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Exploring diverse molecular processes in two genome sequenced hymenopteran model systems: *Nasonia vitripennis*-(non)host interactions and honeybee fertility

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Nasonia vitripennis parasitizing a *Sarcophaga crassipalpis* pupa (© Ellen Formesyn).

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List of Abbreviations

9-0DA	(E)-9-oxodec-2-enoic acid
10-HDA	(E)-10-hydroxy-2-decenoic acid
20E	20-hydroxyecdysone
AARE	Amino acid deprivation response element
ABC	ATP-binding cassette
ADAMTSL-5	A disintegrin and metalloproteinase like with thrombospondin motifs 5
ADHAPS	Alkyldihydroxy-acetonephosphate synthase
AhR	Aryl hydrocarbon receptor
Am	*Apis mellifera*
AMP	Antimicrobial peptide
AN	Annexin V-FITC
AP-1	Activating protein 1
Apaf-1	Apoptotic protease activating factor 1
ARNT	Aryl hydrocarbon receptor nuclear translocator
AS	Asparagine synthase
BAD	Bcl-2-associated death promoter
BAX	Bcl-2-associated X protein
Bcl2	B-cell lymphoma 2
BLASTP	Protein-protein basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
$CaPO_4$	Calcium phosphate
Cbl	Casitas B-lineage lymphoma
cDNA	Complementary desoxyribo nucleic acid
C/EBP	CCAAT-enhancer-binding protein
CHOP	C/EBP homologous protein
CID	Collision-induced dissociation
CMV	Cytomegalovirus
CoA	Coenzyme A
COG	Clusters of orthologous groups
CpG	Cytosine-phosphate-guanosine
CrCRT	Calreticulin-like protein
CRE	cAMP response element
CREB	cAMP response element-binding protein
Ct	Treshold cycle
CTP	Cytidine triphosphate
CYP	Cytochrome P450
Cyto C	Cytochrome c
Daf	Dauer formation
DGP	GTP binding protein
dH2O	Distilled water

DNMT	DNA cytosine-5-methyltransferases
dUTP	Deoxyuridine-triphosphatase
ECM	Extracellular matrix
EF	Elongation factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetra acetic acid
EGR1	Early growth response protein 1
EIF	Eukaryotic translation initiation factor
EpMP	Reprolysin metalloprotease homolog
ERα and β	Estrogen receptor
ER	Endoplasmic reticulum
ERE	Estrogen response elements
ERK	Extracellular signal-regulated kinase
ERSE	ER stress element
ESI	Electrospray Ionization
EST	Expressed sequence tag
FA	Fatty acid
FAB	Fast-atom-bombardment
FACS	Fluorescence-activated cell sorting
Fas	Apoptosis stimulating fragment
FBS	Fetal bovine serum
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
Fl	Fluorescence
FT	Fourier transform
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
γ-GT	γ-glutamyl transpeptidase
GC	Gas Chromatography
GCN2	General control non-derepressible-2
gDNA	Genomic DNA
GEO	Gene expression omnibus
GFP	Green fluorescent protein
GMP	Guanosine monophosphate
GO	Gene ontology
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HBSS	Hank's buffered salt solution
HCl	Hydrogen chloride
HDACi	Histone deacetylase inhibitor
HEK	Human embryonal kidney cells
HIF	Hypoxia-inducible factor
HPLC	High performance liquid chromatography
HSC	Heat shock cognate protein
HSP	Heat shock protein
IAP	Inhibitors of apoptosis proteins

IC	Inhibitory concentration
ICP	Inductively coupled plasma
ICR	Ion cyclotron resonance
ID	Identity
IFN	Interferon
IIS	Insulin-IGF-1 signalling
IL	Interleukin
IP_3	Inositol trisphosphate
IR	Insulin receptor
IRE1	Inositol-requiring enzyme 1
ISB	Insect phosphate saline
ISCDDK	In situ cell death detection kit
IRS	Insulin receptor substrate
JH	Juvenile hormone
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Monopotassium phosphate
KOG	Eukaryotic orthologous groups
LC	Liquid chromatography
L-DOPA	L-3,4-di-hydroxyl-phenylalanine
lrr-pr	Leucine rich repeat protein
LTQ	Linear trap quadrupole
LXR	Liver X receptor
MALDI	Matrix assisted laser desorption ionization
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor-2
miRNA	MicroRNA
$MgCl_2$	Magnesium chloride
mM	Millimolar
MRJP	Major royal jelly proteins
MS	Mass spectrometry
Mst	Mammalian sterile 20-like kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTF1	Metal regulatory transcription factor 1
MWCO	Molecular weight cut off
NAD	Nicotinamide adenine dinucleotide
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Disodium phosphate
NaK-ATPase	Sodium potassium-dependent ATPase
NaCl	Sodium chloride
NEAA	Non-essential amino acids
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer lymphocyte
Nos	nanos

Nore	Novel Ras effector
Nur77	Nerve growth factor IB
Nv	*Nasonia vitripennis*
O_2	oxygen
Obp	Odorant-binding protein
Oct4	Octamer-binding transcription factor 4
P4	Virulence protein 4
PAMPS	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PEI	Polyethylenimine
PERK	Protein kinase-like ER kinase
PI	Propidium iodide
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PO	Phenoloxidase
PPAR	Peroxisome proliferator-activated receptor
Prl	Pleiotropic regulatory locus
pRL	Renilla luciferase plasmid
proPO	Prophenoloxidase
PS	Phosphatidylserine
PSD	Post-source decay
PSI	Position specific iterated
PTEN	Phosphatase and tensin
PTTH	Prothoracicotropic hormone
Q	Quadrupole
QMP	Queen mandibular pheromones
qRT-PCR	Quantitative reverse transcription PCR
QTL	Quantitative trait loci
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RASSF	Ras association domain family
RhoGAP	Ras homologous GTPase Activating Proteins
RLU	Relative light units
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RP	Reversed Phase
RP49	Ribosomal protein 49
Rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
S6K	S6 kinase beta-1
SAPK	Stress-activated protein kinase
Scr	Rous sarcoma oncogene cellular homolog
Sc-SP-3	Secreted chymotrypsin-like serine protease

SCX	Strong cation exchange
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide gel electroforese
Setdb1	histone-lysine N-methyltransferase eggless
Sf	*Spodoptera frugiperda*
sHSP	Small heat shock protein
SID	Surface-induced dissociation
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
SMAC/Diablo	Second mitochondria-derived activator of caspases
SOCS-5	Suppressor of cytokine signaling 5
SRE	Serum response factor element
STAT3	Signal transducer and activator of transcription 3
TdT	Terminal deoxynucletidyl transferase
TNF	Tumor necrosis factor
TOF	Time-of-flight
TOR	Target of rapamycin
TRAF	TNF receptor associated factor
TRE	Transcriptional response element
tRNA	transfer RNA
TUNEL	terminal deoxynucletidyl transferase-mediated dUTP nick end labeling
Ubq	Ubiquitin-conjugating enzyme
UPR	Unfolded protein response
UV	Ultraviolet
Vg	Vitellogenin
XRE	Xenobiotic response element
yp	Yolk protein
Z-VAD-FMK	Benzyloxycarbonyl-Val-Ala-DL-Asp(O-methyl)-fluoromethylketone

PART I

OBJECTIVES AND GENERAL INTRODUCTION

Objectives and outline of this study

Hymenoptera are considered as the most beneficial insect order to humans, as they play an important role in plant pollination and parasitation of harmful insects. The order is best known for its social insects like bees, wasps and ants. However, the majority of the Hymenoptera consists of parasitoids that parasitize both agricultural and medically important pests. Since the completion of the first Dipteran genome, namely that of *Drosophila* in 2000, insect molecular research was lifted to higher levels. However, since the *Drosophila* genome evolves too fast for certain genes, while others are absent, the urge to explore the genomes of other insect orders has grown. This, taking into account with the importance of the Hymenoptera, has led to the sequencing of the first genome of a social insect, *Apis mellifera* in 2006. Three years later, in 2009, the complete genome sequence of the first parasitoid wasp *Nasonia vitripennis* became available. Both insect species are considered as novel model species and good alternatives, which may also complement and expand the existing knowledge, acquired with *Drosophila*. From that point on, advanced research was performed in order to understand the biological processes of several related topics including venomics, reproduction, developmental biology and others.

The Laboratory of Zoophysiology has launched the Hymenoptera venom research 10 years ago and contributed in both genome papers of *A. mellifera* and *N. vitripennis*. This resulted in the identification of the venom components in both insect species and has led to an expansion of the existing research, including several functional studies.

The main part of this PhD encompass studies that were performed using the ectoparasitoid wasp *N. vitripennis*, in order to gain better insights into the functionality of the venom using its natural host (*Sarcophaga crassipalpis*), insect (Sf21) and human (HEK293T) cell lines. In the second part of this PhD, the available genome information was used for a study on *A. mellifera* worker fertility, in collaboration with the KU Leuven.

The introductory **Chapter 1** provides a general overview of gained knowledge about the functionality of *N. vitripennis* venom in natural hosts and lepidopteran cell lines while referring to other parasitoid wasps. In addition, a literature survey on the signaling pathways behind honeybee caste differentiation and worker fertility is provided. In the following chapters, a thorough description of the studies that were performed in the context of this thesis are provided.

Chapter 2: Which genes are differentially expressed in *S. crassipalpis* upon parasitation by *N. vitripennis*?

Chapter 3: Is *N. vitripennis* venom able to target specific tissues and which types of cell death are induced in parasitized *S. crassipalpis*?

Chapter 4: Which venomous proteases are cytotoxic and what kind of programmed cell death do they induce in Sf21 cells?

Chapter 5: Are mammalian cells sensitive for *N. vitripennis* venom and if so which pathways are affected during the first 8h?

Chapter 6: Which downstream molecular mechanism is involved in the switch to honeybee worker reproduction?

Chapter 7: General discussion and future perspectives.

Chapter 1

General introduction

Parts of this chapter are redrafted from:

Formesyn, E.M., Danneels, E.L., de Graaf, D.C., 2011. Proteomics of the venom of the parasitoid *Nasonia vitripennis* (Chapter 19). In: Beckage, N.E., Drezen, J.M. (Eds.), Parasitoid Viruses: Symbionts and Pathogens. Elsevier Inc., pp. 233–246.

Formesyn, E.M., Heyninck, K., de Graaf, D., 2013. The role of serine- and metalloproteases in *Nasonia vitripennis* venom in cell death related processes towards a *Spodoptera frugiperda* Sf21 cell line. Journal of Insect Physiology 59, 795–803.

1. Parasites and parasitoids

Diverse life forms exist that feed on other organisms or hosts. Mostly invertebrates use this survival strategy, which is harmful and/or lethal for its host. Two different lifestyles are used by those organisms, which are known as either parasites or parasitoids. In both lifestyles, the exogenous organism spends a part of its life obtaining nourishment at the expense of the host. As parasites do not intent to kill their hosts, they do display pathological features, resulting in a reduced fitness. However, in the case of parasitoids the outcome of the parasitoid-host relationship is completely different than observed with parasites. Here, the parasitoid demolishes its host as they develop, preventing its reproduction (Whiting, 1967). All parasitoids are invertebrates, mostly insects, which only need a host during the immature stages. A large majority of parasitoids are wasps, which are very important to maintain the balance in nature. Additional information about the taxonomic distribution of the existing families can be found in Box 1.1. Some species parasitize agricultural pest insects and are economically important, while others parasitize disease vectors of humans and animals. Parasitoid wasps deposit their eggs in a broad range of insect hosts. Currently, between 50.000 and 60.000 parasitoid wasps are described, but many more remain to be discovered (Pennisi, 2010).

