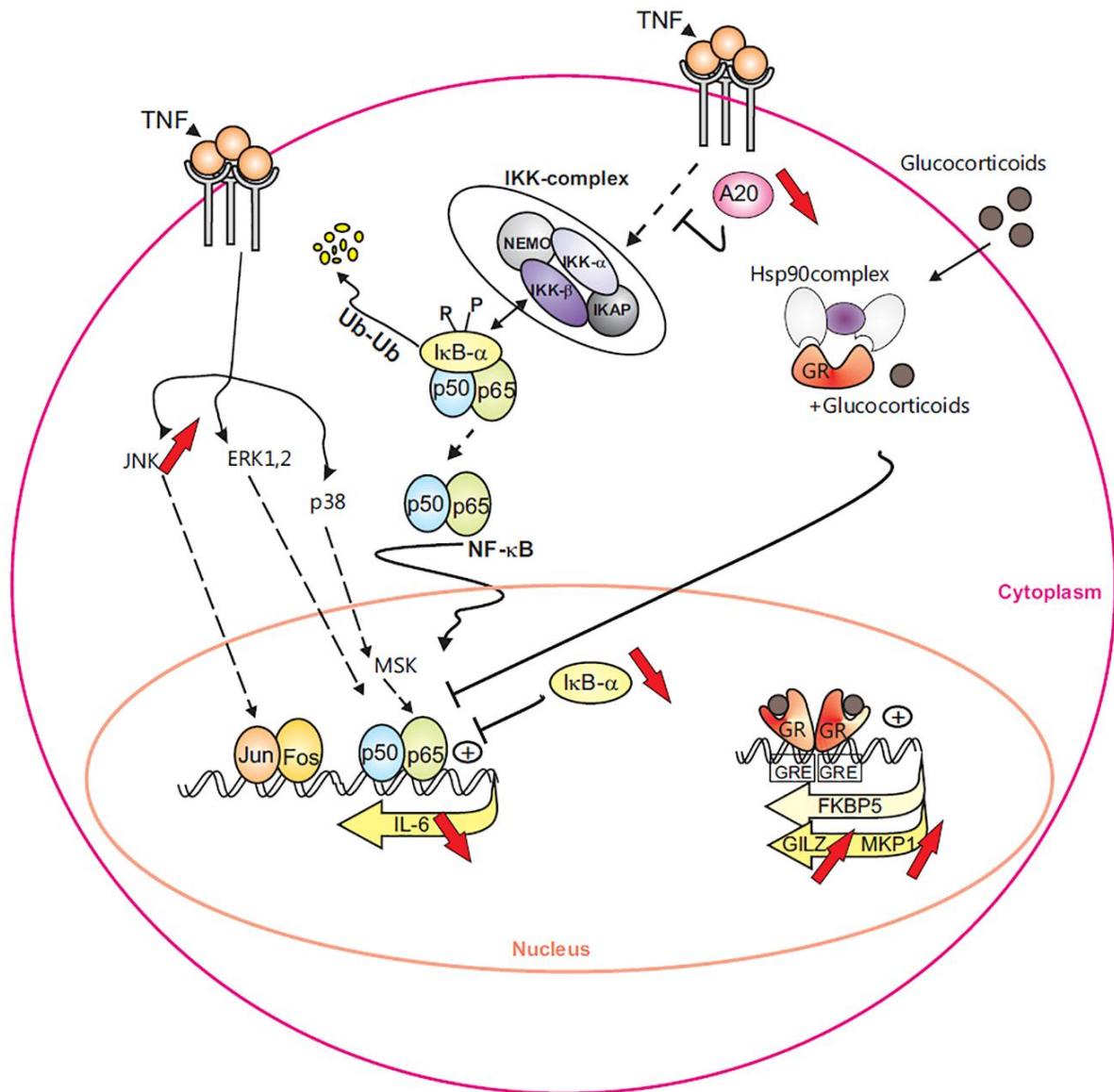


Figure 9: Proposed model of venom interference with the NF-κB and glucocorticoid signalling pathways.

TNF stimulation leads to activation of the canonical NF-κB signal transduction pathway marked by IκBα degradation and translocation of the p65-p50 dimer to the nucleus. Subsequent NF-κB DNA binding enables transcription of different genes, among which the cytokine IL-6. Venom treatment leads to the inhibition of TNF induced IL-6 gene expression. In addition, TNF activates the MAPKs, JNK, ERK1/2 and p38, and pretreatment with venom results in prolonged JNK activation. The negative NF-κB regulators A20 and IκBα are indicated and both were suppressed by the venom. Glucocorticoid binding to the cytosolic Glucocorticoid Receptor (GR) results in the dissociation of chaperoning proteins, followed by GR translocation to the nucleus, where it can interfere with the activity of NF-κB. Activated GR can also directly bind to the DNA, stimulating transcription of FKBP5, MKP1 and GILZ target genes. Only the two latter genes are transcriptionally induced by the venom. The red arrows mark the various levels at which venom of *N. vitripennis* interferes with the represented cellular signalling pathways.

In view of the complexity of the NF- κ B pathway, the identification of specific inhibitors with acceptable side-effects has been challenging, and new molecules are still actively sought after. Perhaps, venom from the ectoparasitoid wasp *N. vitripennis* can meet with these conditions by looking at the results presented here, but for sure, future experiments need to explore this further.

6. Acknowledgments

Authors are thankful to Prof. Dr. Bart Devreese for the use of the CFX96 Real-Time PCR Detection System for performing the RT-qPCR analyses. Special thanks also go out to Kamila Skieterska, Jolien Duchou, Linde Sabbe, Nadia Bougarne and Béatrice Lintermans for their generous help in the lab. Although you are missed deeply, thanks Dieter for your support and our many late-night talks.

7. Supplementary Materials

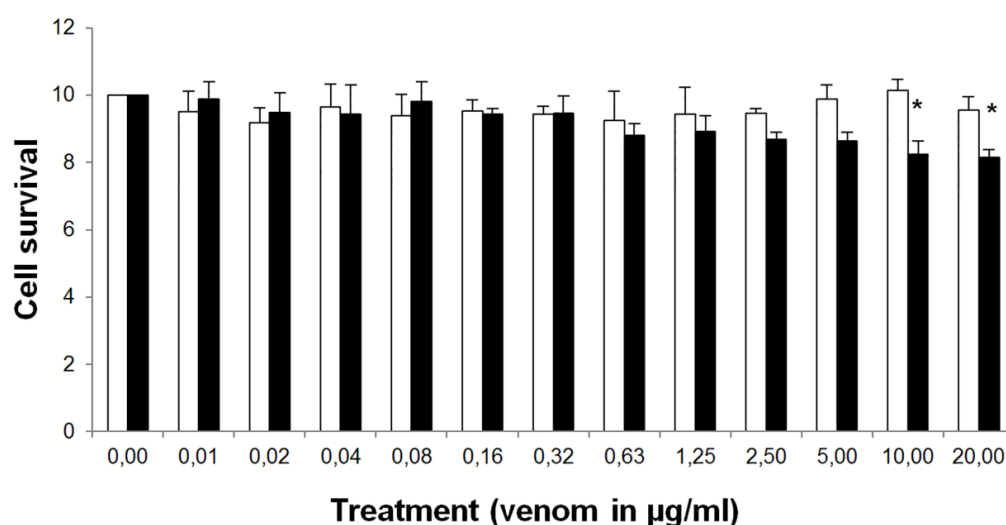


Figure S1: Effect of *N. vitripennis* venom on cell viability in Raw264.7 cells. Viability was measured after 6 and 24 hours venom incubation in an MTT assay by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml) to the cells. This solution was incubated at 37°C until blue deposits were visible. The formazan crystals were then solubilized in SDS/HCl solution and incubated for 5 hours at 37°C. The absorbance was determined colorimetrically at 595 nm. White bars represent 6 hours venom incubation, black bars represent 24 hours venom incubation. Normality was confirmed by a Shapiro-Wilk test ($W=0.9677$). Statistical analysis on 24 hours compared to 6 hours venom incubation, * p -value < 0.05, ANOVA and Dunn's test.

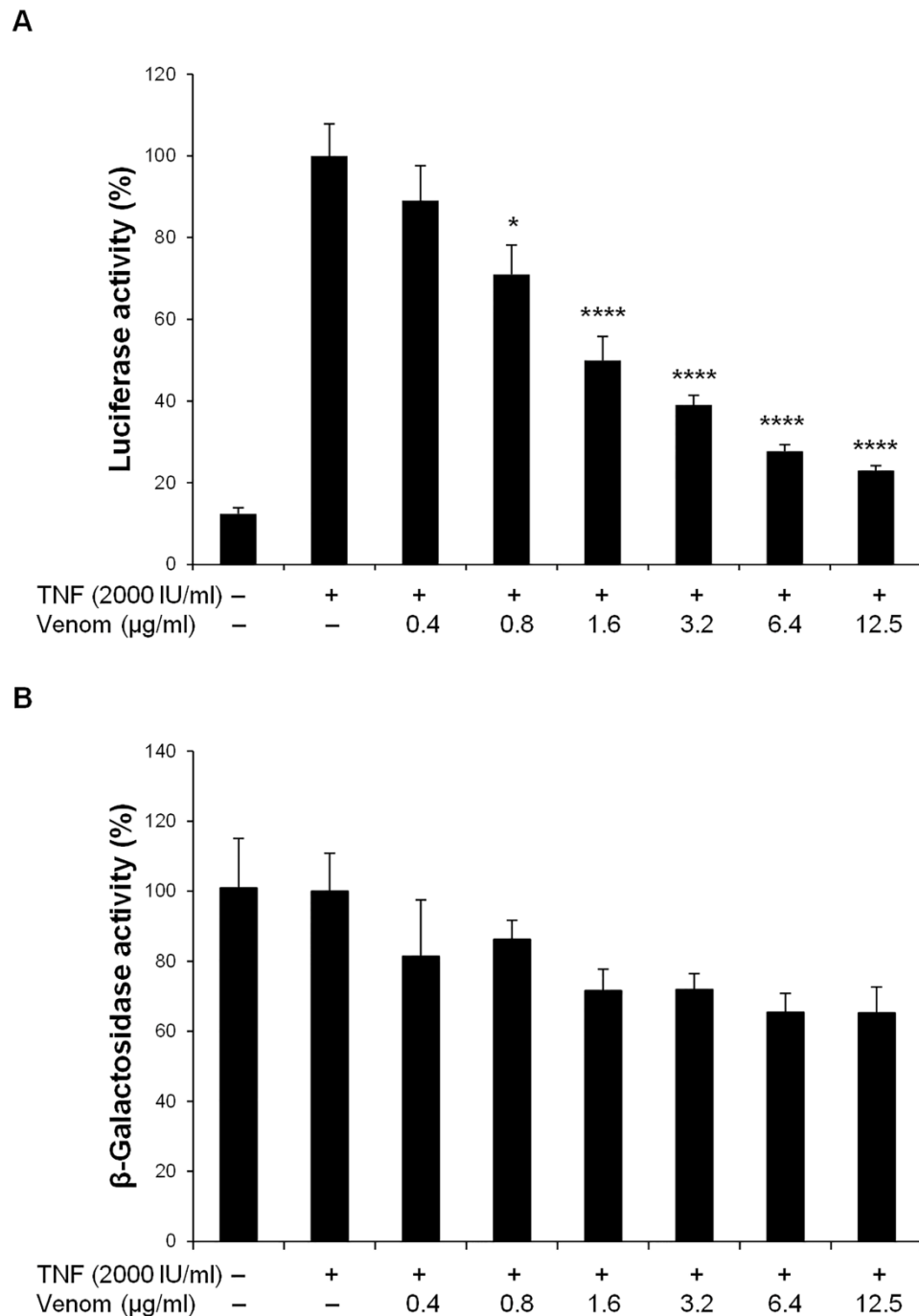


Figure S2: Venom from *N. vitripennis* inhibits TNF-induced expression of a NF-κB-dependent reporter gene: not normalized luciferase and β-galactosidase values. L929sA cells stably transfected with a NF-κB-dependent reporter gene were pretreated with indicated concentrations of venom for 15 minutes followed by stimulation for 6 hours with TNF (2000 IU/ml). A. Luciferase activity. B. β-galactosidase expression. The data are expressed as the mean ± S.D. of three biological replicates. Normality was confirmed by a Shapiro-Wilk test ($W=0.9337$ for A, $W=0.9662$ for B). * $p<0.05$, **** $p<0.0001$ versus TNF alone, ANOVA with Bonferroni posthoc test.