1.1. Endo-and ectoparasitoids

When a female parasitoid finds a suitable host, she will inject venom to subdue the host and subsequently deposit the eggs. However, the position where the eggs are laid and develop differs between species. Endoparasitoids develop inside their host, usually in situations where the host is mobile and exposed (Fig. 1.1B). Therefore most endoparasitoid venoms don't induce paralysis in their hosts. During parasitation, the host is able to proceed its development (koinobiont), until it is completely consumed by the parasitoid larvae. Ectoparasitoids on the other hand, glue their eggs outside the host. Once the larvae are hatched, they use their mouthparts to attach themselves onto the host and continue to feed until the host dies (Fig. 1.1A). Since the larvae develop externally, the host should be immobilized, which is ensured by the paralytic effect of the venom. However, not all ectoparasitoid venoms may induce long-term paralysis and some of them are not even

paralytic. Nevertheless, the induced developmental arrest is permanent in their hosts (idiobiont) (Asgari and Rivers, 2011).

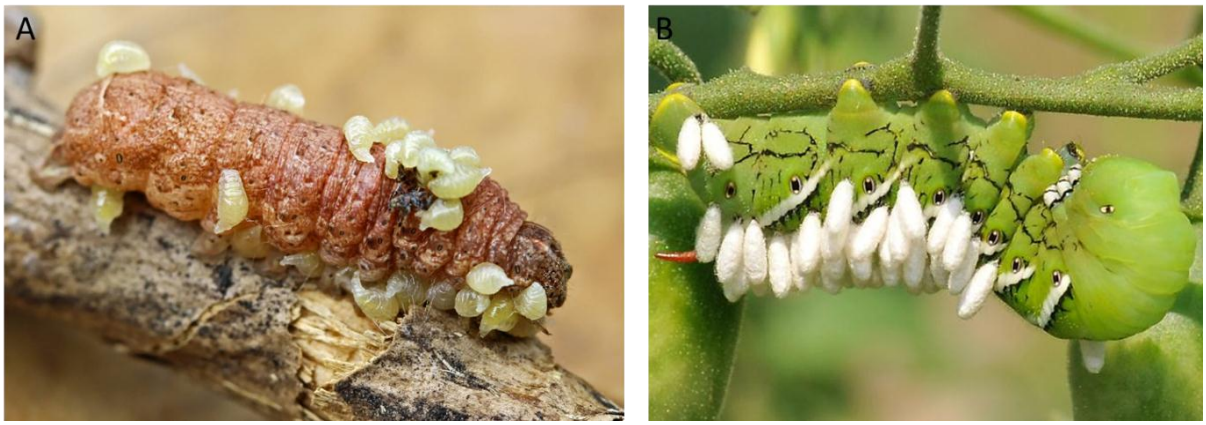
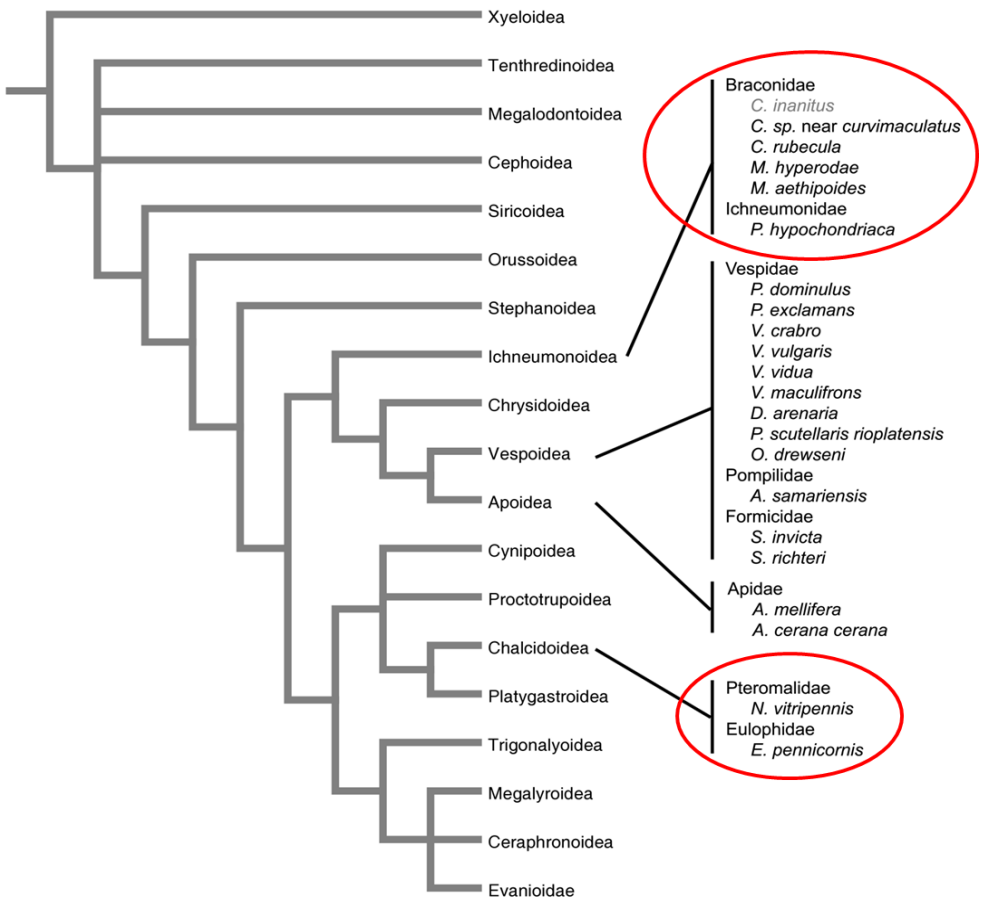


Figure 1.1: A) Ectoparasitoid *Euplectrus sp.* larvae on a Noctuidae caterpillar (©entomart). **B**) Endoparasitoid *Cotesia congregata* pupae on Carolina Sphinx Caterpillar. Adapted from (© Ted Kropiewnicki).



Box 1.1. Taxonomy of parasitoid wasps (Order Hymenoptera).
Overview of the distribution of the two main superfamilies Ichneumonoidea and Chalcidoidea (marked in red). Both families contain parasitoid wasps and are placed in the suborder Apocrita where they are considered as the Parasitica (unranked). *Nasonia vitripennis* is placed in the superfamily of Chalcid wasps which refers to their metallic colour as the main characteristic. The nineteen families contain approximately 22,000 known species. Figure adopted and modified from Vincent *et al.*, 2010.

1.2. The genus *Nasonia*

Nasonia (Hymenoptera: Pteromalidae) are small ectoparasitoid wasps, also known as jewel wasps. The genus consists of four related parasitoid species, *Nasonia vitripennis*, *N. giraulti*, *N. longicornis* and *N. oneida*. The genome sequences of three *Nasonia* species, except *N. Oneida*, are known, which makes them an excellent insect model for several studies. Furthermore, Nasonia are haplodiploid, meaning that haploid males develop from unfertilized eggs, while diploid females originates from fertilized eggs (The Nasonia genome working group, 2010). *Nasonia vitripennis* is a worldwide distributed gregarious parasitoid that can parasitize the pupal stages of several Dipteran families, including Sarcophagidae and Calliphoridae. Other species can also be parasitized, although they are less susceptible to the venom and their sensitivity differs among several life stages (Rivers *et al.,* 1993). Since they parasitize flesh flies, these parasitoid wasps are considered as biological control agents against these pest species. In fact, they are currently commercialized and sold to farmers in the U.S.A. to eradicate pest species in poultry and livestock facilities (Vazirianzadeh *et al.,* 2008; Kaufman *et al.,* 2001a, b). When a *N. vitripennis* female encounters a suitable host (newly pupariated fly larvae) she drills a small hole in the puparium with her ovipositor (Fig. 1.2A). Subsequently, a small amount of venom is injected, followed by the deposition of 20 to 40 eggs on the exterior of the developing fly, but still inside the puparium. After approximately 24 h, the eggs hatch and the larvae start crawling and feeding on the host. Three instar larval and several pupal stages, each with their characteristic colors are described in the literature (Fig. 1.2B) and after two weeks (at 25 °C) the adults emerge from the puparium (Whiting, 1967). Additional information about insect development can be found in Box 1.2.

Box 1.2. Insect development

Due to their though exoskeleton, insect growth is divided into several stages in which the exoskeleton is shedded and replaced. This is called the moulting process or ecdysis and the stages between the moults are referred as the instars. Insect development is not the same in all insects, however the vast majority of them lay eggs. Three types of development can be distinguished, depending on the structural changes that occur in insects during their growth. The most primitive wingless insects do not change much in their form when the young grow into adults (ametabolous). The second group contains the insects that have an incomplete metamorphosis. Here, the nymphs or young insects resembles the adult stage or imago but lack the wings and functional reproductive organs. The nymphs will develop into the adults through gradual changes. Holometabolous insects have a complete metamorphosis with four life stages: the egg, larva, pupa and imago or adult. The larva does not resemble the adult stage and fulfils a complete transformation during the pupal stage.

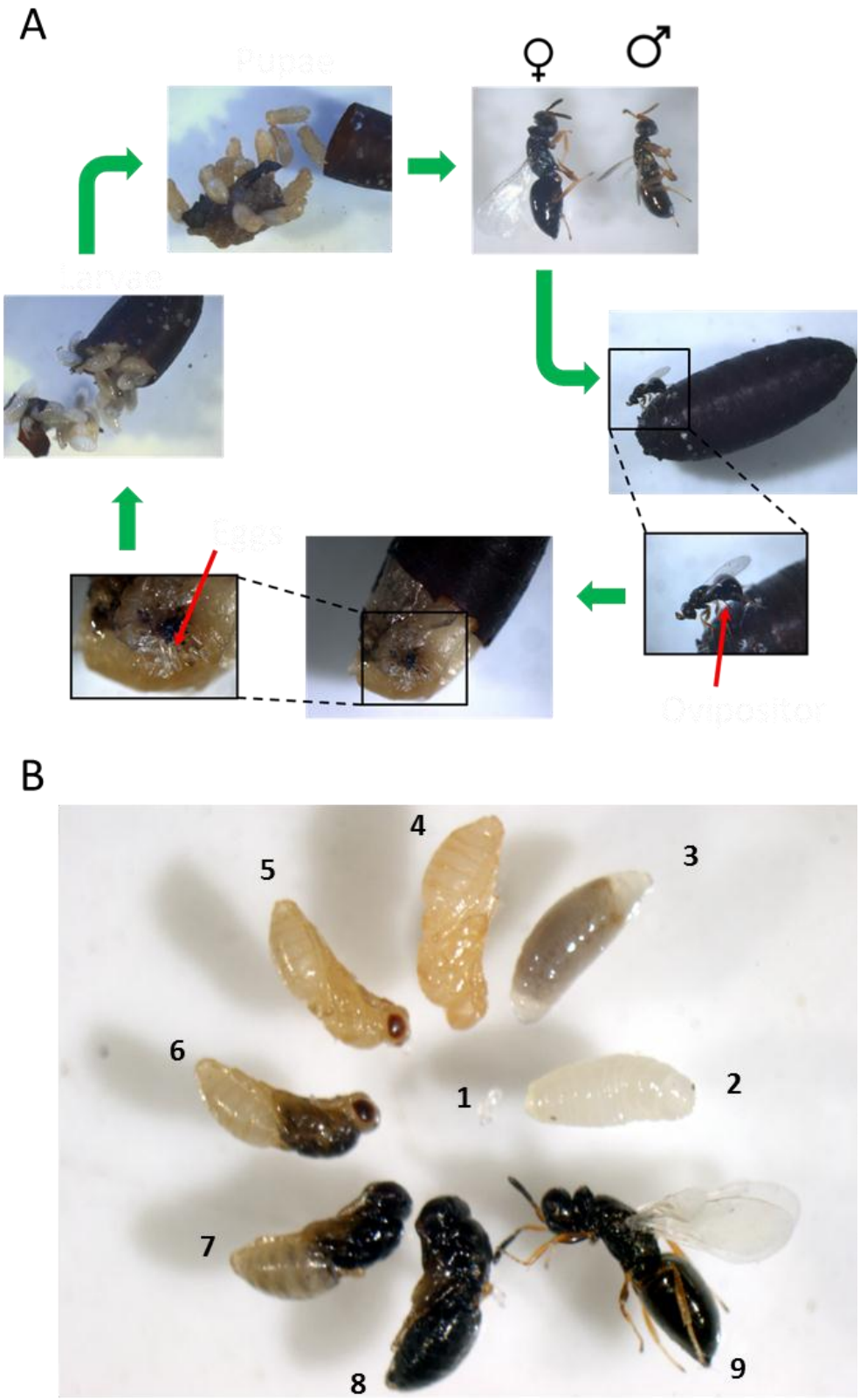


Figure 1.2: A) Life cycle *N. vitripennis* using a *Sarcophaga crassipalpis* host. **B**) Developmental stages of *N. vitripennis*, with eggs (1) and larvae (2-3). After 9 days the larvae will pupate in the host. The white pupae (4) will gradually change in color starting from the eyes which first become red (5). Subsequently the thorax will darken in color (6) followed by the abdomen (7), until the whole pupa is black (8). After 14 days the adults (9) emerge from the host pupa (© Ellen Formesyn).

1.2.1. The venom apparatus

The ovipositor and venom glands of *N. vitripennis* are much smaller than these in honeybees and their structure is slightly different. For instance, the ovipositor of *N. vitripennis* is folded back in the abdomen. The structures that build up the venom system are an elongated acid gland, also known as the venom gland and the venom reservoir that serves as storage room for venom produced in the acid gland (Fig. 1.3).

Both the acid gland and the reservoir produce the toxic proteins that result in diverse modifications to the immune system, physiology and the development of the host (Rivers *et al.,* 2006). The venom gland consists of a long, tubular and folded structure, composed of large columnar cells, which surround the central cuticle-lined lumen. These cells contain secretory granules and invaginations of the apical cell membrane, which are lined with the cuticle of the lumen and end in a vesicular organelle bearing microvilli. This vesicular organelle serves as the secretory site of the gland. Between the columnar cells and the lumen, chitogenous or interstitial cells form a thin layer that most probably produces the lining of the lumen and the basement membrane. The contents of the acid gland are stored in a two-lobed venom reservoir and consist of a two-layered cell wall composed of squamous cells and a muscle sheet on the outside. The squamous cells are on the inside covered with a cuticular lining. The mid-dorsal part of the reservoir forms the region where the acid gland has its entry and is composed of secretory epithelium with numerous vesicles and vacuoles. The apical plasma membrane of these cells possess a cuticular involution, which gives rise to long, apical microvilli that are arranged to form a modified vesicular organelle. Therefore, it is likely that the secretory epithelium can also produce several venom proteins, some of which are not produced elsewhere and many serve to activate the toxins of the acid glands. At the position where the acid gland is connected to the venom reservoir, the ductus divides into several branches through which the secretions flow into the reservoir (Ratcliffe *et al.,* 1969; Whiting, 1967).

The alkaline or Dufour gland, also derived from the female accessory reproduction glands in female Hymenoptera is much shorter than the acid gland. This gland has no vesicular organelles, but the secretory product is delivered into the lumen by accumulation of granular material within the apices of the microvilli. The alkaline gland ends in the discharge

duct that seems to be connected to a chitinous funnel of the vagina and is not connected to the venom reservoir. The viscous contents of this gland are injected independently of the products of the acid gland, do not contain toxic substances and is not considered as a venom gland. Probably, these secretions have a lubricatory function and are involved in greasing the ovipositor components or used to smear the eggs (Ratcliffe *et al.,* 1967; King *et al.,* 1969).

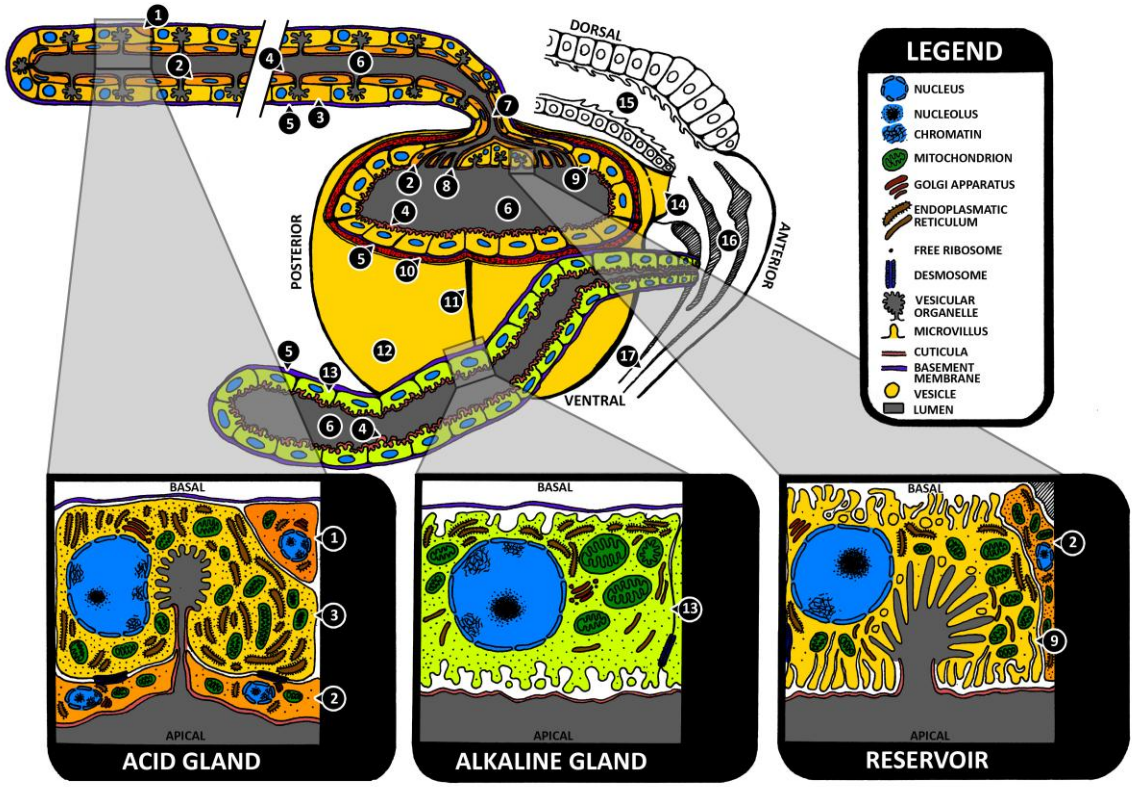


Figure 1.3: Overview of the *Nasonia vitripennis* venom apparatus. (1) Interstitial cell (acid gland), (2) chitogenous cell (acid gland), (3) columnar cell (acid gland), (4) cuticula, (5) basement membrane, (6) lumen, (7) duct, (8) ductile, (9) secretory cell (reservoir), (10) striated muscle (reservoir), (11) medial constriction (reservoir), (12) squamous region (reservoir), (13) secretory cell (alkaline gland), (14) efferent duct, (15) vagina, (16) funnel, (17) ovipositor shaft (Formesyn *et al*., 2011).

1.2.2. Venom collection

Because of the small size of the wasps (1.0 to 3.5 mm for females), it is impossible to employ the manual or electric milking technique performed to collect the venom of for instance honeybees, that delivers substantial quantities of venom (Deyrup *et al.,* 2003; de Graaf *et al.,* 2010). Therefore, dissection of the female wasps is inevitable (Fig. 1.4).

The dissection starts by cutting the abdomen from the thorax with a fine scissor. Subsequently, the abdomen is placed dorsally and held with tweezers at the side where the

thorax was attached. At the posterior side of the abdomen the ovipositor, which is folded back in the abdomen and partly covered by the sterna, is gripped firmly. The venom structures are separated from the surrounding tissue by slowly removing the ovipositor from the abdomen and subsequently the whole structure is placed in a droplet of insect saline buffer (ISB). The acid gland has a characteristic long, white, tubular structure and is often loosely attached to the venom gland. After removing the ovaries and other tissues, the colorless two-lobed venom reservoir and a short alkaline gland become visible. The alkaline gland has a bluish white and shiny appearance, and is firmly attached to the base of the ovipositor. By means of a fine needle, placed under the venom reservoir, the efferent duct from the reservoir is disconnected from the ovipositor. After removal of the acid and alkaline gland, the content of the venom reservoir is liberated by centrifugation in a microtube.

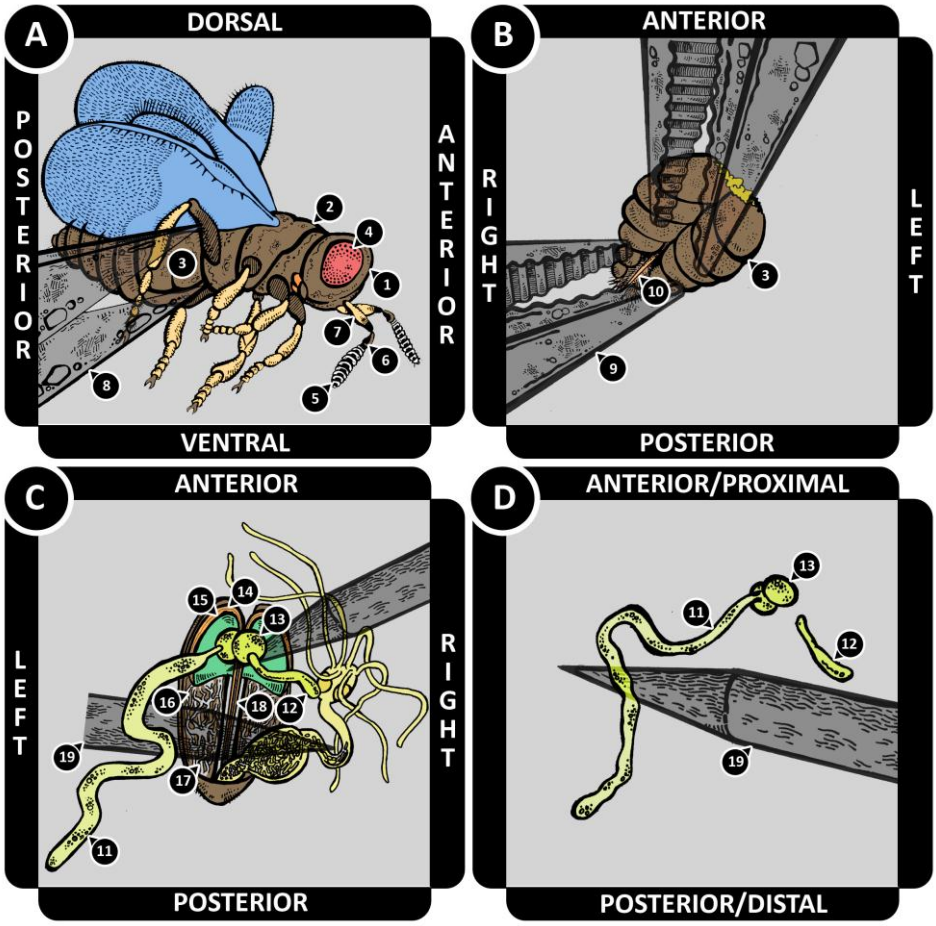


Figure 1.4: Dissection of the venom apparatus in *Nasonia vitripennis* (**A**-**D**). (1) Cephalon, (2) thorax, (3) abdomen, (4) compound eye, (5) flagellum (antenna), (6) pedicel (antenna), (7) scapus (antenna), (8) scissors, (9) forceps, (10) ovipositor, (11) acid gland, (12) alkaline gland, (13) reservoir, (14) stylet, (15) ovipositor ramus, (16) inner ovipositor plate, (17) outer ovipositor plate, (18) sheat and stylets, (19) needle (Formesyn *et al.*, 2011).