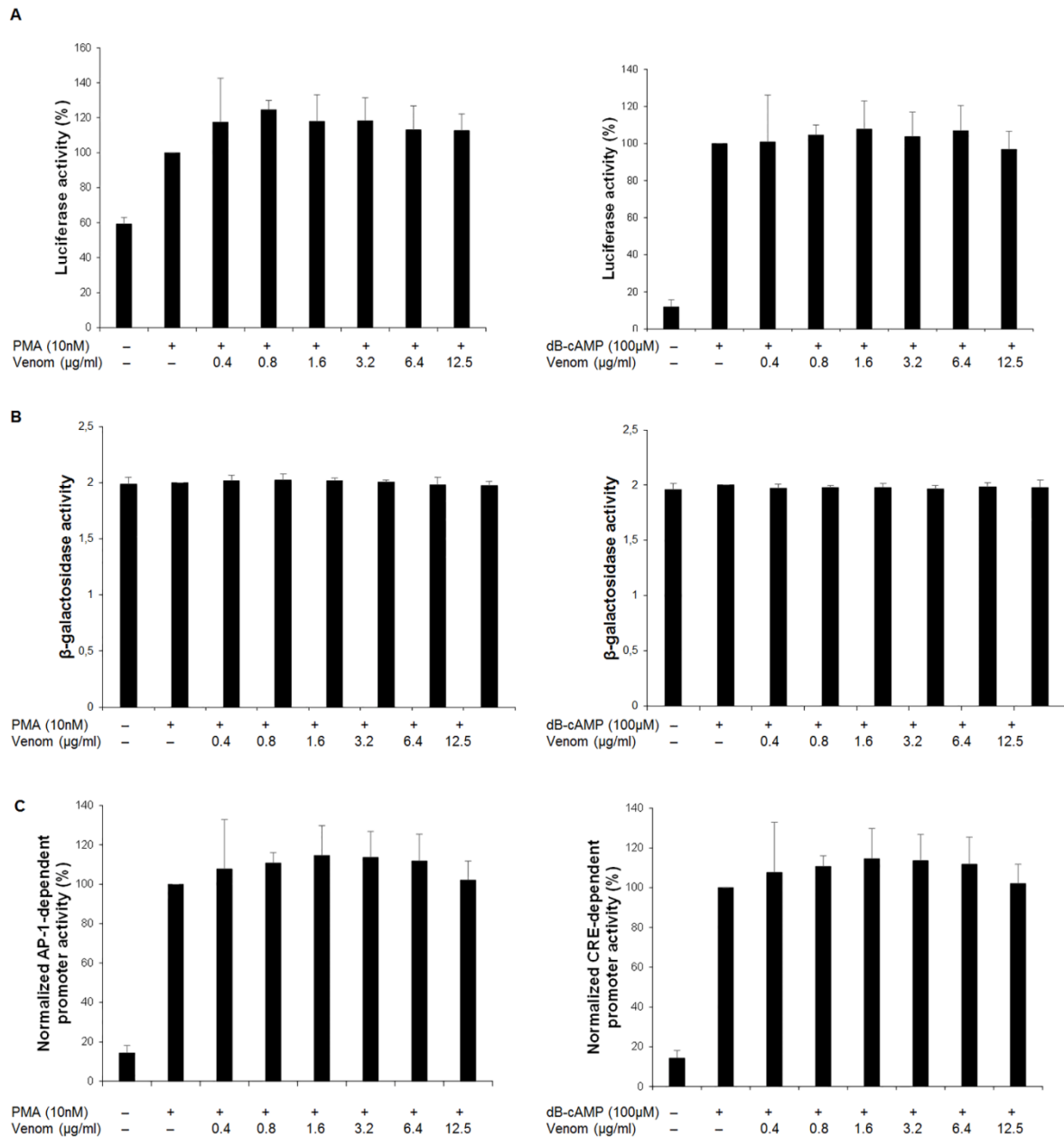


Figure S3: Venom does not inhibit PMA- or dB-cAMP-induced expression of neither an AP-1-dependent reporter gene, nor a CRE-dependent reporter gene respectively. On the left, L929sA cells stably transfected with an AP-1-dependent reporter gene were pre-treated with indicated concentrations of venom for 15 minutes followed by stimulation for 6 hours with phorbol 13-myristate 12-acetate (PMA) (10 nM). On the right, L929sA cells stably transfected with a CRE-dependent reporter gene were pre-treated with indicated concentrations of venom followed by stimulation for 6 hours with N(6),2'-O-dibutyryl adenosine 3':5' cyclic monophosphate (dB-cAMP) (100 µM). A. Luciferase activity. B. β-galactosidase expression. C. Normalized expression. The data are expressed as the mean ± S.D. of three biological replicates. Normality was confirmed by a Shapiro-Wilk test ($W=0.8744$ and $W=0.7985$ for A left and right respectively, $W=0.9124$ and $W=0.9521$ for B left and right respectively, $W=0.9195$ and $W=0.8226$ for C left and right respectively), ANOVA with Bonferroni posthoc test.

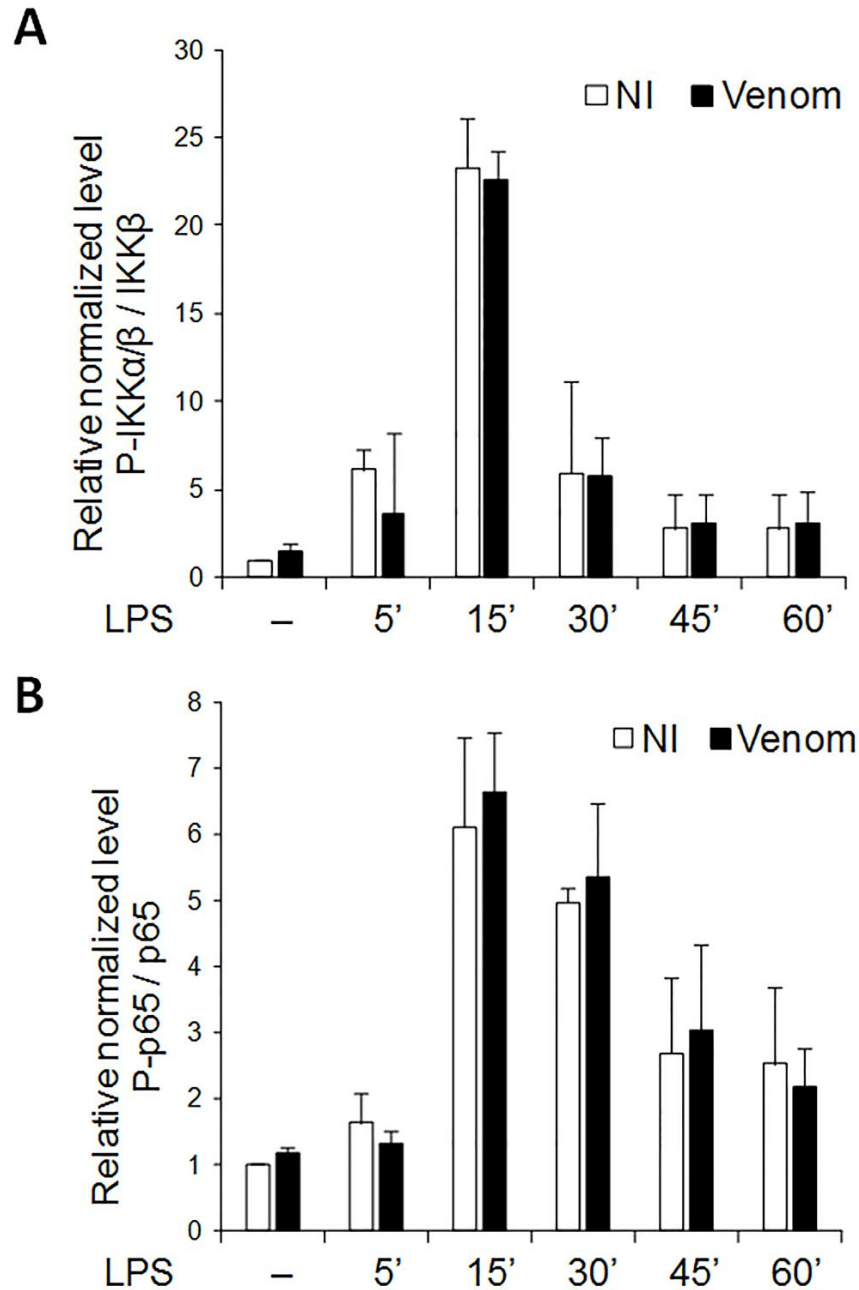


Figure S4: Histograms of Western blots that show the effect of venom on cytosolic protein activity. Raw264.7 cells were left untreated or were pretreated with 5 $\mu\text{g/ml}$ venom for 15 minutes and then stimulated with 1 $\mu\text{g/ml}$ LPS for the indicated times. Total cell extracts were assayed by Western blot analysis using antibodies against indicated proteins: A. P-IKK α/β normalized with the unphosphorylated IKK β . B. P-p65 normalized with the unphosphorylated p65. Bands of these proteins were quantified and data are expressed in histograms as the mean $\pm$ S.D. of three biological replicates. Normality was confirmed by a Shapiro-Wilk test ($W=0.7242$ for A, $W=0.8585$ for B). Statistics were performed by ANOVA with Bonferroni posthoc test. Relative amounts of normalized phosphorylated IKK α/β (A) and p65 (B) levels between venom-treated and not-induced cells were not significant.

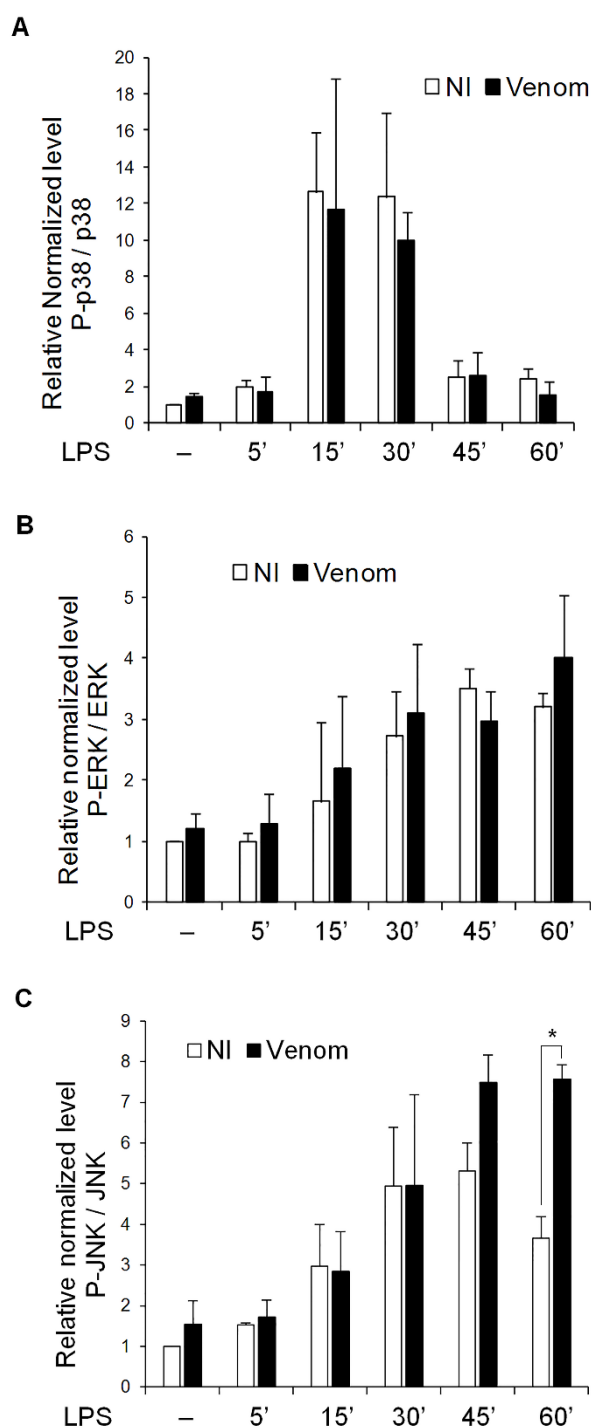


Figure S5: Histograms of Western blots that show the effect of venom on phosphorylation of MAPKs. Raw264.7 cells were left untreated or were pretreated with 5 $\mu\text{g/ml}$ venom for 15 minutes and then stimulated with 1 $\mu\text{g/ml}$ LPS for the indicated times. Total cell extracts were assayed by Western blot analysis using antibodies against indicated proteins. A. P-p38 normalized with p38. B. P-ERK normalized with ERK. C. P-JNK normalized with JNK. Bands of these proteins were quantified and data are expressed in histograms as the mean $\pm$ S.D. of three biological replicates. Normality was confirmed by a Shapiro-Wilk test ($W=0.7085$ for A, $W=0.8703$ for B, $W=0.9012$ for C). Statistical analysis of venom-treated compared with not-induced cells, * $p<0.01$ versus TNF alone, ANOVA with Bonferroni posthoc test.

How the venom from *N. vitripennis* exhibits anti-inflammatory properties

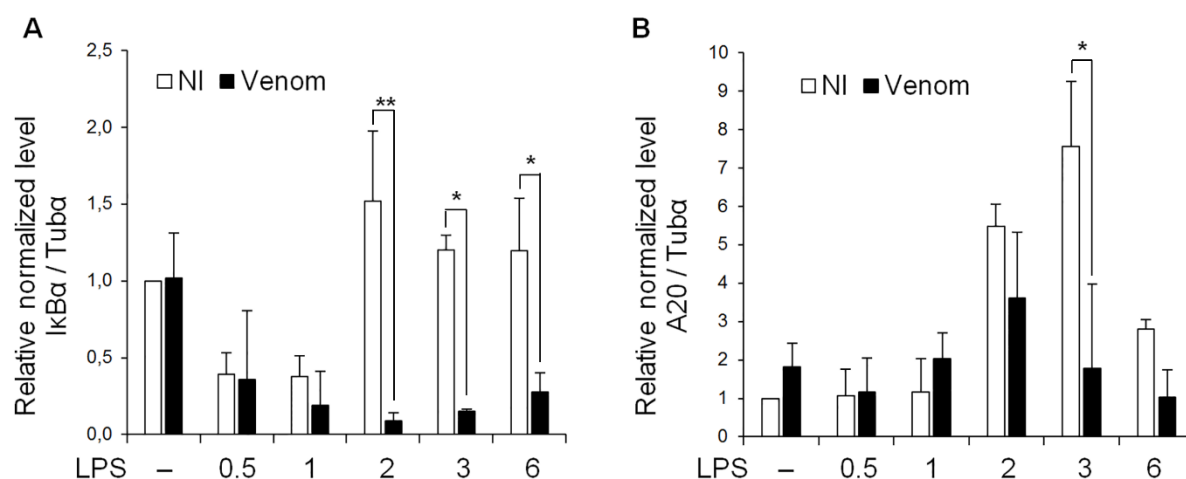


Figure S6: Histograms of Western blots that show the effect of venom on IkBα and A20 protein levels. Raw264.7 cells were left untreated or were pretreated with 5 μg/ml venom for 15 minutes and then stimulated with 1 μg/ml LPS for the indicated times. Total cell extracts were assayed by Western blot analysis using antibodies against indicated proteins. A. IkBα normalized with Tubulin- α. B. A20 normalized with Tubulin-α. Bands of these proteins were quantified and data are expressed in histograms as the mean ± S.D. of three biological replicates. Normality was confirmed by a Shapiro-Wilk test (W=0.9253 for A, W=0.7062 for B). * p<0.01, ** p<0.001 versus TNF alone, ANOVA with Bonferroni posthoc test.

Table S1: Mouse-specific primer sets developed to amplify transcripts by RT-qPCR of immune specific proteins and housekeeping genes.

Primer name	Primer sequence
IL6_qPCR_FWD	5'-GAGGATACCACTCCCAACAGACC-3'
IL6_qPCR_REV	5'-AAGTCATCATCGTTGTTTCATACA-3'
IkBa_qPCR_FWD	5'-TGAAGGACGAGGAGTACGAGC-3'
IkBa_qPCR_REV	5'-TTCGTGGATGATTGCCAAGTG-3'
A20_qPCR_FWD	5'-CCTTGCTTTGAGTCAGGCTGT-3'
A20_qPCR_REV	5'-TAAGGAGAAGCACGAAACATCGA-3'
FKBP5_qPCR_FWD	5'-TGAGGGCACCAGTAACAATGG-3'
FKBP5_qPCR_REV	5'-CAACATCCCTTTGTAGTGGACAT-3'
MKP1_qPCR_FWD	5'-GAGCTGTGCAGCAAACAGTC-3'
MKP1_qPCR_REV	5'-CTCCGAGAAGCGTGATAGG-3'
GILZ_qPCR_FWD	5'-CCAGTGTGCTCCAGAAAGTGAAG-3'
GILZ_qPCR_REV	5'-AGAAGGCTCATTGGCTCAATCTC-3'
Cyclo_qPCR_FWD	5'-GCATACGGGTCCTGGCATCTTGTC-3'
Cyclo_qPCR_REV	5'-ATGGTGATCTTCTTGCTGGTCCTTGC-3'
hPRT1_qPCR_FWD	5'-CCTAAGATGAGCGCAAGTTGAA-3'
hPRT1_qPCR_REV	5'-CCACAGGACTAGAACACCTGCTAA-3'

ADDENDUM TO CHAPTER 4

Effects of *N. vitripennis* venom on glucocorticoid receptor signalling

Personal contribution:

- cell culture
- venom dissections
- sample preparation
- reporter gene analysis
- western blot analysis
- immunofluorescence staining
- functional analysis of the dataset

Macrophages treated with venom from *N. vitripennis* were shown to up-regulate transcription of 2 GR-regulated genes, GILZ and MKP1 (see chapter 4, figure 7B). To follow up on this result, the effect of venom on the GR signalling cascade was further investigated. GR is a ligand-activated transcription factor that must translocate from the cytoplasm to the nucleus and interacts with the promoters of GC responsive genes. Transactivation occurs when liganded GR binds to glucocorticoid-response elements (GREs), activating transcription of GR target genes, like GILZ and MKP1. Possibly, *N. vitripennis* venom alters the transcriptional response elicited by GR, explaining the increase of GR-target genes. Therefore, the effect of venom on GR transactivation on a synthetic GRE-containing promoter reporter was investigated, while using DEX as positive control. HEK293T cells that do not contain functional GR, were used for this experiment. When an increasing amount of GR was transfected into the cells, an increase of transactivation by DEX could be seen, while the venom had no effect on this process (figure 1A).

Furthermore, in order to examine whether the venom causes a change in nuclear localization of GR, fractionation of HEK293T cells was performed followed by western blot analysis of nuclear and cytoplasmic fractions (figure 1B). Hereby, DEX caused a 3-fold increase of GR nuclear localization, while venom showed the same amount of nuclear GR as in un-treated cells. In addition, immunofluorescence staining of GR was performed on HEK293T cells to visualize the trafficking of GR (figure 1C). DEX-treatment resulted in translocation of GR from the cytoplasm to the nucleus, while venom showed no alteration on this process. These results indicate that the venom induced up-regulation of GILZ and MKP1 are not caused by an increase in GR signalling, but are possibly mediated via activation of other transcriptional responses.

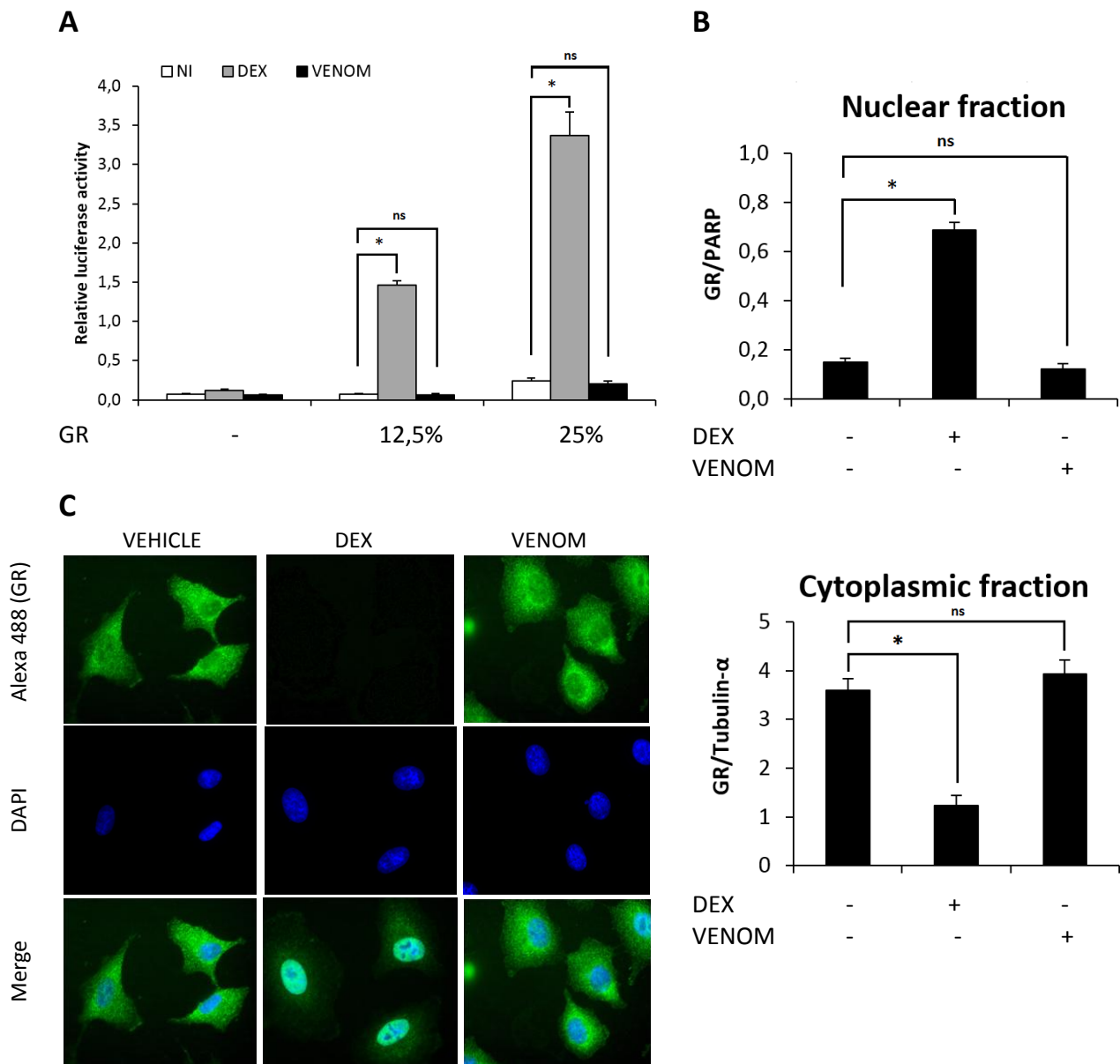


Figure 1: Transcriptional efficacy and nuclear localization of GR after DEX or *N. vitripennis* venom treatment. A. HEK293T cells were transiently transfected by the PEI method with 100 ng of p(GRE)₂-50-luc either or not with two different concentrations of pGR (25 or 50 ng), the total amount of DNA being fixed at 200 ng. 10^{-6} M DEX or 10 μ g/ml venom was added 6 hours before analysis. Lysates were made and the concentration of luc was determined by using β -gal values as a basis for normalization. * $p < 0.0001$, ANOVA with Bonferroni posthoc test. B. Quantification of western blot analysis of nuclear and cytoplasmic extracts from HEK293T cells. Cells were incubated for 1 hour with 10^{-6} M DEX or 10 μ g/ml venom. Subsequently, nuclear and cytoplasmic extracts were subjected to Western blot analysis to determine GR levels. Separation of nuclear and cytoplasmic fractions was verified using PARP and Tubulin- α as respective controls. * $p < 0.0001$, ANOVA with Bonferroni posthoc test. C. HEK293T cells were treated similar as in B, but after the inductions, immunofluorescence staining was performed to visualize the trafficking of GR.

CHAPTER 5

The potential of *N. vitripennis* venom as therapeutic agent

Personal contribution:

- Cell culture
- Venom dissections
- Sample preparation
- RT-qPCR
- Functional analysis of the dataset
- Writing the manuscript

1. Introduction

N. vitripennis venom is known to contain (at least) 80 different proteins, and possibly much more peptides and other bio-molecules. The past century, natural products (NPs) have been the source of inspiration for the majority of FDA approved drugs. This is highlighted by the fact that nearly 50% of all drugs in clinical use is of natural product origin (Paterson & Anderson, 2005). These interesting chemicals are derived from the phenomenon of biodiversity in which the interactions among organisms and their environment formulate the diverse complex chemical entities within the organisms that enhance their survival and competitiveness (Mishra & Tiwari, 2011). The therapeutic areas of infectious diseases and oncology have benefited from the complex molecular scaffolds found in NPs of which the chemical diversity is unmatched by synthetic molecules. Animal venoms are a rich source of NPs that have evolved high affinity and selectivity for a diverse range of biological targets, especially membrane proteins such as ion channels, receptors and transporters. Therefore, venomics has emerged as an important addition to modern drug discovery efforts (Fry *et al.*, 2009). Snake venom is a treasure house of toxins that contributed significantly to the treatment of many medical conditions and presents a great potential as anti-tumour agent (Vyas *et al.*, 2013). The venom and its constituents from honey bees has many therapeutic applications ranging from anti-arthritis and pain-releasing to anti-cancer effects (Son *et al.*, 2007). Venoms from parasitoid wasps contain a staggering amount of toxins, and because they can manipulate cell physiology in diverse ways (see chapter 1, 3.2. Mode of actions), their therapeutic potential seems interesting to dig into.

In chapter 4, *N. vitripennis* venom was shown to exert a suppressive action on the NF- κ B pathway in murine macrophages. This important transcription factor regulates a large number of target genes involved in multiple cellular processes including inflammation, immunity and stress responses. Dysregulation of this signal transduction pathway has been associated with inflammatory or autoimmune diseases and cancer. The analysis of the expression of nearly hundred key genes responsive to NF- κ B signal transduction by the NF- κ B Signalling Targets PCR Array (SABiosciences, Qiagen) can result in a broader understanding of the effects of *N. vitripennis* venom on the immune response. Next to this,

alterations of NF- κ B signalling target genes with a role in inflammatory diseases and oncology could hint at potential therapeutic lead compounds present in the venom.

In 1999, Pahl listed over 150 target genes known to be expressed by the active NF- κ B transcription factor (Pahl, 1999). To date, this list has been extended by more than 250 extra investigated NF- κ B target genes and even more than 300 genes are predicted by computer-based methods to have composite NF- κ B regulatory sites (<http://www.bu.edu/nf-kb/gene-resources/target-genes/>). The majority of proteins encoded by NF- κ B target genes participate in the host inflammatory and immune responses, which include cytokines and chemokines, as well as receptors required for immune recognition, proteins involved in antigen presentation, acute phase proteins and cell adhesion molecules. Many of them are induced by exposure to a wide variety of bacteria, hosts of viruses and their respective products. NF- κ B, however, is involved in the control of transcription of many genes whose functions extend beyond the immune response, but are involved in more general stress responses. Various physiological stress conditions such as liver regeneration and hemorrhagic shock can activate NF- κ B. Also physical stress in the form of irradiation as well as oxidative stress to cells induce NF- κ B, that in turn activates a large variety of stress response genes. In fact, NF- κ B relays the information of an imminent stress and at the same time enacts a response by promoting the transcription of genes whose products alleviate the stress condition. The human body is also exposed to environmental hazards and therapeutic drugs, activating NF- κ B that in turn activates its target genes including many cell surface receptors. Several stimuli, among them the cytokine TNF α , can lead to NF- κ B activation that exerts anti-apoptotic activities. On the other hand, there is ample evidence for apoptosis-promoting functions of NF- κ B as well (Kaltschmidt *et al.*, 2000; Bednarski *et al.*, 2009). The nature of the apoptotic stimulus determines the pro- or anti-apoptotic function of NF- κ B. Activation of NF- κ B can lead to the transcriptional induction of various transcription factor genes, even members of the own Rel/NF- κ B/I κ B family. The enormous amount of NF- κ B target genes can be categorized in the different groups mentioned above, which has been done for the 84 tested genes in the NF- κ B Signalling Targets PCR Array.