1.3. The species *Sarcophaga crassipalpis*

Sarcophaga crassipalpis (Diptera: Sarcophagidae), is a flesh fly species which is commonly used to study insect pupal diapause (Denlinger *et al.,* 1972a; Pavlides *et al.,* 2011). Furthermore, this species is commonly utilized as a host for *N. vitripennis*. In addition, an EST databank of this fly was established in 2009, which makes this species even more suitable for research (Hahn *et al.,* 2009).

To complete their life cycle, they depend on dead or living tissues and are frequently found in poultry and livestock facilities, where they live in the open wounds of animals. For this reason they are considered as a detrimental species. *S. crassipalpis* are larviparous, meaning that the eggs develop internally and first-instar larvae hatch inside the female, just before deposition. The first-instar stadium is followed by a second and third-instar stadium in which the larvae are becoming gradually larger. When the larvae are approximately 112 h old (at 25 °C), the third-instar larvae start to pupariate and enter the prepupal stadium. Twelve days after larvae deposition, *S. crassipalpis* is considered to be in the pupal stadium (Fig. 1.5B). Seventeen days later, the adult flies emerge from the pupae and the life cycle is repeated (Fig. 1.5A). Additional information about insect development can be found in Box 1.2.

Diptera

Sarcophagidae

Sarcophaga

Sarcophaga crassipalpis

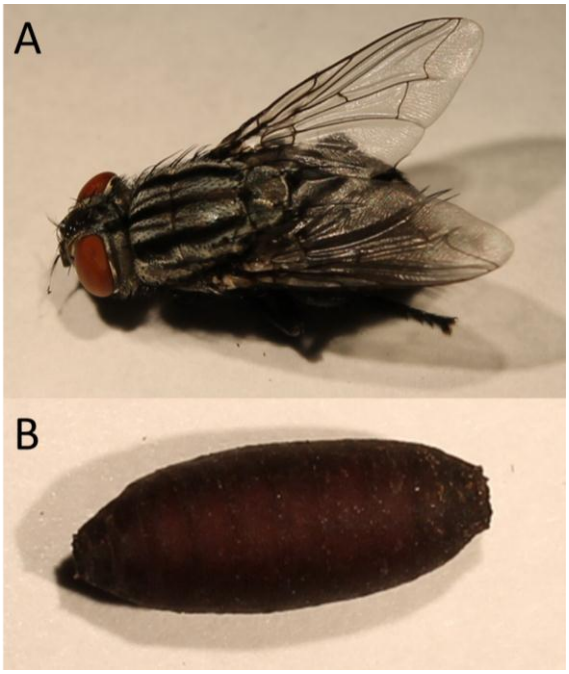


Figure 1.5: Classification of *Sarcophaga crassipalpis*. **A**) Adult fly and **B**) pupa (© Ellen Formesyn).

1.4. Venom characterization of parasitoid wasps

Animal venoms contain a complex mixture of proteins, enzymes, peptides and small organic compounds. Venomics, the analysis of venom proteomes, has gained a lot of interest because venoms are used for predation or defense and contain toxic compounds that are associated with specific pathologies.

In the past, identification of venom components in parasitoid wasps was restricted due to the technological limitation and lack of genome sequences. Intensive studies of the parasitoid *Pimpla hypochondriaca* could be launched once a cDNA library from its venom gland was constructed (Parkinson *et al.,* 2001). Thereafter, cDNA libraries became available from venom glands of several other parasitoid wasps: *Cotesia rubecula* (Asgari *et al.,* 2003a), *Aphidius ervi* (Falabella *et al.,* 2007), *Microctonus hyperodae* (Crawford *et al.,* 2008), *Eulophus pennicornis* (Price *et al.,* 2009), *Pteromalus puparum* (Zhu *et al.,* 2010) and *Eumenes pomiformis* (Baek *et al.,* 2010). Nevertheless, most studies on parasitoid venoms were directed towards a limited number of components, like for instance a calreticulin-like protein (Zhang *et al.,* 2006), a serine proteinase homolog venom protein (Asgari *et al.,* 2003b) and a phenoloxidase (PO) (Parkinson *et al.,* 2001), and until 2009 none of the parasitoids' venomes were identified completely. The first genome and hence venom characterization of a parasitoid wasp was performed on *N. vitripennis* in 2009 (The Nasonia genome consortium group, 2010; de Graaf *et al.,* 2010). This resulted in the identification of its complete venome and subsequently made it possible to lift the current venom research to other levels.

1.4.1. Venom composition of *N. vitripennis* in the pre-genome area

Venoms of parasitoid wasps contain a complex mixture of peptides and proteins, which are mostly acidic in the Ichneumonoidea superfamily (Moreau *et al.,* 2005). Recent analysis with infrared spectroscopy revealed the proteinaceous and acidic nature of *N. vitripennis* venom. The absence of the absorption bands at position 3600 (OH peak), 2900 (C-H stretching) and 1700 (C=O stretching), referred as wave number (cm^{-1}), indicates that the venom is unglycosylated, a quite uncommon feature for parasitoid venoms. This property was also found with a periodic acid Schiff staining performed in the laboratory of Zoophysiology. Failure to stain venom spots with this technique demonstrates the absence of some

carbohydrates including polysaccharides, mucopolysaccharides, glycoproteins and glycolipids. Furthermore, the venom is composed of mid to high molecular weight proteins, ranging from 13 to 200 kilodalton (kDa) and it is most likely that multiple proteins are involved in the modification of host development and physiology (Rivers *et al.,* 2006).

High performance liquid chromatography (HPLC) analysis of crude venom revealed the presence of two major components of crude venom, namely apamin and histamine. An enzymatic property of crude venom concerns the PO activity. Two venom proteins of 68 and 160 kDa are found to perform this PO activity, through sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent oxidation of L - 3,4 - di - hydroxyl - phenylalanine (L-DOPA) as substrate (Abt *et al.,* 2007). Recently two laccase proteins were found to be present in the venom using a bioinformatic approach. Probably one of these proteins, with a molecular weight of 68 kDa, is performing this L-DOPA oxidizing activity (de Graaf *et al.,* 2010). Venom of the endoparasitoid wasp *P. hypochondriaca* also possesses this PO activity, which is performed by three genes (Parkinson *et al.,* 2001). Rivers and colleagues detected also a calreticulin-like protein with an approximate molecular weight of 68 kDa in the venom (Rivers and Brogan, 2008).

1.4.2. Venom analysis using bioinformatics and proteomic approaches

For several years, research towards arthropod venoms is performed with several proteomic tools, in order to reveal the structure and biological role of these venomous components. The use of different mass spectrometric analyses, enabling high sample throughput and sensitivity resulted in the venom characterization of many hymenopteran venoms (Table S1.1; supplemental materials can be obtained by contacting Prof. de Graaf).

A proteomic study was done on the content of 10 *N. vitripennis* venom reservoirs using two different ionization methods and tandem mass spectrometry (MS) instruments; i.e. Matrix Assisted Laser Desorption Ionization - Time-Of-Flight/ Time-Of-Flight - MS (MALDI-TOF/TOF-MS) and Electrospray Ionization - Fourier Transform - Ion Cyclotron Resonance - MS (ESI-FT-ICR-MS). Prior to MS analysis, samples underwent a tryptic digest and subsequent 2D-LC separation. The MALDI TOF/TOF MS analysis contributed to the identification of 29 peptides, representing 14 proteins. For the LC-ESI-FT-MS analysis of *N. vitripennis* venom nine selected SID fractions were used, which resulted in the identification of 258 unique peptides. In this

way the analysis contributed to the identification of 61 venom proteins including the 14 proteins previously identified with MALDI TOF/TOF analysis. However, this resulted also in the identification of 15 additional nonsecretory proteins.

In order to identify potential venom proteins of *N. vitripennis*, the genome was screened for homologues using the BLASTP program, with a query file that contained 383 known Hymenopteran venom proteins. The found hits were further narrowed down to a set of 59 protein sequences by an in depth study of their conserved domain architecture and presence of a signal peptide. Twenty one of them could be confirmed by reverse-transcription PCR (de Graaf *et al.,* 2010).

Both MS analysis yielded 52 proteins that were not found with the bioinformatic study, while nine proteins found with bioinformatics could be confirmed using MS. This unique attempt to unravel the complete venom composition of an ectoparasitoid wasp resulted in the identification of 79 venom proteins, of which proteases were the predominant venom components. Surprisingly, 23 unknown venom proteins were also detected. However, these proteins do not have any similarities with other known insect venom components and hence their functions remain unknown (de Graaf *et al.,* 2010). In addition, a defensin protein was found in *N. vitripennis* venom using Edman degradation and MALDI-TOF-MS, which brings the total count on 80 identified venom proteins (Ye *et al.,* 2010).

1.5. Mode of action of the venom

Many parasitoids and in particular endoparasitoids, coinject a mix of venom, calyx fluid, polydnaviruses and virus-like particles to assure the successful development of the offspring. There are several records demonstrating that the injected substances alone are not sufficient enough to subdue the host. Indeed, teratocytes coming from endoparasitoid eggs seem 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). In addition, the feeding larvae secrete factors that affect metamorphosis, development, hemocytes and other immune reactions (Richards *et al.,* 2001, 2002a, b; Edwards *et al.,* 2006; Price *et al.,* 2009). The main insights into the functionality of the *N. vitripennis* venom were obtained using bioassays and suggest that, in contrast to some endoparasitoid wasps, the venom on its own is able to modify the host physiology, immune responses and biochemical profile.

1.5.1. Metabolism

In order to create the optimal food conditions for the developing larvae, the host metabolism is modified and synchronized with the development of the wasps. Several metabolic changes are observed during parasitism and seem to depend on the life stages involved. Soon after venom injection, the trehalose levels will drop significantly whereas the glycogen levels will briefly rise up to two hours after envenomation. The latter increase is probably induced by the insertion of the ovipositor and within the following days these glycogen levels steadily decrease again. Venom proteins that may be involved in these processes are acid phosphatases, trehalase, α-esterase, lipases and a lipase-like venom protein, which are also present in venom of the endoparasitoid *P. hypochondriaca* (de Graaf *et al.,* 2010; Danneels *et al.,* 2010; Dani *et al.,* 2005). Envenomated pharate adults also display a decrease in the rate of their oxygen consumption following the first 12 hours, which remains suppressed afterwards. This response is not affected by the wound made during envenomation but is probably caused by the venom. On the long term the levels of pyruvate will increase, whereas whole body lipid levels will rise during the first four days, followed by a decrease, eight days after envenomation. These high lipid levels are the most striking changes observed and seem to be synchronized with the development of the parasitoid larvae (Rivers *et al.,* 1994a, b, 1995). Lipase and a lipase-like venom protein in *N. vitripennis* venom may function to liberate host nutrients (de Graaf *et al.,* 2010; Danneels *et al.,* 2010). Many ecto- and endoparasitoid venoms regulate their host nutritional milieu by digesting fat body cells and stimulation of host nutrient synthesis, allowing nutrients to flow into the hemolymph were they can be consumed by the developing larvae (Nakamatsu *et al.,* 2004). Increased lipid levels were also observed in other parasitoid-host interactions, including the ectoparasitoid *Euplectrus separatae* and its lepidopteran host *Pseudaletia separata* (Nurullahoglu *et al.,* 2004; Nakamatsu *et al.,* 2003; 2004) and parasitation of *Psuedoplusia includes* by *Microplitis demolitor* (Pruijssers *et al.,* 2009). Moreover, adult *N. vitripennis* wasps seem to have lost their ability to synthesize lipids *de novo* and become completely dependent on the lipids of the host (Rivero *et al.,* 2002; Visser *et al.,* 2012). Interestingly, venom of *Cotesia vestalis* (Braconidae), *Diadegma semiclausum* (Ichneumonidae) and *A. Ervi* (Braconidae) is able to induce host castration (Bai *et al.,* 2009; Falabella *et al.,* 2007). Degeneration of host tissues and in particular host reproductive

tissues, may be a strategy to decrease the competition between the host and the developing parasitoids for available host nutrients (Falabella *et al.,* 2005). The resulting altered nutrient flow may be linked with the developmental arrest observed in parasitoids (Pennacchio and Strand, 2006).

1.5.2. Development

Parasitoid venoms are known to induce host paralysis and developmental arrest in order to provide the best conditions for their progeny. These responses will eventually elicit death of the host. Both responses are regularly seen in ectoparasitoids, in which paralysis is used to immobilize the host and the induction of a developmental arrest is necessary to prevent elimination of the larvae during molting. The developmental arrest induced by endoparasitoids is more subtle and can be evoked by the venom, polydnaviruses or teratocytes (Rivers *et al.,* 2009; Reed *et al.,* 1997; Dahlman *et al.,* 2003). However, unlike other ectoparasitoids, the venom of *N. vitripennis* seems to be nonparalytic in non-target flies and its preferred hosts, but young pupae of *Trichoplusia ni* and *Tenebrio molitor* (Lepidoptera) display a dose-dependent loss of abdominal mobility (Rivers *et al.,* 1993).

The venom of *N. vitripennis* is known to induce a developmental arrest of the immobile host. Previous research demonstrated that this phenomenon could not be reversed by applying exogenous ecdysteroids, but it is possible that metabolic changes are involved in this arrest (Rivers *et al.,* 1994). However, in many host-parasitoid associations, host arrest is the result of a hormone imbalance, caused by a disruption or deterioration of endocrine tissues, or inhibition of target tissues to respond to hormone signals (Asgari and Rivers, 2011). 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.,* 2006; Rivers *et al.,* 2011). Recent studies revealed that under this arrest, *N. vitripennis* venom causes incomplete development of bristles and eye pigment deposition, which is probably under the influence of venom calreticulin and phenoloxidase (Rivers and Brogan, 2008).

1.5.3. Immunity

When an appropriate host encounters a parasitoid, it will mount an innate immune response. Additional information about insect immunity can be found in Box 1.3.

Box 1.3. Insect immunity
Unlike mammals that have both an innate and acquired immune system, insects only have an innate immune system which contains two main responses. Upon a septic injury the humoral response is induced and several antimicrobial peptides are synthesized. Furthermore, proteolytic cascades such as the phenoloxidase cascade are induced that will result in clotting and melanization. In addition cellular responses are also activated and include phagocytosis, cell aggregation, nodulation and encapsulation. These processes are modulated by several hemocyte types such as the plasmatocytes (encapsulation, phagocytosis and cell aggregation) and crytal cells (melanization) (Hoffmann, 1995, Lemaitre and Hoffmann, 2007).

These innate immune responses must be evaded by the parasitoid in order to secure the development of the progeny. This is achieved by injecting fluids that alter the immune response. Melanization, an important process of the defense system, is controlled by the PO cascade. This cascade is stimulated after injury and detection of pathogen associated molecular patterns (PAMPS) and is mainly mediated by a subset of the haemocyte population, the crystal cells. Furthermore, the PO cascade plays a role in wound healing, encapsulation and the production of toxic intermediates. Melanization occurs when prophenoloxidase is activated proteolytic by a serine proteinase, which results in the formation of melanin and toxic phenolic compounds. Venom of *C. rubecula* was found to inhibit the proPO activity and subsequent melanization in its host *Pieris rapae* (Asgari *et al.,* 2003a). Coagulation, another immune process involved in wound healing is supported by plasmatocytes that carry out a permanent surveillance in circulation. Encapsulation is used to prevent the invasion of parasites, which starts with the detection and attachment of plasmatocytes around the particle. During the first hours, the lymph gland will increase the proliferation and differentiation of hemocytes into lamellocytes, which are subsequently released to form a multilayered capsule around the particle (Lemaitre *et al.,* 2007; Cerenius *et al.,* 2008). Suppression of host immunity is a common characteristic induced by venom fluids of parasitoid wasps. Encapsulation is frequently inhibited in endoparasitoids that lay their eggs into the hemocoel of their host (Lu *et al.,* 2006; Parkinson *et al.,* 2002c). However, the ectoparasitic wasp *E. pennicornis* is also able to suppress this encapsulation in its lepidopteran host *Lacanobia oleracea*. In this way wound healing cannot occur and the larvae are able to continue their feeding (Richards *et al.,* 2002b). Many parasitoids are

known to inhibit these processes when their venom intermingles with host hemolymph (Richards *et al.,* 2000; Zhang *et al.,* 2004; Ergin *et al.,* 2006).

Although, venom of *N. vitripennis* is able to suppress these immune responses, which makes feeding possible for female wasps and larvae, melanization is often observed at the wound site. However, a defensin-like peptide from *N. vitripennis* venom was found to inhibit host melanization and parasitoid serine proteases and protease inhibitors are also able to prevent this process (Tian *et al.,* 2010; Colinet *et al.,* 2009; 2013). Furthermore, *in vitro* assays with hemocytes showed that the venom is able to inhibit host hemolymph PO activity and encapsulation processes, affecting the suppression of adhesive and spreading behavior of granular cells and plasmatocytes. These plasmatocytes and other susceptible cells are found to retract their pseudopods, have a spherical shape, a granular cytosol and display plasma membrane blebbing. The two cell types involved in wound healing are plasmatocytes and granular cells. In fact, plasmatocytes recruit granular cells at the wound site using chemo-attractant molecules. However, only plasmatocytes undergo venom-induced cell death by lysis (Rivers *et al.,* 2002a). This is also seen with the venoms of *P. hypochondriaca* and *E. pennicornis*, which affect haemocyte morphology, behavior and viability (Rivers *et al.,* 2009; Richards *et al.,* 2002a). The same responses were observed during parasitation of *P. separata* by *Meteorus pulchricornis* (Suzuki *et al.,* 2008) and *Galleria mellonella* exposed to *Pimpla turionellae* (Er *et al.,* 2010, 2011). In many parasitoid-host relationships, the total number of hemocytes declined due to cell death, particularly by apoptosis, a process that is largely tolerogenic (Suzuki and Tanaka, 2006; Teramoto and Tanaka, 2004; Richards and Edwards, 2002). Additional information about apoptosis can be found in Box 1.4. This type of programmed cell death was also found to be one of the dominant mechanisms in BTI-TN-5B1 cells (Lepidoptera: Noctuidae) exposed to venom of *N. vitripennis* (Rivers *et al.,* 2010). Furthermore, previous studies demonstrated that the venom-induced cell death is a result of Ca^{2+} mobilization from intracellular stores (e.g. mitochondria and endoplasmic reticulum) into the cytosol, through G-protein dependent signaling. This Ca^{2+} release is stimulated by venom calreticulin in *N. vitripennis* and can lead to irreversible cell damage. This calreticulin was detected by immunoblotting (Rivers and Brogan, 2008). A calreticulin-like protein (CrCRT) in *C. rubecula* has the ability to suppress the spreading behavior of host hemocytes, thereby preventing encapsulation. This protein might be an antagonist of host calreticulin

and possibly competes for the same binding sites (Zhang *et al.,* 2006). Studies with *P. hypochondriaca* venom also indicated that a calreticulin-like protein targets host hemocytes by inducing cell death and possibly affects cellular calcium homeostasis (Rivers *et al.,* 2009). The observed PO activity of the venom can cause the same cell damage as seen with calreticulin (Rivers *et al.,* 2005). Probably, this Ca^{2+} mobilization is controlled by phospholipase C (PLC) and inositol trisphosphate (IP_3), which subsequently activate phospholipase A_2 (PLA_2) (see 1.5.5.1). The latter might be involved in the regulation of fatty acid synthesis and release, which can lead to toxic accumulation and induce death on the long term (Rivers *et al.,* 2002b). Several *N. vitripennis* venom proteins are identified with putative functions in host immunity (de Graaf *et al.,* 2010; Danneels *et al.,* 2010).

Box 1.4. Cell death

There are different types of cell death which can be classified by several criteria including cell morphology and biochemical events. Apoptosis is one of the most studied programmed cell death types because it plays a role in many biological processes such as development and immunity. Apoptosis involves several morphological features such as plasma membrane blebbing, rounding up of the cell, apoptotic bodies, phosphatidyl serine externalization, nuclear fragmentation and engulfment by resident phagocytes. It is also an energy dependent process that involves the activation of caspases that will eventually lead to cell demise. Another important cell death type is necrosis, which was long considered as an accidental process, but evidence is accumulating that this is also a tightly regulated process. Necrosis is morphological characterized by cell swelling (oncosis), plasma membrane rupture and loss of intracellular contents (Kroemer *et al*., 2009).

1.5.4. Stress response

Parasitized hosts also display a molecular response that results in an altered gene expression of proteins, including parasitism specific proteins and stress proteins. These proteins are part of the stress response and can be up or down regulated in envenomated hosts. Heat shock proteins (hsp) are induced upon stress and exert protective functions. Observations of the expression profiles of stress proteins in *S. crassipalpis*, using northern blot hybridizations 13 hours after envenomation, revealed the upregulation of both *hsp 23* and *hsp70*. Additionally, *hsp90* and heat-shock cognate protein 70 (*hsc70*) transcripts are downregulated. These transcriptional changes are possibly involved in the immune response or developmental arrest following envenomation (Rinehart *et al.,* 2002). A similar response is observed after envenomation of the ectoparasitoid *Bracon hebetor* and its lepidopteran host *Plodia interpunctella*. In this case, *hsc70* and small heat shock protein (*shsp*) transcripts are up regulated while *hsp90* is down regulated (Shim *et al.,* 2008). Furthermore, parasitation of

Pieris rapae by the endoparasitoid *P. puparum* also altered gene expression, resulting in an upregulation of *hsp20* and downregulation of *hsp75* and *hsp90* (Zhu *et al.,* 2013).

1.5.5. Cytotoxic effect of the venom

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 (Fig. 1.5), which is often seen in host-parasitoid relationships and associated with immunosuppression of the host (Uckan *et al.,* 2004; Suzuki *et al.,* 2009; Parkinson and Weaver, 1999; Asgari and Rivers 2011). Furthermore, this type of cell death may be involved in inducing the developmental arrest seen in *N. vitripennis* hosts by targeting brain tissues, and the altered metabolism in order to release nutrients for the developing larvae (Rivers *et al.,* 2006, 2011, 1994b, 1995). Under normal conditions, apoptotic cells are phagocytized, resulting in little or no immune responses such as inflammation, which occurs during injurious stimuli (Elmore, 2007). Consequently, apoptosis induction is also a useful survival strategy applied by parasitoids that reside in/or attached to their host (Schmidt *et al.,* 2001).