Since it is now known that venom from *N. vitripennis* suppresses activation of NF- κ B signalling (see chapter 4), it seems interesting to investigate the consequences of this

inhibition on the expression of its signalling targets. Certain pathologies, like cancer or inflammatory diseases, can cause great alterations in expression levels of NF- κ B target genes. If a certain product can specifically interrupt this alteration, it holds great promise as therapeutic agent. This was investigated by applying *N. vitripennis* venom on macrophage cells after treatment with LPS, presenting the cells with an immune challenge. By analysing the effects of this venom on NF- κ B signalling targets, its possible biomedical application can be revealed.

2. Materials and methods

2.1. Isolation of crude wasp venom

See Materials and methods, Chapter 4.

2.2. Cell culture and treatment

Mouse macrophage-like Raw264.7 cells were maintained in RPMI 1640 medium at 37°C in 5% CO₂ humidified air. Medium was supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 0,1 mg/ml streptomycin. Twenty-four hours before induction, cells were seeded in multiwell dishes so that they were confluent at the time of the experiment. Raw264.7 cells were submitted to the following different treatments: untreated; 6-hour LPS induction (1 μ g/ml); 6 hours and 15 minutes incubation with 10 μ g/ml of *N. vitripennis* venom; 6 hours LPS induction and 6hours and 15 minutes venom incubation. The 4 treatments were performed in duplicate.

2.3. Total RNA extraction and reverse transcription

RNA was isolated by using TRIzol Reagent (Invitrogen) as described previously (De Bosscher *et al.*, 2005). An on-column DNase digestion was performed and the concentration of RNA in all 8 samples was measured. Equal amounts of RNA were reverse-transcribed into cDNA using the RT² First Strand Kit (Qiagen) following the manufacturer's instructions.

2.4. Real-time PCR-based array analysis

The relative expression of NF- κ B signalling target genes was determined in each of the 8 samples by RT-qPCR (RT² Profiler Mouse NF- κ B Signalling Targets PCR Array; SABiosciences Corp., Frederick, MD) using the qPCR master mix (RT² SYBR Green; SABiosciences Corp.) according to the supplier's directions. Mixes were pipetted into 384-well PCR array plates to evaluate the expression of 84 NF- κ B signalling target genes. RT-qPCR was performed in technical duplicates (Roche LightCycler 480, 384-well block). Raw data from the real-time PCR were uploaded using a PCR array data analysis template available at <http://www.sabiosciences.com/pcr/arrayanalysis.php>. Quality controls included within the array plates confirmed the lack of DNA contamination and successfully tested for RNA quality and PCR performance. The integrated Web-based software package for the PCR array system automatically performed all comparative threshold cycle ($\Delta\Delta C_t$)-based fold-change calculations from the uploaded data. For these calculations, the average expression of 3 housekeeping genes (β -actin, glyceraldehydes-3-phosphate dehydrogenase and β 2-microglobulin) was used for normalization of the data. After normalization, the relative expression of each gene was averaged for the 2 samples in each condition. Fold regulations in average gene expression were expressed as the difference in expression of venom-treated compared with untreated cells, or of venom- and LPS-treated compared with only LPS-treated cells. A fold change ≥ 2.5 with $p \leq 0.05$ was considered significant.

3. Results and discussion

To gain better insight in the NF- κ B regulating effects of the *N. vitripennis* venom, a PCR array experiment was set up to evaluate the expression of some NF- κ B target genes under different conditions. Thereto Raw264.7 cells were treated with *N. vitripennis* venom, with or without LPS stimulation. Significant relative expression of 84 genes was evaluated by RT-qPCR as presented in table S1. The genes that show a significantly differential expression of more than 2.5-fold higher or lower in the comparisons of venom-treatment versus no treatment and venom- and LPS-treatment versus LPS-treatment alone are summarized in table 1 in which the NF- κ B target genes are classified according to their properties and/or functions.

The LPS stimulation increased transcription of 36 NF- κ B target genes and down-regulated the expression level of 1. The highest up-regulated genes can be found in the groups of the cytokines/chemokines, stress response genes and growth factors. Several transcription factors show an elevated transcription level, among them TFs that belong to the own NF- κ B cascade. In chapter 4, RT-qPCR on Raw264.7 cells stimulated with *N. vitripennis* venom showed no significant effect in mRNA levels of I κ B α and A20, that are both early response genes. The NF- κ B Signalling Targets PCR Array resulted in 1 NF- κ B target gene that showed down-regulated expression, and 9 that were up-regulated after venom treatment. Remarkable is the up-regulation of Relb expression by venom treatment, which could possibly lead to further elevation in expression of other NF- κ B target genes. On the other hand, Reporter Array performed after venom treatment on human embryonal kidney cells (HEK293T) resulted in the induction of several transcription factors, like ATF2/3/4, C/EBP, AP-1 and MEF2 (figure S1; Formesyn, 2013c) potentially attributing to the up-regulated expression levels shown here.

Before cells are stimulated with LPS, NF- κ B subunits like p50 and p52 can already be bound to NF- κ B binding sites in the promoters of a number of genes, exhibiting a certain range of expression levels (Schreiber *et al.*, 2006). After cellular stimulation with LPS, other NF- κ B family members enter the nucleus and bind to those genes and to other genes, leading to enhanced gene transcription. *N. vitripennis* venom could influence this process before cells are stimulated with LPS resulting in the down-regulated expression level of NF- κ B target gene, cyclin D1.

Transcription of the stress response gene NAD(P)H dehydrogenase quinone 1 (Nqo1) is almost 25 times up-regulated after venom treatment. This gene has been demonstrated to play an important role in protecting cells against oxidative stress (Dinkova-Kostova & Talalay, 2000). Venoms of several animals, including the parasitoid wasp *Aphidius ervi*, are known to cause oxidative stress in their host organism (Falabella *et al.*, 2007; Katkar *et al.*, 2014). The elevation of the Nqo1 gene expression after venom treatment in macrophage cells could therefore suggest a protective function. Remarkably, the venom induced transcription of 2 growth factors, colony stimulating factor 1 and 3 (Csf1 and Csf3) that stimulate the bone marrow progenitor cells to differentiate into macrophages or granulocytes respectively.

These proteins have an important role in innate immunity and inflammation (Takahashi *et al.*, 1996). Especially transcription of *Csf1* is highly up-regulated (nearly 56 times), which even doubles when the cells are immune challenged with LPS.

However, most differentially expressed genes could be observed when venom was added to the macrophage cells, combined with an immune challenge of LPS. Transcription of two of the NF- κ B target genes tested by the NF- κ B Signalling Target PCR Array were previously tested by RT-qPCR: I κ B α and IL-6 (see chapter 4). Although the suppression of I κ B α transcription (see figure 7A in chapter 4) could not be validated in this experiment, the 2-fold inhibition of IL-6 transcription on the other hand (see figure 2B in chapter 4) could be confirmed by a 1.5-fold down-regulation in the PCR Array. Transcription of the cytokine IL-1 β , produced by activated macrophages and an important mediator of inflammatory response, shows a nearly 6-fold up-regulation after venom-treatment, while being 4.3 times down-regulated after co-treatment of venom and LPS. Apparently, the cells need to be immune challenged, in this case by LPS, in order for the venom to be able to suppress the inflammatory response.

In the previous chapter, we focussed on the inhibitory effect of venom on the canonical NF- κ B pathway, in which IKK β phosphorylates I κ Bs at N-terminal sites to trigger their ubiquitin-dependent degradation and induce nuclear entry of RelA:p50 dimers (Karin & Ben-Neriah, 2000). The remark has to be made that next to this classical NF- κ B signalling pathway, an alternative non-canonical signalling cascade has been identified that activates NF- κ B signalling based on processing of the NF κ B2:p100 precursor protein by IKK α (Xiao *et al.*, 2001, figure 1). In this alternative NF- κ B pathway, the stimulation by LPS (among others) results in the nuclear translocation of the dimer RelB:p52 (Mordmuller *et al.*, 2003). While many target genes are shared between the canonical and the non-canonical pathways, some promoters of NF- κ B target genes are only recognized by RelB:p52 dimers and not by RelA:p50 dimers (Bonizzi *et al.*, 2004;Schreiber *et al.*, 2006). Several NF- κ B target genes can bind multiple members of the NF- κ B family, suggesting that they can be activated by both the canonical and the non-canonical pathways. For instance, *Icam1* and *Gadd45b* can bind all 5 members of the NF- κ B family (p52, p50, RelA, Relb and c-Rel) in U937 cells after LPS stimulation (Schreiber *et al.*, 2006). When Raw264.7 cells were treated with *N. vitripennis*

venom, transcription of *Icam1* and *Gadd45b* were up-regulated respectively 17.63 and 10.99 times. Therefore, it can be suggested that *N. vitripennis* venom potentially has an inhibitory effect on the canonical pathway (see chapter 4), but a stimulatory effect on the alternative NF- κ B signalling pathway explaining the up-regulation of some inflammatory genes. This hypothesis however needs to be further investigated. Interestingly, *N. vitripennis* venom up-regulated transcription of 4 different NF- κ B subunits when cells were co-treated with LPS, of which *Nfkb2* (p52) and *Relb*, that take part in the non-canonical NF- κ B pathway, show the highest elevations.

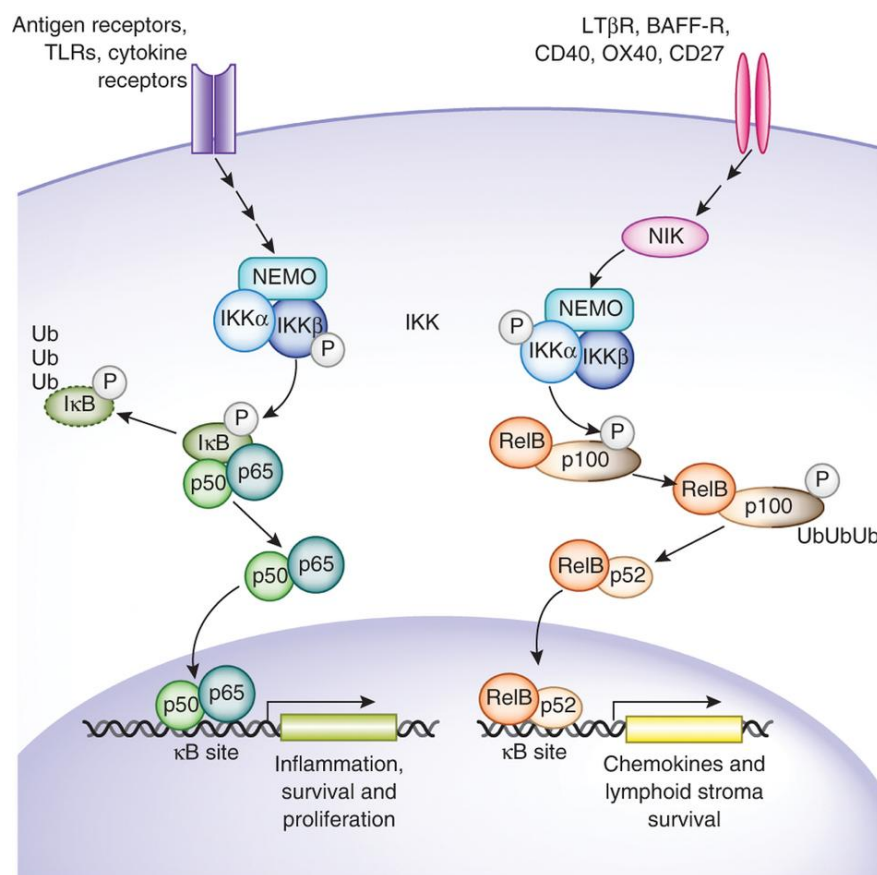


Figure 1: The canonical NF- κ B pathway (left) induced by signals including antigens, TLR ligands and cytokines such as TNF uses a wide variety of signalling adaptors to engage and activate the IKK β subunit of the IKK complex. IKK β phosphorylation of classical I κ B proteins bound to NF- κ B dimers such as p50-p65 results in ubiquitination (Ub) of I κ B and proteasome-induced degradation. This allows NF- κ B to enter the nucleus where it binds specific DNA sequences (κ B sites). The noncanonical pathway (right) engaged by members of the TNF-like family of cytokines requires NIK to activate IKK α , which then phosphorylates p100 (NF- κ B2), triggering its proteosomal processing needed for the activation of p52-RelB dimers. (figure from Gerondakis *et al.*, 2014)

Table 1: Differentially expressed NF- κ B signalling target genes in Raw264.7 cells after treatment with *N. vitripennis* venom, either induced with LPS or not. Gene fold regulations for the comparison of venom- and LPS-treatment versus LPS-treatment alone are presented in the third column (Venom+LPS/LPS). The last column contains differentially expressed genes for the comparison of venom-treatment versus no treatment (Venom/veh). When no number is inserted, the fold regulation was not significant ($p \leq 0.05$ or/and $FR \geq -2.5$ or ≤ 2.5). (Abb = abbreviation; FR = fold regulation)

Differentially expressed NF- κ B target genes	Abb	FR (Venom+ LPS/LPS)	FR (Venom/ veh)
<i>Cytokines, chemokines and their modulators</i>			
Interleukin 15	Il15	13.41	
Interleukin 1 beta	Il1b	-4.39	5.88
<i>Immunoreceptors</i>			
CD40 antigen	Cd40		9.93
CD83 antigen	Cd83	29.40	
<i>Cell adhesion molecules</i>			
Intercellular adhesion molecule 1	Icam1	17.63	
Vascular cell adhesion molecule 1	Vcam1	3.48	
<i>Stress response genes</i>			
NAD(P)H dehydrogenase, quinone 1	Nqo1		24.64
<i>Regulators of apoptosis</i>			
B-cell leukemia/lymphoma 2 related protein A1a	Bcl2a1a		5.03
Baculoviral IAP repeat-containing 2	Birc2	6.29	
Baculoviral IAP repeat-containing 3	Birc3	3.60	
Fas (TNF receptor superfamily member 6)	Fas	6.86	3.26
Tnf receptor-associated factor 2	Traf2	5.11	
<i>Growth factors, ligands and their modulators</i>			
Colony stimulating factor 1 (macrophage)	Csf1	117.79	55.85
Colony stimulating factor 3 (granulocyte)	Csf3	-31.72	5.49
<i>Transcription factors and their modulators</i>			
Interferon regulatory factor 1	Irf1	13.95	
Microphthalmia-associated transcription factor	Mitf	7.25	
Myelocytomatosis oncogene	Myc		17.90
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	Nfkb1	3.40	
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p52/p100	Nfkb2	10.25	
Reticuloendotheliosis oncogene	Rel	3.86	
Avian reticuloendotheliosis viral (v-rel) oncogene related B	Relb	8.68	3.59
<i>Miscellaneous</i>			
Cyclin D1	Ccnd1	-7.44	-2.67
Growth arrest and DNA-damage-inducible 45 beta	Gadd45b	10.99	
Matrix metalloproteinase 9	Mmp9	-5.80	

The potential of *N. vitripennis* venom as therapeutic agent

Interesting is the number of up-regulated NF- κ B target genes involved in apoptosis regulation after venom and LPS co-treatment. Formesyn and colleagues have previously proven that serine proteases and metalloproteases in *N. vitripennis* venom cause apoptosis in non-host insect cells and suggested their possible role in immune related processes (Formesyn *et al.*, 2013b). In this PCR Array on murine macrophages, pro- as well as anti-apoptotic mediators were differentially expressed: Fas activation induces apoptosis (Huang *et al.*, 1999), while Birc2, Birc3 and Traf2 are known for their anti-apoptotic effects (Wang *et al.*, 2012; Lin *et al.*, 2003).

An acute stimulation, for instance by LPS, is known to create two distinct waves of NF- κ B recruitment to target promoters: a fast recruitment to immediately accessible promoters and a late recruitment to promoters requiring stimulus-dependent modifications in chromatin structure to make NF- κ B sites accessible (Saccani *et al.*, 2001). A relatively long (6 hours) LPS-treatment was chosen in the PCR Array experiment, so the data are in relation to the results presented in chapter 4. Unfortunately, possible transient effects caused by *N. vitripennis* venom, due to the short mRNA half life of many of the early response genes, cannot be observed by this experiment and time kinetics would need to be performed in order to have a glimpse on the complete venom profile.

Besides an NF- κ B binding site, the promoter of the genes tested by this PCR Array may also contain binding sites for other transcription factors (TFs). Instead of an up-regulated transcription by NF- κ B binding to the promoter, expression of these genes can also be targeted by other TFs. When performing a TFSEARCH, the promoter sequence of a gene of interest can be screened for possible TF binding sites by correlating this sequence against the TRANSFAC MATRIX database. The upstream gene sequence 2000 nucleotides before and 50 nucleotides after the start of the gene (where the promoter sequence is assumed to be located) is inserted into the TFSEARCH program and a 85.0 threshold is used. This was done for 5 genes that showed the highest significant up-regulated fold regulation (FR) after venom-and LPS-treatment, and for 4 genes that had a negative FR after venom- and LPS-treatment. All 9 genes obviously contained the NF- κ B TF binding site, as the genes included in the array were previously demonstrated to be NF- κ B target genes. The TF binding sites that were predicted by the program to be present in all 5 up-regulated genes were listed in table

2. This means that the up-regulated transcription of these genes, next to NF- κ B binding to their promoter, could also be the result of binding of these other TFs to their promoters. When looking at these TF binding sites in the promoters of the 4 down-regulated genes, cAMP response element-binding protein (CRE-BP) can be a possible candidate for causing the synthesis of several of the tested genes while at the same time not having an increasing effect in transcription of *Csf3*, *Mmp9* and *Ccnd1*, since these genes do not contain a binding site for CRE-BP. On the other hand, a role of CCAAT-enhancer-binding proteins (C/EBPs) in the activation of NF- κ B target genes can also be proposed. C/EBP binding sites can be found in promoter regions of several tested genes with an up-regulated transcription, while being absent in the promoters of some of the down-regulated genes (see table 2). Additionally, C/EBPs can interact with p50 homodimers activating NF- κ B target genes, even in the absence of canonical NF- κ B activation (Dooher *et al.*, 2011). Interestingly, the reporter array performed on HEK293T cells treated with *N. vitripennis* venom, also showed significant up-regulation of the C/EBP reporter (figure S1). The presence (or absence) of many other TF binding sites in specific gene promoters could possibly aid to explaining the apparent contradiction between the up-regulation of several inflammatory genes by *N. vitripennis* venom, but at the same time the anti-inflammatory activity of the venom on the other hand (see chapter 4). Other mechanisms can play a role in the change of gene expression: microRNAs can post-transcriptionally bind to the 3'-UTR (untranslated region) of their target mRNAs and repress protein production (Cannell *et al.*, 2008), or epigenetic phenomena like DNA methylation or histone modification can cause differential gene expression (Jaenisch & Bird, 2003). Until now, none of these processes have been investigated for the gene alterations caused by parasitoid venoms.

The remark has to be made that venom from *N. vitripennis* is a complex mixture of at least 80 different proteins. Maybe even more other venom compounds like peptides or other biomolecules could be present, all having their own effects on gene regulation. Regarding to venomous effects on the complex apoptosis process, it would be interesting to see how the separate venom compounds, especially the serine proteases and metalloproteases as tested in insect cells (Formesyn *et al.*, 2013b), would affect the mammalian cell death process. But it would also be interesting to investigate what the effects of individual venom compounds would be on mammalian cells, concerning gene regulation.

Table 2: Transcription factor binding sites that are commonly present in the promoters of 5 up-regulated genes tested by the NF- κ B Signalling Targets PCR Array, predicted by TFSEARCH (threshold 85.0). The presence of these TF binding sites is shown for 4 down-regulated genes when cells were treated with venom and LPS. 'x' represents the presence of the respective TF site in the promoter region of that particular gene. At the bottom are the fold regulations presented of the selected genes.

NF- κ B signalling target genes	Cd83	Csf1	IL15	Irf1	Icam1	Il1b	Csf3	Mmp9	Ccnd1
<i>TF binding site</i>									
NF-Kap	x	x	x	x	x	x	x	x	x
C/EBP	x	x	x	x	x	x	x	x	
C/EBPa	x	x	x		x		x	x	
AML-1a	x	x	x	x	x	x	x	x	x
CdxA	x	x	x	x	x	x	x	x	x
CRE-BP	x	x	x	x	x	x			
deltaE	x	x	x	x	x	x	x	x	x
GATA-1	x	x	x	x	x	x	x	x	x
GATA-2	x	x	x	x	x	x	x	x	x
GATA-3	x	x	x	x	x		x	x	x
GATA-X	x	x	x	x	x	x	x		x
HSF2	x	x	x	x	x	x		x	
MZF1	x	x	x	x	x	x	x	x	x
Nkx-2.	x	x	x	x	x	x	x	x	x
Oct-1	x	x	x	x	x	x	x	x	
SRY	x	x	x	x	x	x	x	x	x
TATA	x	x	x	x	x	x		x	x
<i>Fold regulation</i>									
venom- and LPS- vs LPS-treated	29.4	117.8	13.4	14.0	17.6	-4.4	-31.7	-5.8	-7.4
venom-treated vs untreated	3.0	55.9	2.5	2.0	1.2	5.9	5.5	1.6	-2.7

Many of the target genes of NF- κ B signalling are involved in multiple inflammatory or autoimmune diseases. Some of them are promising targets in certain therapies (Dziadziuszko & Jassem, 2012; Lawlor *et al.*, 2004), while others can themselves be used as agents in processes involved in human diseases (Murray, 1994; Bouker *et al.*, 2005). Interestingly, *N. vitripennis* venom significantly altered the expression of some of these possible drug targets, presenting the venom with an exciting potential as therapeutic in several diseases (table 3). The second highest up-regulated NF- κ B target gene transcription tested after the addition of solely venom on the cells, was NAD(P)H:quinone oxidoreductase 1 (Nqo1). This enzyme detoxifies quinones and reduces oxidative stress. Low activity of this enzyme is associated with increased risk of acute leukemia in adults (Smith *et al.*, 2001). Inducing this

detoxification enzyme in certain mammalian cell types could potentially benefit patients suffering from acute leukemia.

For the other venom targets with potential therapeutic application, the cells needed to be immune challenged with LPS, offering a possible use in diseases characterized by constitutive activity of NF- κ B, like autoimmune diseases (Ellrichmann *et al.*, 2012) or many cancers (Voboril & Weberova-Voborilova, 2006). With regard to anti-inflammatory drugs, colony stimulating factor 3 (Csf3) with a nearly 32-fold decrease in transcription after venom and LPS incubation compared to LPS induction, seems the most interesting to further investigate. This regulator of granulopoiesis has a critical role in driving joint inflammatory diseases, like rheumatoid arthritis (RA), and its antagonists may be of therapeutical value (Lawlor *et al.*, 2004). Anti-IL-1 β is a commonly used drug with the name canakinumab used in several auto inflammatory diseases (Dinarello, 2010). Previously mentioned inhibition of IL-1 β transcription by venom- and LPS-treatment may therefore hint at a therapeutic role of *N. vitripennis* venom.

Intriguingly, the possible anti-cancer role of *N. vitripennis* venom is displayed by the regulation of different NF- κ B targets. Transcription of Cyclin D1, involved in the G1-S phase transition of cells, is significantly suppressed by *N. vitripennis* venom, with and without LPS-treatment. Since pharmacological inhibition of cyclin D1/CDK4 complexes is suggested to be a useful strategy to inhibit the growth of tumours, the potential of *N. vitripennis* venom in cancer treatments is worthwhile to further investigate. In contrast, the expression of Irf1 is significantly up-regulated after the co-treatment of venom and LPS. A functional role of Irf1 was established in the growth suppression of breast cancer cells and it was implicated to act as a tumor suppressor gene in breast cancer by controlling apoptosis (Bouker *et al.*, 2005). The potential of *N. vitripennis* venom as therapeutic agent in several oncogenetic diseases is therefore interesting to dig into.

The potential of *N. vitripennis* venom as therapeutic agent

Table 3: NF- κ B target genes that were differentially transcribed and can be a possible drug target of *N. vitripennis* venom. Three different comparisons are presented: venom-treated versus no treatment, venom- and LPS-treatment versus LPS-treated cells and LPS-treatment versus no treatment. In bold is the fold regulation that lead to the assumption of the venom being a possible drug target. (Abb = abbreviation; FR = fold regulation) Fold regulations are only mentioned when $p \leq 0,05$ and $FR \geq 2,5$ and $FR \leq -2,5$

Possible drug targets of venom	Abb	FR venom (untreated control)	FR venom+LPS (LPS control)	FR LPS (untreated control)	Potential targeted diseases	Reference
NAD(P)H dehydrogenase, quinone 1	Nqo1	24.64			acute leukemia	(Smith <i>et al.</i> , 2001)
Cyclin D1	Ccnd1	-2.67	-7.44	-5.05	breast cancer	(Grillo <i>et al.</i> , 2006)
Interferon regulatory factor 1	Irf1		13.95	2.95	breast cancer	(Bouker <i>et al.</i> , 2005)
Matrix metalloproteinase 9	Mmp9		-5.80	17.82	cancer	(Hua <i>et al.</i> , 2011)
Colony stimulating factor 3 (granulocyte)	Csf3	5.49	-31.72	15821.68	inflammatory arthritis	(Lawlor <i>et al.</i> , 2004)
Interleukin 1 beta	Il1b	5.88	-4.39	15647.33	autoinflammatory diseases	(Dinarello, 2010)
Complement factor B	Cfb		-4.20	14.68	complement mediated inflammatory diseases	(Ruiz-Gomez <i>et al.</i> , 2009)

4. Conclusion

The NF- κ B Signalling Target PCR Array performed on Raw264.7 macrophages treated with *N. vitripennis* venom and/or LPS uncovered several new ideas on how the venom exerts its complex effects. The inhibition of the canonical NF- κ B pathway by the venom described in chapter 4 is however not that obvious when looking at the expression of a large number of NF- κ B target genes. Several aspects encourage us to be cautious in interpreting the results. The complexity of the venom mixture applied on the cells creates multiple crosstalk effects, that could be circumvented when working with separate venom compounds. The fact that one time-point was used, only gives a glimpse of the complete picture. Additionally, changes in transcription expression do not always translate into the same changes in protein level, since alterations in translation efficiency and post-translational modifications can lead to a different end-result. Possible effects on the non-canonical NF- κ B pathway next to the canonical pathway, in addition to the presence of multiple TF sites in the promoter of NF- κ B target genes, makes the puzzle extra hard to solve. However, keeping all these remarks in mind, still several interesting hints for future research could be noted, which was the original intent of this experiment. Some NF- κ B target genes that were differentially expressed by the addition of *N. vitripennis* venom, with or without LPS-treatment, are drug targets of major diseases, hinting to possible future biomedical application as therapeutic agent in several human diseases. Performing time kinetics and dose-responses by the separated venom compounds seems the logical next step.