1.5.5.1. Cell death pathways induced by parasitoid venoms

The cytotoxic action of parasitoid venoms generally involves several cell death pathways, such as apoptosis, oncosis or necrosis. The latter is assumed to be the end result of the complete apoptotic pathway that is displayed in *in vitro* cell cultures, but less is known about these associated pathways. Apoptosis is a very complex process that involves an energy-dependent cascade of molecular events (Fig. 1.6). Two main apoptotic pathways exist, the extrinsic pathway that initiates with cell surface/death receptors (i.e. apoptosis stimulating fragment, Fas) using secondary messengers such as Ca^{2+}, IP_3, or cyclic adenosine monophosphate (cAMP) to activate caspases and the intrinsic pathway that involves mitochondrial release of caspase activators (Asgari and Rivers, 2011). The latter is activated upon stimuli such as the absence of certain growth factors, radiation, toxins or hypoxia and involves the loss of apoptotic suppression and subsequent activation of apoptosis. These stimuli cause the loss of mitochondrial transmembrane potential and result in the release of cytochrome c and second mitochondria-derived activator of caspases (Smac/Diablo). Subsequently, cytochrome c will bind and activate Apoptotic protease activating factor 1

(Apaf-1) and procaspase-9, forming the apoptosome, which activates caspase-9. Smac/Diablo promote apoptosis by inhibition of IAP (Inhibitors of Apoptosis Proteins). Both intrinsic and extrinsic pathways are linked to each other and converge at the execution pathway which is initiated by the cleavage of caspase-3, resulting in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cytoskeletal reorganization and formation of apoptotic bodies (Elmore, 2007). A third pathway involves the endoplasmic reticulum (ER) which results in the activation of several caspases.

Cell death induced by *N. vitripennis* venom may involve G-protein sensitive receptors. Upon venom induction, several events are initiated such as the loss of mitochondrial membrane potential (ΔΨm), subsequently an uncontrolled [Ca^{2+}] efflux into the cytosol occurs, which is followed by the increase of cytoplasmic cAMP levels that promotes additional [Ca^{2+}] release from intracellular stores (Rivers *et al.,* 1999, Rivers *et al.,* 2002b and Rivers *et al.,* 2005). Treatment of BTI-TN-5B1-4 cells with the caspase 3 inhibitor Z-VAD-FMK was also able to rescue a large part of the cells treated with *N. vitripennis* venom (Rivers *et al.,* 2010). In addition, studies with *P. hypochondriaca* showed that the ΔΨm drops in venom treated cells, while [Ca^{2+}] levels increase (Rivers *et al.,* 2009). Furthermore, *N. vitripennis* venom can induce changes in plasma membrane permeability, resulting in an influx of [Na^{+}], which could activate PLC followed by IP_3 formation and the release of [Ca^{2+}] from the mitochondria. The loss of membrane integrity and subsequent calcium increase was also observed in BTI-TN-5B1-4 cells treated with *P. turionellae* venom (Keenan *et al.,* 2007). This calcium release is able to trigger PLA_2 activation which may stimulate fatty acid (FA) synthesis in the host fat body. Excessive accumulation of these fatty acids can become toxic and induce cell death (Rivers *et al.,* 2002b). When calcium homeostasis is disrupted, cells may die by apoptosis or oncosis (Rivers *et al.,* 2006; Asgari and Rivers, 2011). Oncotic cell death is the result of a disrupted homeostasis of the intracellular environment by an altered plasma membrane integrity. During this process, macromolecules and ions enter the cell in an unregulated way, followed by water. Venoms of *P. hypochondriaca*, *P. turionellae* and *N. vitripennis* have the ability to disrupt the plasma membrane in host cells, resulting in membrane blebbing, rounding, swelling and eventually cell lysis (Ergin *et al.,* 2006; Keenan *et al.,* 2007; Rivers *et al.,* 2006).

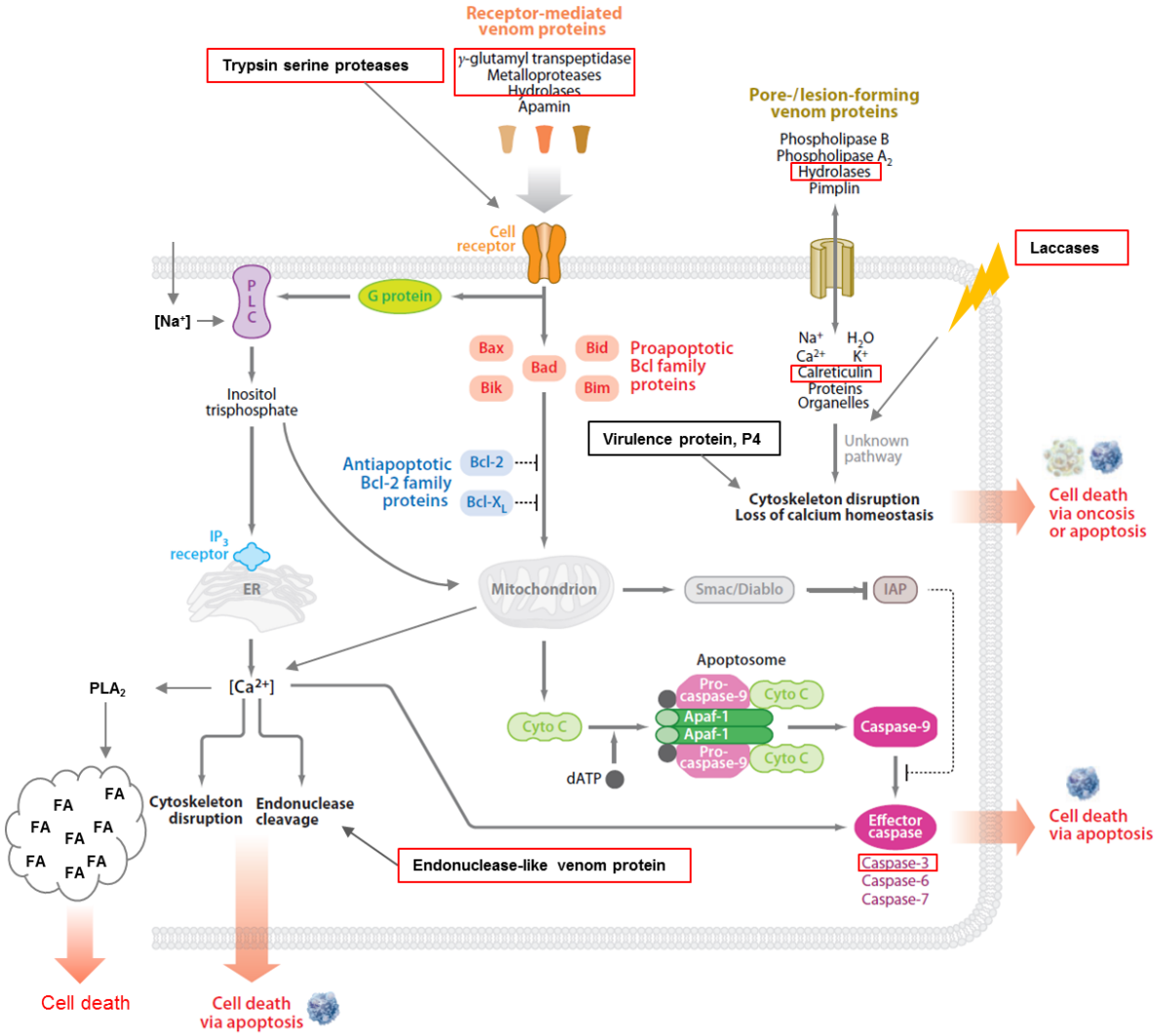


Figure 1.6: Potential mode of action of parasitoid venom proteins in evoking cell death in hosts. Both apoptotic and oncotic/necrotic pathways may be induced in endo and ectoparasitoid wasps and are based on biochemical and morphological observations described in the text. Venom proteins present in *N. vitripennis* are given in red boxes. Virulence protein 4 (P4) is found in *L. boulardi*, contains a RhoGAP domain (Ras homologous GTPase Activating Proteins) and may regulate actin cytoskeleton changes (Labrosse *et al.,* 2005). Abbreviations: Apaf-1, apoptotic protease activating factor 1; Cyto C, Cytochrome c; ER, endoplasmic reticulum; IAP, inhibitor of apoptosis; IP_3, inositol triphosphate; PLC, phospholipase C; Smac (second mitochondria-derived activator of caspases)/Diablo, a mitochondrial protein that binds to IAP and relieves IAP inhibition of caspases; FA, fatty acids (figure adapted and modified from Asgari and Rivers, 2011).

1.5.5.2. Venomous proteases involved in cell death

Sixteen serine proteases (± 20% of the protein ID's) and two metalloproteases were discovered in *N. vitripennis* venom (de Graaf et al., 2010). Members of these protease protein families were also identified in *P. puparum* (Zhu *et al.,* 2010), *Chelonus inanitus* (Vincent *et al.,* 2010), *H. didymator* (Dorémus *et al.,* 2013) and *P. hypochondriaca* (Parkinson *et al.,* 2002a, 2002c). One of these identified metalloproteases in *N. vitripennis* venom is an

angiotensin-converting enzyme (ACE). This enzyme is a cell membrane associated zinc dependent carboxyl dipeptidase that acts as an ectoenzyme which have their enzymatically active site outside the plasma membrane and are also known for their role in the processing of peptides for the major histocompatibility complex (MHC) class I molecules (Shen *et al.,* 2011). This enzyme is also present in other insects and may function in the brain, insect reproduction and development (Schoofs *et al.,* 1998; Lamango *et al.,* 1997). Furthermore, ACE was found to have a wide tissue distribution in *Locusta migratoria* and its expression in hemocytes is stimulated upon injection of bacterial lipopolysaccharides, which suggest its involvement in immune processes (Macours *et al.,* 2003). The second is a member of a disintegrin and metalloproteinase with a thrombospondin motif 5-like (ADAMTSL-5) and a reprolysin domain. Reprolysin metalloprotease homologs EpMP1-3 are also identified in the venom of *E. pennicornis*. Recombinant EpMP3 was found to be toxic in *L. oleracea* and may have a role in manipulation of host development (Price *et al.,* 2009; Asgari and Rivers, 2011). Proteomic analysis of the *Hyposoter didymator* venome also resulted in the identification of a disintegrin and metalloproteinase with thrombospondin motifs (Dorémus *et al.,* 2013). Reprolysin metalloproteases may also be involved in the developmental arrest of the host, since they are key players in the regulation of several developmental events in *Drosophila melanogaster* (Sotillos *et al.,* 1997). These metalloproteases are well represented in several snake and spider venoms, and play major roles in immunity and apoptosis related processes. Furthermore, metalloproteases of the ADAM family are known as potent inhibitors of both platelet aggregation and integrin-dependent cell adhesion. These enzymes are able to hydrolyze both extracellular matrix (ECM) proteins and cell surface integrin receptors, which mediate cell attachment (You *et al.,* 2003; Takeda *et al.,* 2012).