5. Supplementary materials

Table S1: Effect of *N. vitripennis* venom in Raw264.7 cells, either induced with LPS or not, on NF- κ B signalling targets. Fold regulation of all tested NF- κ B signalling target genes are presented in the different comparisons with either LPS-treated or untreated cells as control. When no fold regulation is inserted, $p > 0,05$ (Abb = abbreviation; FR = fold regulation)

NF- κ B signalling target genes	Abb	FR (LPS versus untreated control)	FR (venom versus untreated control)	FR (LPS- and venom versus LPS control)
<i>Cytokines/chemokines and their modulators</i>				
Chemokine (C-C motif) ligand 22	Ccl22	917.635		
Chemokine (C-C motif) ligand 5	Ccl5	1254.881		
Chemokine (C-C motif) receptor 5	Ccr5	1.625		
Chemokine (C-X-C motif) ligand 10	Cxcl10	483.835		
Chemokine (C-X-C motif) ligand 3	Cxcl3	111.806		
Interleukin 15	Il15			13.408
Interleukin 1 alpha	Il1a	1273.082		
Interleukin 1 beta	Il1b	15647.327	5.885	-4.392
Interleukin 1 receptor antagonist	Il1rn	26.052		
Interleukin 6	Il6	525.452		-1.472
Lymphotoxin A	Lta	25.056		
Tumor necrosis factor	Tnf	36.399		-1.193
<i>Immunoreceptors</i>				
CD40 antigen	Cd40	68.505	9.933	
CD80 antigen	Cd80	3.113		
CD83 antigen	Cd83			29.395
Tumor necrosis factor receptor superfamily, member 1b	Tnfrsf1b	22.445		
<i>Proteins involved in antigen presentation</i>				
Complement component 3	C3	3.651		
Complement factor B	Cfb	14.677		-4.199
<i>Cell adhesion molecules</i>				
Intercellular adhesion molecule 1	Icam1			17.631
Vascular cell adhesion molecule 1	Vcam1			3.481
<i>Acute phase proteins</i>				
Coagulation factor III	F3	41.407		
<i>Stress response genes</i>				
NAD(P)H dehydrogenase, quinone 1	Nqo1		24.637	
Prostaglandin-endoperoxide synthase 2	Ptgs2	698.790		
Superoxide dismutase 2, mitochondrial	Sod2	4.919	-1.052	
<i>Regulators of apoptosis</i>				
B-cell leukemia/lymphoma 2 related protein A1a	Bcl2a1a	33.896	5.027	
Bcl2-like 1	Bcl2l1	2.677		

Baculoviral IAP repeat-containing 2	Birc2			6.288
Baculoviral IAP repeat-containing 3	Birc3	1.874		3.599
Fas (TNF receptor superfamily member 6)	Fas	11.621	3.261	6.857
Tnf receptor-associated factor 2	Traf2		1.983	5.107
<i>Growth factors, ligands and their modulators</i>				
Colony stimulating factor 1 (macrophage)	Csf1	35.675	55.854	117.792
Colony stimulating factor 2 (granulocyte-macrophage)	Csf2	530.000		
Colony stimulating factor 3 (granulocyte)	Csf3	15821.676	5.486	-31.724
Platelet derived growth factor, B polypeptide	Pdgfb	3.395		
<i>Transcription factors and regulators</i>				
Interferon regulatory factor 1	Irf1	2.945		13.953
Microphthalmia-associated transcription factor	Mitf			7.247
Myelocytomatosis oncogene	Myc	25.814	17.898	
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	Nfkb1	4.526		3.399
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	Nfkb2			10.255
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Nfkbia	7.963		
Reticuloendotheliosis oncogene	Rel	2.664		3.864
Avian reticuloendotheliosis viral (v-rel) oncogene related B	Relb		3.591	8.675
Signal transducer and activator of transcription 1	Stat1	2.855	-1.257	
Signal transducer and activator of transcription 3	Stat3	1.517	-1.104	-1.444
<i>Miscellaneous</i>				
Cyclin D1	Ccnd1	-5.051	-2.667	-7.439
Growth arrest and DNA-damage-inducible 45 beta	Gadd45b	8.330		10.985
Matrix metalloproteinase 9	Mmp9	17.819		-5.805

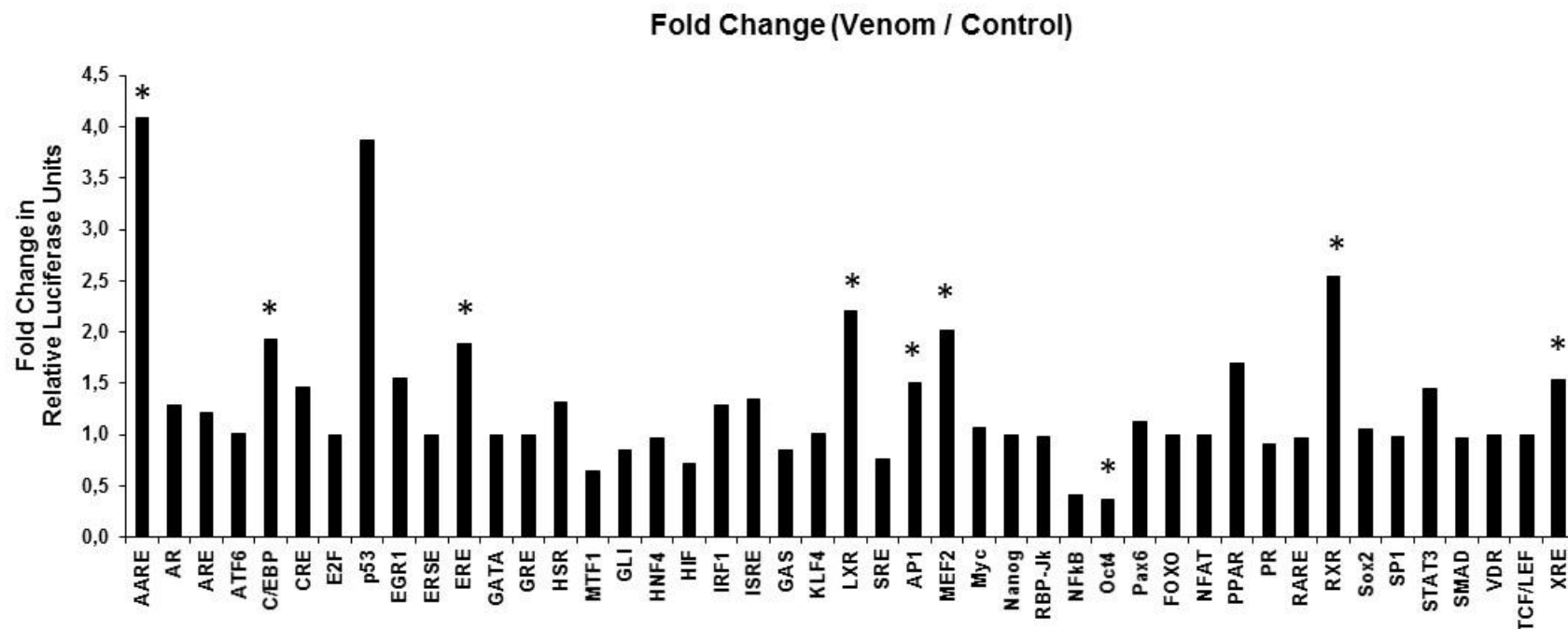


Figure S1: Selective up- and down-regulation of several pathways after *N. vitripennis* venom treatment in HEK293T cells tested by Cignal Finder™ 45-Pathway Reporter Array. Normalized values of both test and control samples were used to calculate the mean fold change of pathway (in)activation. A fold change of 1 indicates no differences in pathway activation between test and control samples. A fold change < 1 indicates a pathway down regulation in venom treated cells, up-regulation of pathways upon venom treatment is indicated by a fold change > 1. Asterisks indicate the significant up- and down-regulation of pathways when comparing relative luciferase units of venom-treated samples with untreated samples, ANOVA with Bonferroni posthoc test, * $p < 0.05$.

PART IV

DISCUSSION

CHAPTER 6

General discussion and future perspectives

1. Molecular traits of the parasitoid-host interaction

Attack and oviposition by parasitic wasps induce vigorous physiological alterations in the host insect. Although endo- and ectoparasitoids differ in venom composition and mode of egg-laying, successful parasitization by both results in alterations of host development, immunity and metabolism (Asgari & Rivers, 2011; Danneels *et al.*, 2010). In the end, hosts are completely consumed by the developing wasp larvae, but the different strategies used by female wasps varies extensively. Since endoparasitoids lay their eggs inside the host body tissues, effective ways to evade host defence responses are crucial. *Asobara tabida* for instance, an endoparasitoid of *D. melanogaster*, lays eggs that adhere to the fat body and other internal organs of the host. This results in incomplete formation of a capsule, which allows the parasitoid eggs to hatch and escape encapsulation (Wertheim *et al.*, 2005). Because ectoparasitoids lay their eggs on the outside of hosts, less extensive suppression of host immune systems can be expected. On contrary, *Nasonia vitripennis* lays its eggs on the outside of the host outer integument, and was proven to affect host hemocytes mediating cellular and humoral immune responses (Rivers *et al.*, 2002b; Qian *et al.*, 2013). To offer the best possible environment for the offspring, parasitoids use an extensive arsenal of virulence factors: polydnviruses, virus-like particles, venom, teratocytes and proteins secreted from larvae (Beckage & Gelman, 2004). Interestingly, *N. vitripennis* only has venom and larval secretions at its disposal to ensure successful parasitization. This means that before the eggs hatch and larvae actively start feeding, host physiology can only be channelled by maternally derived venom. Aiming at understanding this fascinating interaction, we performed a transcriptomic study on parasitized flesh fly pupae targeting the early stages of host response to envenomation (chapter 3). Since *N. vitripennis* larvae start host feeding 24 hours after egg-laying, time-points explicitly before that stage were chosen. The study revealed that only 1 transcript was differentially expressed in *S. crassipalpis* pupae 3 hours post-parasitization by *N. vitripennis*. This is in contrast with results from a transcriptome study on hemocytes from *Musca domestica* treated with extracted venom from *N. vitripennis*. One hour after venom treatment, differentially expressed transcripts revealed effects related to immune and stress response, apoptosis, metabolism, transport and transcription/translation regulation (Qian *et al.*, 2013). Furthermore, other studies using host hemocytes of *S. bullata* or specific tissues such as the fat body and brains, showed that envenomation by *N.*

vitripennis induces alterations in cell behaviour and toxicity, which were already visible after 1 hour (Rivers *et al.*, 2002b). Since whole bodies of parasitized flesh fly pupae were used for the transcriptome study, transcripts with various regulation in different cell types or tissues could be ruled out. All tissues and cell types are included in the RNA sample, possibly resulting in the absence of specific transcripts. Nonetheless, this approach in which unnecessary manipulations of tissue dissections are avoided, was still found favourable, ensuring unharmed samples. With this scope in mind, successful parasitization by *N. vitripennis* in the flesh fly host also must be verified without opening the pupae in an attempt to search for parasitoid eggs. Therefore, a screening tool was developed that targets *Nos* mRNA in parasitoid eggs by the use of RT-qPCR.

Twenty-five hours after envenomation, a lot more genes were differentially expressed compared to 3 hours post parasitization. In total, 19 genes were significantly down-regulated and 109 up-regulated. By clustering these genes in groups relating to their possible function, 3 major host processes appeared to be changed by parasitization: the immune system, host development and metabolism. Concerning host immunity, several suggestions can now be made of potential interactions between venom and host pathways. Imbalance in Ca^{2+} homeostasis in the mitochondria caused by *N. vitripennis* venom appears to facilitate immune modulation by promoting apoptosis in hemocytes. Next to this, Rho signalling pathway members were found to be differentially expressed. Activation of this pathway promotes blebbing by actin cytoskeleton reorganisation, hereby reducing hemocytes to produce pseudopodial projections critical for spreading responses. MAPK signalling seems a promising candidate for regulating both cellular immune response to envenomation as well as hemocyte apoptosis. Since envenomation by *N. vitripennis* has long been associated with decreased melanisation of *Sarcophaga* host hemolymph (Rivers *et al.*, 2002b; Abt & Rivers, 2007), it was not surprising to find up-regulated transcripts that potentially impede with pro-phenoloxidase activity and host melanisation response. Interestingly, not a single antimicrobial peptide transcript was found to be differentially expressed, which is consistent with another study on host-endoparasitoid interaction that shows minimal AMP up-regulated expression (Wertheim *et al.*, 2005). We have to remark however, that these transcriptomic studies cannot disentangle the host defence response from the manipulations caused by the parasitoid. Therefore, further studies investigating specific

General discussion and future perspectives

immune responses of the host to different threats (antimicrobial, parasites, viruses), compared to the additional infection with parasitoids, could aid to filter out the exact manipulations caused solely by the parasitoid.

Molecular techniques in science are constantly evolving. When this transcriptomic study was planned in 2008, microarray was widely-used and had proven to be successful in reaching publishable results. Next generation sequencing had only recently made its entrance and was a lot more expensive at that time. Nowadays, this technique became increasingly affordable and would probably now be the preferred approach, since only an EST database is available for the host *S. crassipalpis*. The group of John Werren at the University of Rochester has recently performed an elaborate transcriptomic study on *S. bullata* pupae parasitized by *N. vitripennis* at different time points by RNA sequencing (unpublished data). It would be interesting to see whether their results can confirm current data and maybe new interactions have come to light.

Following on the transcriptomic study, the obvious next quest is to unravel what venom compounds are causing the effects. The combination of bioinformatic and proteomic studies has revealed 80 proteins in the venom of *N. vitripennis* (de Graaf *et al.*, 2010;Ye *et al.*, 2010). Twenty-three of these proteins showed no homology to any known protein. Computational tools failed to designate a function to these proteins based on sequence and structural homology of proteins with known functions. Since a vast majority of proteins interacts with others for proper biological activation, the function of the novel protein may be extrapolated by knowing the function of its binding partners (Phizicky & Fields, 1995). Investigating the interacting partner of *N. vitripennis* venom compounds would therefore be one approach. Another method that has already been used for parasitoid wasps to assign functions to venom proteins, is the injection of recombinant expressed venom protein into the host (Price *et al.*, 2009;Wang *et al.*, 2013;Colinet *et al.*, 2011). Yet, a completely different approach to tackle this functionality research, is silencing one (or more) venom protein(s) in the female *N. vitripennis* wasp. After both injection of recombinant expressed venom compounds and silencing the compound in the wasp, specific bio-assays need to be performed in order to investigate the effect of this compound (or the lacking of this compound) on the host physiology. Mostly, general effects caused by natural parasitization

are known, presenting several possibilities of processes that the single compound could be involved in. For the interaction of *N. vitripennis* with its host, several bio-assays are extensively described and already performed on naturally parasitized hosts concerning developmental arrest (Rivers & Denlinger, 1994a), apoptosis (Rivers *et al.*, 2009), melanisation (Abt & Rivers, 2007), encapsulation (Rivers *et al.*, 2002b) and hemocyte spreading (Rivers *et al.*, 2010). Both approaches to unravel functionalities of *N. vitripennis* venom compounds were tested. Unfortunately, due to practical difficulties, both techniques could not have been followed through successfully. The preliminary studies that have been executed and possible solutions for the road blocks we ran into, are suggested in the addendum to chapter 3.

2. Biomedical application of *N. vitripennis* venom

Working with *N. vitripennis* in the laboratory has many practical advantages: short generation time, availability during the whole year, large amount of offspring and many more. One unfortunate disadvantage however, is the small size of these wasps together with the low quantity of venom they produce. When performing experiments to investigate the mode of action of this venom, dissecting the venom glands out of the female wasps is inevitable. Luckily, extracted *N. vitripennis* venom samples can be stored for a period of time in -20°C remaining its biological activity, making it possible to perform the dissections several weeks before the planned assays. All experiments to investigate the biomedical application of *N. vitripennis* venom were performed with manually dissected extracts. The discovery of the exact venom compound that is causing the anti-inflammatory effects on mammalian cells, would imply that laborious dissections can be replaced by use of the recombinantly expressed protein or synthesised peptide, depending on the nature of the specific compound.

The fact that venom of parasitoid wasps suppresses the immune system of the host, is no secret for several decades now. Even the fact that these parasitoids can interfere with the insect NF- κ B signalling cascade by injecting virulence factors into the host, is known. In endoparasitoids, polydnviruses injected along with the wasp eggs into the host are responsible for this specific immune suppression (Thoetkiattikul *et al.*, 2005; Kroemer &

Webb, 2005; Bae & Kim, 2009; Gundersen-Rindal & Pedroni, 2006; Gueguen *et al.*, 2013). Instead of replicating in the host, these viruses interfere with the immune system. Polydnviruses can code for proteins that destroy hemocytes (Kumar & Kim, 2014) or can interfere with the production of phenoloxidase, suppressing host melanisation (Lu *et al.*, 2010). Finally, polydnviruses can encode a family of genes with homology to I κ B proteins from insects and mammals. These I κ B proteins have posterior ankyrin repeats that act as irreversible inhibitors to NF- κ B signalling in the host, hereby suppressing the insect immune response. Surprisingly, ectoparasitoids that only possess venom as virulence factor are also able to effectively suppress host immunity without the use of polydnviruses. An endogenous venom compound must therefore be the responsible agent in the inhibition of host defence system by the ectoparasitoid wasp *N. vitripennis*.

Targeting immune pathways of enemies by immune-suppressive molecules is a general strategy for success among Hymenopteran insects (Jang *et al.*, 2006). Components in honeybee venom protect bees against arthropod and vertebrate predators. Unintentionally, however, bee venom also has an anti-inflammatory mode of action and is an ancient therapy for chronic inflammation and pain relief (Cherniack, 2010). Viral ankyrins (vankyrins) in endoparasitoids were reported to inhibit NF- κ B signalling, presenting one possible mechanism by which anti-inflammatory effects of Hymenopteran products may be realized (Gueguen *et al.*, 2013). Since inhibition of NF- κ B signalling continues to be a significant area of research for strategic development of drug targets for human diseases (Gilmore & Garbati, 2011), possible other mechanisms of anti-inflammation used by ectoparasitoids may provide potential for the treatment of inflammation-based human diseases from arthritis to cancer. Therefore, extracted *N. vitripennis* venom was applied on different types of mammalian cells in order to further investigate how this immune suppressive action in insects translates to higher evolved metazoans.

When cytotoxicity towards *N. vitripennis* venom was estimated by MTT assay, the insect cell line Sf21 showed first cytotoxic effects 6 hours after treatment that became more distinct after 24 hours induction (Formesyn *et al.*, 2013b), while in the human cell line HEK293T, it took at least 48 hours until venom resulted into first signs of cytotoxicity (Formesyn, 2013c). Even though low toxicity appeared in HEK293T cells after venom treatment, several studies

revealed that the cells suffered from an increase in stress responses affected by the venom. The highest up-regulated reporter tested by the Signal Finder™ 45-Pathway Reporter Arrays on HEK293T cells after *N. vitripennis* treatment, was the amino acid response element (AARE) reporter, which is known to be an early response upon amino acid deprivation of ER stress (chapter 5, figure S1; Formesyn, 2013c; Chen *et al.*, 2005). AARE contains a C/EBP-ATF composite site that is composed of a half-site for the C/EBP family of TFs and a half-site for the ATF family of TFs (Schneider *et al.*, 2012). ATF4 is an important regulator of several ER stress target genes, amino acid transporters and antioxidants (Saito *et al.*, 2011). By binding to the C/EBP-ATF composite site within the AARE, ATF4 transactivates C/EBP protein-homologous protein (CHOP). CHOP is a multifunctional TF in the ER stress response, involved in apoptosis and inflammation (Nishitoh, 2012). Interestingly, the C/EBP reporter was also significantly up-regulated in the performed Reporter Array after venom treatment, suggesting the likely activation of ER stress by venom. In addition, possible involvement of C/EBP in transcriptional regulation by *N. vitripennis* venom has also been proposed in chapter 5 when Raw264.7 macrophages were co-treated with LPS and venom, as analysed by PCR Array. The presence of C/EBP binding sites in promoter regions of several venom up-regulated genes, together with its absence in promoters of some down-regulated genes, suggested its role in the seemingly contradictory up-regulation of inflammatory genes. In addition, ER stress also induces the IRE1-TRAF2-ASK1-JNK pathway that contributes to CHOP-mediated macrophage cell death (Sano & Reed, 2013). Both TRAF2 transcription and JNK phosphorylation were found to be enhanced by co-treatment of *N. vitripennis* venom and LPS on macrophages, respectively detected by PCR Array (chapter 5, table 1) and western blotting (chapter 4, figure 6). Based on the results of current experimental data, we hypothesize that *N. vitripennis* venom possibly causes ER stress, characterized by alterations in the AARE and C/EBP reporters, together with enhanced TRAF2 transcription and prolonged JNK activation after co-treatment with LPS, eventually leading to cell death.

Multiple experiments undoubtedly have proven that *N. vitripennis* venom suppresses mammalian NF- κ B activity. TNF-stimulated NF- κ B activation was significantly suppressed in L929sA fibrosarcoma cells, while LPS-stimulated Raw264.7 macrophages showed a decrease in protein secretion and gene transcription of the NF- κ B regulated cytokine IL-6. In addition, transcription and translation of 2 negative feedback regulators that prevent excessive NF- κ B

activation, I κ B α and A20, were both significantly down-regulated, probably as a direct result of inhibition of NF- κ B activity by the venom. Prolonged JNK activation, seen after co-treatment with *N. vitripennis* venom and LPS in macrophages, could potentially lead to cell death and is probably a secondary effect indirectly caused by suppression of NF- κ B activation. The canonical cascade in the cytoplasm and nuclear translocation of the p65 subunit appeared to be unaffected by the venom.

The assembly of these results only leave few options open for venom intervention on the NF- κ B pathway. One of them is an alteration in the general transcriptional machinery, preventing effective transcription of NF- κ B target genes. The complete transcription regulation in eukaryotes is a very complex process that is composed out of several processes (Bhatt & Ghosh, 2014). First, the core promoter serves as a platform for the assembly of different transcription factors, RNA polymerase II and other general TFs. Next to that, co-factors can transmit regulatory signals between gene-specific activators and the general transcription machinery. As for other transcription factors, NF- κ B activity can be drastically altered by affecting the general transcription machinery and this might be a possible place of intervention by *N. vitripennis* venom. Also interference with other TFs is a well-known event for transcriptional regulation. The probably most studied mechanism of repression of NF- κ B activity is via glucocorticoids. Through binding with glucocorticoids, GRs act strongly anti-inflammatory and thereby GCs are widely used in the clinic to treat both chronic inflammation and cancer. GRs can modulate their anti-inflammatory activity via multiple mechanisms (Ratman *et al.*, 2013). Possible mechanisms of GCs/GR interfering with NF- κ B signalling, is via protein-protein interaction, of which several different models have been proposed (Almawi & Melemedjian, 2002). In the composite as well as the competition model, NF- κ B binds with its DNA binding site, but transactivation of NF- κ B is inhibited: in the composite model, GR associates with the trans-activating domain of p65, while in the competition model, GR competes with NF- κ B for nuclear coactivators. However, in the Gal4-p65 assay (see chapter 4, figure 5), where the (Gal)2-50hu.IL6P-luc+ plasmid was transiently transfected in HEK293T cells, no effect of *N. vitripennis* venom on GAL4-p65 activity involving the general transcription machinery could be observed. Thereby, both models of GR activity cannot explain the possible suppression of NF- κ B activity by *N. vitripennis* venom. Furthermore, venom did not inhibit PMA- and dB-cAMP-induced luciferase expression of

respectively the AP-1-dependent reporter gene, nor the CRE-dependent reporter gene (see chapter 4, figure S3), making the option that venom affects NF- κ B dependent gene regulation via targeting of the general transcription machinery including RNA polymerase II and its co-actors rather unlikely. Furthermore the latter data underline the specificity of venom activity.

Based on these observations, the most plausible scenario for venom activity implies the prevention of NF- κ B binding to its DNA binding site. A large group of NF- κ B inhibitors specifically blocks the binding between NF- κ B and its cognate DNA binding site in the nucleus (Gilmore & Herscovitch, 2006). This specific process was not investigated by the Gal4-p65 assay, since the binding between DNA and the Gal4-p65 fusion protein takes place between the DNA-binding domain of the yeast nuclear protein Gal4 and the two Gal4-binding DNA sequence elements that precede the luciferase reporter gene. Therefore, the lacking effect of venom on the transactivation process of p65 independent from the promoter context, analysed by this experiment, does not exclude the fact that venom potentially interrupts direct binding between p65 and its DNA-binding site. Interestingly, incubation of Raw264.7 cells with *N. vitripennis* venom for 6 hours showed significant up-regulation of GILZ transcription (see chapter 4, figure 7B). GILZ was previously demonstrated to physically bind the p65-subunit and thereby prevent the NF- κ B-mediated activation of transcription (Riccardi *et al.*, 1999; Cheng *et al.*, 2013b). Indeed exogenous GILZ in endothelial cells inhibited TNF-induced p65 DNA binding, which occurred in the absence of an effect on p65 nuclear translocation (Cheng *et al.*, 2013b). Similarly, *N. vitripennis* venom could inhibit NF- κ B activation in Raw264.7 macrophages by the up-regulation of GILZ expression, followed by the prevention of NF- κ B binding to its DNA binding site. A possible scenario for the up-regulation of GILZ can be found in the transcription activating action of glucocorticoid receptor (GR) signalling. Investigating the crosstalk between TNF and GR signalling pathways, many possible mechanisms of interference of GCs/GR in TNF signalling were revealed (Van Bogaert *et al.*, 2010), among which the up-regulation of GILZ. Unfortunately, the venom showed no change in GR transactivation on a glucocorticoid responsive element (GRE)-containing promoter reporter (see addendum to chapter 4, figure 1A) and no alteration on translocation of GR from the cytoplasm to the nucleus was caused by *N. vitripennis* venom, which did occur when dexamethasone was added (see addendum

to chapter 4, figure 1B and C). Therefore, we would assume that this up-regulation of GILZ expression is possibly mediated via activation of other transcriptional responses. Indeed also the b/HLH/Z TF upstream stimulatory factors, USF1 and USF2, as well as the mineralocorticoid receptor have previously been demonstrated to regulate GILZ expression (Koberle *et al.*, 2012; Ueda *et al.*, 2014). So, in order to understand what *N. vitripennis* venom is eliciting with regard to the GILZ signalling pathway, further investigation of transcriptional regulation of GILZ by the venom is required. However, in a similar manner as for NF- κ B also AP-1 activity could be repressed by GILZ (Mittelstadt & Ashwell, 2001), which could not be observed for the *N. vitripennis* venom in the L929sA reporter assay system. Therefore, knock down studies for GILZ should reveal its contribution to the NF- κ B repressing effects of the venom.