The serine proteases found in *N. vitripennis* venom all belong to the trypsin serine protease family (de Graaf *et al.,* 2010). They have diverse functions and are involved in digestion, coagulation, moulting, larval development and ECM degradation. Furthermore, a secreted chymotrypsin-like serine protease Sc-SP-3 in the nematode *Steinernema carpocapsae* induces apoptosis in Sf9 cells, affects hemocyte spreading and suppresses prophenoloxidase activity in *G. mellonella* (Balasubramanian *et al.,* 2010; Toubarro *et al.,* 2009). In *C. rubecula*, a serine protease homolog venom protein was found to inhibit melanization in its host *Pieris rapae* (Asgari *et al.,* 2003).

1.5.5.3. Other venom compounds with possible roles in cell death

However, since the venom consists of a complex mixture of 80 venom proteins, other venom compounds may also be involved in cell death. Some of these proteins (γ-glutamyl transpeptidase-like venom protein 1 & 2 and a γ-glutamyl cyclotransferase-like venom protein) have assigned functions in the glutathione metabolism, a process that plays a role in apoptotic cell death (Franco and Cidlowski, 2009; Circu and Aw, 2008). In *A. Ervi*, an γ-glutamyl transpeptidase (γ-GT) was found to induce apoptosis in the ovaries, which results in castration of its host *Acyrthosiphon pisum*. This γ-GT may disrupt the glutathione balance, resulting in oxidative stress and subsequently induce apoptosis (Falabella *et al.,* 2007). *Nasonia vitripennis* venom also includes two laccases and an endonuclease-like venom protein with possible functions in programmed cell death (Danneels *et al.,* 2010). Venom of the endoparasitoid *P. hypochondriaca* also contains a laccase and three PPO proteins (Parkinson *et al.,* 2002). Proteins with phenoloxidase activity, including laccases, have the ability to disrupt plasma membranes of cells (Abt and Rivers, 2007). Calreticulin, another *N. vitripennis* venom protein is also involved in cytotoxic processes (de Graaf *et al.,* 2010; Asgari and Rivers, 2011; Rivers and Brogan, 2008) and is present in the venoms of *Microctonus spp.* (Crawford *et al.,* 2008), *Pteromalus puparum* (Zhu *et al.,* 2010) *and C. rubecula* (Zhang *et al.,* 2006). Treatment of *P. hypochondriaca* venom with an anticalreticulin antibody was also able to block cell death in host hemocytes. Although this calreticulin venom protein is not yet identified in *P. hypochondriaca*, results indicate that a compound is present that affects cellular calcium homeostasis (Rivers *et al.,* 2009). Hydrolases are known for their cytotoxic and cytolytic functions. In parasitoid species they may enable venom distribution and interfere with host immunity (Moreau and Guillot, 2005; Xia *et al.,* 2000). Although they are present in the venoms of *P. hypochondriaca*, *P. puparum* and *N. vitripennis* their exact functions remain unknown (Asgari and Rivers, 2011). Furthermore, 23 unknown venom proteins are present in *N. vitripennis* venom and although their exact functions are not identified yet, some of them could play possible roles in cell death.

1.6. Arthropod derived compounds as a source for novel therapeutic agents

Arthropods are one of the largest and widely distributed groups of animals. Many representatives including spiders, scorpions, bees, wasps and ants contain venoms and other

secretions which are used for feeding and defense. Arthropods are recognized as one of the greatest recourses of biologically active molecules, especially venom compounds. Still, many compounds present in arthropods remain unidentified. Nowadays it is obvious that arthropod venoms and other substances such as hemolymph contain a plethora of molecules with interesting properties and in the past, most studies were focused on venoms of dangerous species. Besides the well-known insecticidal functions of many arthropod venoms, numerous spider and scorpion venom compounds were found to have antimicrobial and antitumor properties (Schwartz *et al.,* 2012; Quintero-Hernandez *et al.,* 2011; Garcia *et al.,* 2013; Liu *et al.,* 2003).

1.6.1. Insect compounds

The insect class, which is the largest class in the arthropod phylum, contains many promising bioactive peptides and proteins with intriguing functions. Several insect antimicrobial peptides (AMPs) which are important compounds of the innate immune system have the potential to display specific tumoricidal activity by disrupting the membrane of cancer cells. Alloferon, a peptide isolated from bacteria-challenged *Calliphora vicina* larvae was found to stimulate natural killer lymphocyte (NK) activity and interferon (IFN) synthesis in animal and human cell lines involved in cancer by activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) signaling pathway. The peptide also enhanced antiviral and antitumor activities in mice (Chernysh et al, 2002; Ryu *et al.,* 2008). Furthermore, *Heliothis virescens* insect hemolymph contains a N-myristoylated-peptide, which was found to have antitumor activity against 34 human tumor cell lines, including lung cancer, leukemia and melanoma (Ourth, 2011). Cecropins, orignially found in *Hyalophora cecropia* hemolymph, are AMPs with the ability to form ion-permeable channels subsequently resulting in cell depolarization, irreversible cytolysis and finally cell death. Interestingly, members of the Cecropin family exert tumoricidal activity against several cancer cell lines, including bladder cancer, leukemia and lymphoma (Suttmann *et al.,* 2008; Hui *et al.,* 2002; Winder *et al.,* 1998). Enantiomeric 9-mer peptides derived from 43-mer insect defensins of *Oryctes rhinoceros* and *Allomyrina dichotoma* beetles were also found to display selective cytotoxic activities against a mouse myeloma cell line (Iwasaki *et al.,* 2009). These studies confirm that insects, indeed produce interesting compounds which may be used as a novel source for therapeutic agents.

1.6.2. Hymenoptera venoms

Hymenoptera are well known for their venom producing species. Many venoms of bees, bumblebees, wasps and ants are extensively studied for their pathological functions and possible medical use in humans for treatment of infections or diseases such as cancer (Dkhil *et al.,* 2010; Heinen *et al.,* 2011; Son *et al.,* 2007). Most venom compounds, such as honeybee melittin are promising venom proteins because of their ability to induce several types of cell death in, for example cancer cells (Oršolić, 2003; 2012). Until now, venoms of parasitoid wasps remained unexploited regarding to this research area. Nevertheless, the availability of the complete venom composition of *N. vitripennis* has been a springboard for the research of other parasitoid venoms and several attempts have been made to identify new venom proteins and elucidate their functions. These group of insect venoms may provide us a new source of pharmacologically active components. Although parasitoid venoms are mainly investigated in their natural hosts or in surrogate species within the same class of their usual hosts, their impact on mammalian cells, including human cell lines, and their possible antimicrobial activity in mammals remains unknown. However, since parasitoid venoms are known for their cytotoxic properties in natural hosts and many of their target pathways are conserved throughout evolution, especially those regarding to apoptosis and immune processes, they may fulfill promising, undiscovered functions in other organisms (Belvin, 1996; Meier *et al.,* 2000a).

Some protein families, present in arthropod venoms are known for their functions in immune, inflammation or cytotoxic processes and interestingly they also have representatives in *N. vitripennis* venom. Although, the complete venom composition of *N. vitripennis* is known, less is known about the functionality of similar venom compounds of other hymenopteran species and more specific their mode of action in humans. Antimicrobial peptides are present in many venoms, including parasitoid wasps, where they are used to disinfect the host after injecting venom or eggs and protect the offspring against bacteria and fungi (Moreau, 2013). Defensins are found in many organisms, including mammals, insects and plants. Despite their antimicrobial activity against plant and insect pathogens they are also efficacious against human fungal pathogens, including *Candida spp.* and *Aspergillus spp.* (Thevissen *et al.,* 2007). These antimicrobial effector molecules of innate immunity also provide an efficient initial defense against infectious pathogens and

some are toxic for mosquito stages of the *Plasmodium gallinaceum* parasite (Ganz, 1999; Bulet *et al.,* 1999; Vizioli and Salzet, 2002). A defensin-like peptide present in *N. vitripennis* venom was found to have strong antimicrobial activity against the fungus *Candida albicans* and several Gram-positive and Gram-negative bacteria (Ye *et al.,* 2010). Serine proteases and serine protease inhibitors are of broad interest, because of their diverse physiological functions in digestion, hemostasis, fibrinolysis, inflammation, blood coagulation, and ion channel blocking (Neurath, 1984; Chen *et al.,* 2012). Trypsin serine proteases are the most abundant group of venom proteins present in *N. vitripennis* and are accompanied with several serine inhibitors (de Graaf *et al.,* 2010). Serine proteases present in other hymenopteran species were already discovered to have interesting functions including the fibrin(ogen)olytic activity of bee and bumble bee venom serine proteases (Choo *et al.,* 2010; Qiu *et al.,* 2011). The Kunitz-type venom protein found in *N. vitripennis* venom is also present in bumble bee venom and acts as an plasmin inhibitor, suggesting its antifibrinolytic role (Choo *et al.,* 2012).

2. Introduction on honeybee reproduction

Highly eusocial insects are characterized by their reproductive division of labor within the colony and hence the occurrence of distinct morphological phenotypes. Since the availability of the complete genome sequence of the eusocial insect, *Apis mellifera* in 2006 (The Honeybee Genome Sequencing Consortium), the honeybee has become a key model system. A honeybee colony is characterized by its queen-worker caste determination, which is a well-studied insect polyphenism. This is not only reflected in a different phenotype, whereby workers are smaller than the queen and do not reproduce, but also in behavioral differences (Fig. 1.7).

Hymenoptera
Apocrita
Aculeata
Apoidea
Apidae Apinae
Apis
Apis mellifera
Apis mellifera carnica

Figure 1.7: Classification of *Apis mellifera carnica*. Honeybee workers and the queen (numbered) on a brood comb (adopted from beeinformed, by Rob Snyder).

Although adult queens and workers differ in their behavior, morphology and physiology, genetic predisposition is not assumed to be involved in this process (Evans *et al.,* 1999; Maleszka, 2008). In contrast, this remarkable difference in phenotype is mainly influenced by environmental cues that the larvae encounter during their development. Queen-destined larvae are fed on a much richer diet, that includes secretions from the hypopharyngeal and mandibular glands of nurse workers. These secretions are generally known as royal jelly and consists of proteins, essential amino acids, lipids, vitamins and other compounds (Maleszka, 2008). This diet results in hypertrophy of the reproductive systems, an enlarged body size and a prolonged lifespan in both honeybee and the *Drosophila* larvae tested (Kamakura, 2011). Larvae destined to become workers, are fed on a restricted diet, starting from the 3rd instar and have smaller body sizes and reduced ovaries (Mutti *et al.,* 2011a). Furthermore,

workers also respond to environmental cues. Pheromones produced by the queen and brood are known to inhibit worker ovary activation (Tan *et al.,* 2012). As a result, only the queen acquires the monopoly to reproduce. In all honeybee species, the queen is inseminated by multiple males (polyandry), while workers are responsible for food collection, protection of the beehive and rearing the offspring. This reproductive division of labor and hence the altruistic behavior of workers is predominantly explained by the inclusive fitness theory, which is the sum of the direct and indirect fitness. The latter states that the level of kinship determines the amount of supporting behavior by the workers. Despite the high family relatedness in a honeybee colony, individual selfishness can occur, resulting in rogue workers that do not work for the benefit of the colony. These workers do have activated ovaries and try to lay unfertilized eggs, which become males. In queen right colonies, less than 1% of the *A. mellifera* workers have activated ovaries. Furthermore, ovary activation rates differ between species, which is about 5% in *A. cerana* workers (Velthuis, 1970; Oldroyd *et al.,* 2001). Interestingly, reproductive activity of workers increases and task performance reduces when the mating frequency of the queen drops (Mattila *et al.,* 2012). This can be explained by the fact that, when the queen is inseminated by multiple males, workers are on average more related to their brothers, than to the sons of other workers (van Zweden *et al.,* 2012). In queenless colonies or caged workers up to 30% of *A. mellifera* workers and 70% of *A. cerana* workers have activated ovaries (Tan *et al.,* 2009). One week after losing their queen and without the possibility of requeening, workers are able to lay male eggs. However, a special case is that of the rare anarchistic honeybees which can appear in a queenright colony. Here, workers have higher ovary activation rates in both queenless and queenright colonies compared to normal workers and lay large numbers of male eggs. Furthermore, another unusual form of reproduction exist in *A. mellifera capensis* colonies. In contrast to the arrhenotokous reproduction of haploid male eggs observed in other honeybees, workers of this species do lay eggs under normal conditions. This form of reproduction is performed by thelytoky, producing diploid female eggs (Barron *et al.,* 2001). Despite these exceptions, normal workers do not usually activate their ovaries and several mechanisms exist to suppress worker reproduction. In the subsequent paragraphs, the existing strategies concerning the suppression of worker reproduction, as well as the molecular mechanisms of ovary (in)activation during larval development and adult life, are further reviewed.