In another GR anti-inflammatory action model, called the simple model, hormone-activated GR does not affect the availability or translocation of NF- κ B nor its function, but rather binds to NF- κ B in the nucleus and/or cytosol, thereby forming a complex which fails to bind to DNA. This scenario could fit with the results of *N. vitripennis* venom effects in mammalian cells mentioned in chapter 4 and would imply that venom binds to NF- κ B, still allows NF- κ B to translocate to the nucleus, but prevents NF- κ B from binding its DNA binding site. Since hormone-activated GR binds to NF- κ B, forming a GR-NF- κ B-complex that does not bind DNA, transactivation through GRE-binding is interrupted (see addendum to chapter 4, figure 1A). It would however not explain the unaffected translocation of GR to the nucleus (see addendum to chapter 4, figure 1B and C). In addition, for this scenario to persist, *N. vitripennis* venom needs to contain a possible ligand of GR, in order to activate its binding to NF- κ B. Since no steroid hormones have been found in venoms in general, this train of thoughts seems highly unlikely. Alternatively, the possibility that a compound in the venom from *N. vitripennis* directly binds to NF- κ B, preventing it from binding DNA, still remains and requires further investigation. First results investigating possible interaction of *N. vitripennis* venom on p65-DNA binding by electrophoretic mobility shift assay (EMSA) using a non-radioactive p65 probe on cell-lysates of HEK293T cells overexpressing p65, seem promising. However, optimisation of this EMSA is required.

Besides general effects on NF- κ B DNA binding, the effects of *N. vitripennis* venom might be more gene specifically regulated, as suggested by the results from the PCR array performed on venom treated Raw264.7 cells (chapter 5). Chromatin can be remodelled by histone modifications like lysine acetylation and methylation as well as genomic DNA methylation, in order to make binding sites accessible to their cognate DNA-binding proteins. For instance, in case of NF- κ B transcriptional regulation, differences in promoter accessibility have been demonstrated to create 2 waves of NF- κ B recruitment to target promoters resulting in immediate early responses and late NF- κ B responses (Sacconi *et al.*, 2001). Therefore, Chromatin Immunoprecipitation (ChIP) to determine what specific regions in the promoter associated with p65 are affected by the venom, need to be performed to fully disentangle the effect of *N. vitripennis* venom on the NF- κ B pathway.

Described results seem promising to use *N. vitripennis* venom as anti-inflammatory agent for chronic inflammatory conditions, auto-immune diseases and cancer. In order to reach that goal, first the exact mode of action of venom needs to be fully unravelled and the venom compound responsible for this activity needs to be identified. Fractionation of the venom by HPLC followed by identification and production of the compound performing anti-inflammatory action is therefore the strategy for the coming years.

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CURRICULUM VITAE

PERSONAL DETAILS

Name	Ellen Danneels
Date of birth	30/10/1985
Nationality	Belgian
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EDUCATION

2008-2014	Assistant and Ph.D. candidate, Biochemistry and Biotechnology, Ghent University • PhD thesis: The venom of <i>Nasonia vitripennis</i>: involvement in the host-parasitoid interaction and putative biomedical application. • Advisor: Prof. Dr. Dirk de Graaf ;co-advisors: Prof. Dr. Kathleen Van Craenenbroeck and Em. Prof. Dr. Frans Jacobs
2005-2007	Master in Science (MSc), Biology, KU Leuven (magna cum laude) • Master thesis: De knock-down van type 2 joodthyroninedejodase met behulp van morfolino's bij de embryonale zebravis (<i>Danio rerio</i>) • Advisor: Prof. Dr. Veerle Darras
2003-2005	Bachelor in Science (BSc), Biology, KU Leuven (cum laude)
1996-2003	Secondary school: Latin-Science, Bisschoppelijk College der Onbevleete Ontvangenis, Veurne

INTERNSHIPS

13/01/2013 - 14/02/2013: Centre for Ecological and Evolutionary Studies, University of Groningen, The Netherlands

SCIENTIFIC OUTPUT

A1 peer reviewed publications

1. Peiren Nico, de Graaf C. Dirk, Vanrobaeys Frank, Danneels L. Ellen, Devreese Bart, Van Beeumen Jozef, Jacobs J. Frans (2008). Proteomic analysis of the honey bee worker venom gland focusing on the mechanisms of protection against tissue damage. *Toxicon* 52: 72-83
2. de Graaf C. Dirk, Aerts Maarten, Danneels Ellen, Devreese Bart (2009). Bee, wasp and ant venomomics pave the way for a component-resolved diagnosis of sting allergy. *Journal of Proteomics* 72: 145-154
3. Danneels L. Ellen, Rivers B. David, de Graaf C. Dirk (2010). Venom proteins of the parasitoid wasp *Nasonia vitripennis*: recent discovery of an untapped pharmacopee. *Toxins* 2: 494-516
4. Cardoen Dries, Wenseleers Tom, Ulrich R. Ernst, Danneels L. Ellen, Laget Dries, de Graaf C. Dirk, Schoofs Liliane, Verleyen Peter (2010). Genome-wide analysis of alternative reproductive phenotypes in honeybee workers. *Molecular Ecology* 20: 4070-4084
6. Danneels L. Ellen*, Formesyn M. Ellen*, Hahn A. Daniel, Denlinger L. David, Cardoen Dries, Wenseleers Tom, Schoofs Liliane, de Graaf C. Dirk (2013). Early changes in the pupal transcriptome of the flesh fly *Sarcophaga crassipalpis* to parasitization by the ectoparasitic wasp, *Nasonia vitripennis*. *Insect Biochemistry and Molecular Biology* 43: 1189-1200
(* shared first authors)
7. Formesyn M. Ellen, Cardoen Dries, Ernst Uli, Danneels L. Ellen, Van Vaerenbergh Matthias, De Koker Dieter, Verleyen Peter, Wenseleers Tom, Schoofs Liliane, de Graaf C. Dirk (2014). Reproduction of honeybee workers is regulated by epidermal growth factor receptor signalling. *General and Comparative Endocrinology* 197: 1-4
8. Danneels L. Ellen, Gerlo Sarah, Heyninck Karen, Van Craenenbroeck Kathleen, De Bosscher Karolien, Haegeman Guy, de Graaf C. Dirk (2014). How the venom from the ectoparasitoid wasp *Nasonia vitripennis* exhibits anti-inflammatory properties on mammalian cell lines. *PLoS ONE* 9(5): e96825

B2 Book chapter

1. Formesyn M. Ellen, Danneels L. Ellen, de Graaf C. Dirk (2012). Proteomics of the venom of the parasitoid *Nasonia vitripennis*. In: Beckage E. Nancy & Drezen Jean-Michel (Editors), *Parasitoid viruses: Symbionts and pathogens*, 233-246

PARTICIPATION AT CONFERENCES

1. de Graaf C.D., Danneels E.L., Brunain M., Jacobs F.J. (2009) – poster. New insights into the composition of bee, wasp and ant venoms and how it can contribute to a better therapy of patients suffering sting allergy. Apimondia, Montpellier, France, September 15th -20th 2009, p. 168.
2. Danneels E.L., Formesyn E., Jacobs F.J. & de Graaf D.C. (2010) - poster. Exploring the functionality of *Nasonia vitripennis*' unknown venom proteins. *Bee-together: conference on pollinators with emphasis to stimulate interactions in the field*, Ghent, Belgium, December 21th 2010, p. 25.
3. Danneels E.L., Formesyn E., Jacobs F.J. & de Graaf D.C. (2010) – poster. Evaluation of host immune responses to envenomation by the ectoparasitic wasp *Nasonia vitripennis* with antibacterial assays. 17th Benelux Congress of Zoology, Ghent, Belgium, October 22th - 23th 2010, p. 121.
4. Danneels E.L., Gerlo S. & de Graaf D.C. (2011) – oral presentation. Exploring the effect of venom from the ectoparasitic wasp *Nasonia vitripennis* on NF-κB-like pathways in mammalian and insect cell-lines. *Nasonia meeting 2011*, Nashville, USA, June 14th -17th 2011, p. 8.
5. Formesyn E.M., Danneels E.L., de Graaf D.C. (2012) – poster. Involvement of several venomous proteases in the viability of the Sf21 cell line. 60th Annual Meeting of the Entomological Society of America (Entomology 2012), November 11th-14th 2012, p. 141.
6. Danneels E.L., Formesyn E.M., Hahn D.A., Denlinger D.L., Cardoen, D., Verleyen P., Wenseleers T., Schoofs L. & de Graaf D.C. – oral presentation. Unveiling the early responses in the pupal transcriptome of *Sarcophaga crassipalpis* after parasitization by *Nasonia vitripennis*. *Nasonia meeting 2013*, Wageningen, The Netherlands, June 17th -19th 2013.
7. Danneels E.L., Gerlo S., Heyninck K., Van Craenenbroeck K., De Bosscher K., Haegeman G. & de Graaf D.C. – poster. How the venom from the ectoparasitoid wasp *Nasonia vitripennis* exhibits anti-inflammatory properties on mammalian cell lines. *Seventh International Symposium on Molecular Insect Science*, Amsterdam, The Netherlands, July 13th -16th 2014.

PARTICIPATION AT WORKSHOPS AND COURSES

1. Workshop on Data Mining & Bioinformatics for Proteomics - 08/09/2009, Antwerp University, Campus Middelheim

2. Danneels E.L., Formesyn E., Jacobs F.J. & de Graaf D.C. (2010) – poster. Exploring the impact of venom from the ectoparasitic wasp *Nasonia vitripennis* on host immunity with an agar diffusion test. *Diagnostics in honeybees: from sampling to data analysis*, Ghent, Belgium, August 30th – September 1st 2010, p. 19.
3. Summer course “Beneficial, pest and vector insects” – June 11th-15th 2012, KU Leuven
4. Basisassistententraining - November 2008, UGent
5. Assistententraining: het feedbackgesprek- Februari 2010, UGent
6. Course “Advanced Academic English: Conference skills – Academic Posters” – November 3rd -10th, UGent
7. Course ‘Advanced Academic English: Conference skills – English Proficiency for Presentations’ – October 6th – 27th, UGent

GUIDANCE OF STUDENTS

Practical courses

1. First Bachelor of Medicine and Dentistry: Aspects of peripheral blood research.
Academic years from 2008 until 2012
2. Second Bachelor of Biochemistry and Biotechnology: Practical exercises of biomedical physiology.
Academic years from 2007 until 2014
3. First Bachelor of Biochemistry and Biotechnology: Practical exercises of Biodiversity of animals.
Academic years from 2012 until 2014

Bachelor project

1. Bepaling van de eigen antistofopbouw tegenover bijengif in relatie tot de steekfrequentie in het verleden. (2008-2009: Maria Gekhman, Steven Timmermans, Merlijn Morisse, Mieke Vandewalle, Charlotte Peeters, Simon Devos, Ben Helsen, Bert Waelkens, Gert-Jan Van Landeghem, Ajay Kakad)
2. Bepaling van de eigen antistofopbouw tegenover bijengif in relatie tot de steekfrequentie in het verleden. (2009-2010: Toon Babylon, Kenneth Goossens, Alexander Reinartz, Merten De Smet, Erwin Pannecoucke, Annelien Vanderhaeghe, Karen Dendoncker, Ellen Lambrecht, Dagmar Poelemans)

3. Effect van het gif van *Nasonia vitripennis* op de NF-κB pathway in een muizencellijn. (2010-2011: Nathalie Cornillie, Barbare De Smet, Marie-Laure Erffelinck, Valerie Van Ruyskesnvelde)

Master project

1. Recombinante expressie van gifcomponenten van de ectoparasitoïde wasp *Nasonia vitripennis*. (2009-2010: Ajay Kakad)
2. RACE technologie en recombinante expressie van gifcomponenten van de parasitoïde wasp *Nasonia vitripennis*. (2010-2011: Hannes Demaré, Ine Opsteyn)
3. Determination of housekeeping genes in the fleshfly *Sarcophaga crassipalpis* whether or not envenomated by *Nasonia vitripennis*. (2011-2012: Nazim Enes Altan)

Master thesis

1. Het gif van de parasitoïde wasp *Nasonia vitripennis*: recombinante expressie van nieuwe gifcomponenten en effect op de humorale immuunrespons van de gastheer. (2009-2010: Dieter de Koker)
2. Het gif van de parasitoïde wasp *Nasonia vitripennis*: recombinante expressie van nieuwe gifcomponenten en effect op de cellulaire immuunrespons van de gastheer. (2009-2010: Wim Jonckheere)
3. Gif van de parasitoïde wasp *Nasonia vitripennis*: invloed van recombinant geëxprimeerde gifcomponenten op het immuunantwoord van de gastheer. (2012-2013: Cédric Fichet)
4. Biological function of chemical biomolecules in the venom of *Nasonia vitripennis*. (2013-2014: Jonas De Saeger)