2.1. Regulation of honeybee caste differentiation

Despite having the same genetic background, caste differentiation in honeybees is influenced by a nutritional dichotomy between the queen and workers during larval development. Queen destined larvae receive a much richer diet throughout the larval stages, consisting of the nutrient-rich royal jelly. This nutrient-rich diet results in an accelerated larval growth, hypertrophy of the reproductive systems, an enlarged body size and a lifespan which is 20 times longer than that of a normal worker bee. This is in contrast to worker larvae that receive a less nutritious diet of worker jelly, consisting of glandular secretions, nectar and pollen, which start from the 3rd instar, and results in small-bodied workers with reduced ovaries and a short lifespan (Maleszka, 2008; Mutti *et al.,* 2011a). Furthermore, many genes are found to be differentially expressed in developing queens and workers (Evans and Wheeler, 1999). Worker larvae were found to up regulate more genes involved in the development of caste-biased structures, including brain, legs and ovaries. In addition, several juvenile hormone (JH) responsive genes are differentially expressed upon JH treatment of worker larvae. In queen destined larvae, a large proportion of physiometabolic genes are upregulated that contribute to mass-transforming processes and growth, including target of rapamycin (*tor*) (Barchuk *et al.,* 2007).

2.1.1. Juvenile hormone in the developing honeybee larvae

Royal jelly is metabolized in the fat body where it activates nutrient-sensing pathways that lead to the release of JH by the *corpora allata* in the brains. Since long, the importance of JH in insect development is extensively studied. The involvement of JH in caste differentiation was confirmed by administering synthetic JH to larvae which consequently developed queen-like treats (Rembold *et al.,* 1974). Queen larvae have the highest JH expression and titers during the 4^{th} instar. Larvae acquiring a restricted diet, will have low JH titers and eventually develop into workers. Ovary size is determined during the 3th larval instar, when JH levels start to rise in queen destine larvae. Since JH is known to control programmed cell death in larval ovaries, low JH titers in worker destined larvae will induce the degeneration from 150-200 ovariole primordia to less than 10. High JH titers in queen larvae may prevent this process which is regulated by ecdysteroids in *Drosophila* (Fig. 1.8). Consequently, from

the fourth larval instar until the prepupal stage, programmed cell death reduces the number of ovariole primordia in workers (Capella and Hartfelder, 1998; Soller *et al.,* 1999).

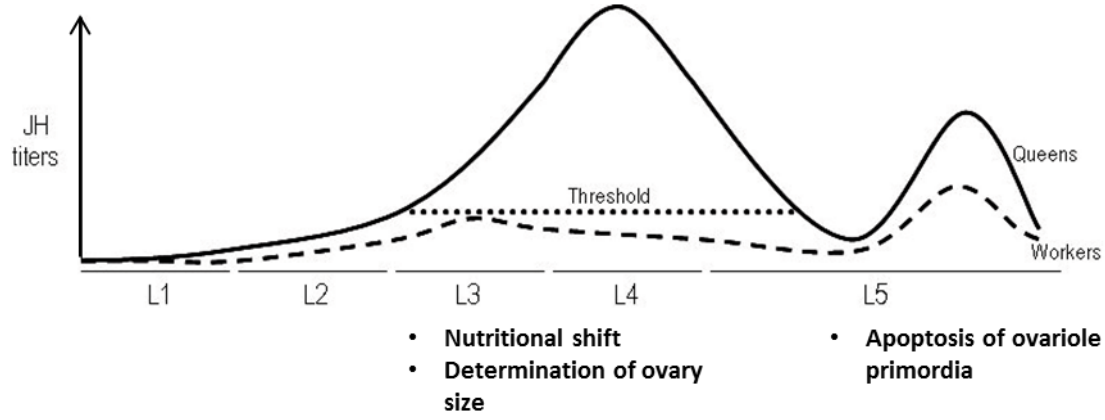


Figure 1.8: JH titres during larval development (L1–L5). Reaching of the juvenile hormone (JH) threshold in developing females, as a consequence of the nutritional shift at the 3th larval instar, allows general body growth and ovary development. The development of worker like characteristics are inhibited, including apoptosis of the ovariole primordia during the 5th larval instar. Modified from Barchuk *et al.,* 2007.

2.1.2. Key players in caste differentiation

2.1.2.1. Insulin signaling and TOR pathway

The nutrient-sensing pathways affecting the release of JH are known as the insulin-IGF-1 signalling (IIS) pathway, which subsequently activates the target of rapamycin (TOR) pathway (Rachinsky *et al.,* 1990; Kapahi *et al.,* 2004). Target of rapamycin (TOR) is a protein kinase involved in nutrient-sensing, growth control and is also known as a negative regulator of autophagic cell death. Furthermore, TOR plays an important role in triggering JH synthesis and can facilitate vitellogenin (Vg) expression (Barchuk *et al.,* 2007). Vitellogenin, a precursor of yolk protein (yp) is synthesized in the fat body and plays an important role in the egg production, behavior and immunity. Furthermore, it is well known for its antioxidant activity and involvement in the longevity of queen bees (Corona *et al.,* 2007). Suppression of TOR in *Drosophila* leads to smaller larval and adult body sizes (Baehrecke, 2005; Colombani *et al.,* 2003). In honey bees, high *amTOR* expression levels, especially in 4th instar larvae, are found to be necessary for queen development. Queen destined 4th and 5th instar larvae treated with rapamycin, a TOR inhibitor, displayed a worker-like phenotype, however, ovary morphology remained queen-like. In addition, knockdown of *amTOR* prevented queen development resulting in workers (Patel *et al.,* 2007). The IIS pathway is evolutionary

conserved and plays a role in development, body size, reproduction and longevity in many taxa (Oldham and Hafen, 2003). Downregulation of the IIS pathway is associated with an increased longevity and decreased fertility in both *Caenorhabditis elegans* and *D. melanogaster* (Finch and Ruvkun, 2001). In honeybees, nutrition quality, quantity and larval developmental status affects the expression of several genes involved in the IIS pathway. For instance, two insulin-like peptides were found to have differential expression patterns with respect to the developmental status of both queen and worker larvae, which seem to be regulated by JH (de Azevedo and Hartfelder, 2008). Interestingly, although the insulin receptor *AmIR-1* transcript levels are 30-fold higher in 3rd instar queens compared to worker larvae, ovaries of 4th instar worker larvae have higher levels which increases towards the prepupal stage. These changes may be the result of the nutritional shift during larval development (Wheeler *et al.,* 2006; de Azevedo and Hartfelder, 2008). *Drosophila* larvae treated to overexpress the insulin receptor (*IR*) or silencing of the IIS inhibitors were found to have an altered onset of metamorphosis (Colombani *et al.,* 2005). Furthermore, *Drosophila IR* mutants have a reduced body size and weight, however, when reared on royal jelly these mutants display larger body sizes and shorter developmental times (Kamakura, 2011). RNAi knockdown of the insulin receptor substrate (*IRS*) in larvae receiving a rich diet resulted in a worker phenotype with smaller ovaries. These findings suggest that both the insulin-IGF-1 signalling (IIS) pathway and the rapamycin (TOR) pathway are linked together and influence caste differentiation. Indeed, knockdown of the insulin receptors also resulted in decreased levels of *amTOR* mRNA expression. However, downregulation of *amTOR* did not result in lower *IRS* expression. Both knockdown of *IRS* and *amTOR* reduced JH levels in 5th instar larvae, which are high in developing queens. Application of JH was able to rescue the queen phenotype in both knockdowns. In addition, several proteomic changes were reported upon *IRS* knockdown, including lower levels of hexamerin 110, a storage protein also found in low levels in worker destined larvae and involved in caste differentiation. (Wolschin *et al.,* 2011; Evans and Wheeler, 1999; Mutti *et al.,* 2011a).

2.1.2.2. Interplay of royalactin and the epidermal growth factor receptor (EGFR) in caste differentiation

One of the main compounds of royal jelly are the major royal jelly proteins (MRJPs) which are part of the yellow family and known for their diverse functions related to development

and sexual maturation in insects (Drapeau *et al.,* 2006). However, overexpression of MRJP2-5 in *Drosophila* did not affect the phenotype. Interestingly, a 57 kDa monomer of MRJP1, known as royalactin is able to induce a queen phenotype in worker destined larvae, which results in larger ovaries and shorter developmental times. Royalactin was found to increase JH titers (Kamakura, 2011). The epidermal growth factor receptor (EGFR) is expressed in the fat body of insects and is part of a conserved signal transduction pathway that regulates many developmental processes, including cell proliferation, survival, fate specification and differentiation (Vivekanand and Rebay, 2006). EGFR signaling is known to be involved in both embryonic gonad development (Weyers *et al.,* 2011) and oocyte maturation in adult individuals in other invertebrate model organisms (Shilo and Raz, 1991; Poulton and Deng, 2006; Van Buskirk and Schüpbach, 1999) and is also involved in the negative regulation of apoptosis (Parker, 2006; Bogdan and Klämbt, 2001). In addition, both EGFR and IIS signaling were found to modulate social behavior in adult worker bees upon phosphatase and tensin (PTEN) knockdown in honeybee larvae. PTEN is a negative regulator of the IIS and EGFR pathways in *D. melanogaster* and may also play a central role during the development of queen and worker bees (Mutti *et al.,* 2011b). Furthermore, the importance of both royalactin and EGFR in caste differentiation, including regulation of lifespan, developmental time and body size was revealed in experiments performed by Kamakura (2011) using both *D. melanogaster* and *A. mellifera*. *Drosophila* (*EGFR*) mutants and knockdown of EGFR in both *D. melanogaster* and queen-destined honeybee larvae resulted in reduced adult and ovary sizes, and a prolonged developmental time, which could not be restored by royal jelly. EGFR was found to mediate mitogen-activated protein kinase (MAPK) and ribosomal protein S6 kinase beta-1 (S6K) activation upon royalactin ingestion in both *Drosophila* and queen larvae, which results in an increased 20-hydroxyecdysone (20E) and JH titer and *Vg* expression (Fig. 1.9). These changes are inhibited by *EGFR* RNAi. S6K was found to be involved in body size, while MAPK acts downstream of EGFR in increasing 20E synthesis (which regulate Vg synthesis in honeybee larvae) and shortening developmental time (Kamakura, 2011; Barchuk *et al.,* 2002). Although, ecdysteroid titers differ between the castes in larval and pupal stages, their contribution in reproduction and division of labour is rather minimal (Rachinsky et al. 1990; Pinto *et al.,* 2002; Hartfelder *et al.,* 2002). These findings prove that royalactin is the major compound involved in caste differentiation, which acts trough EGFR (Kamakura, 2011). Since JH is increased downstream of EGFR signaling, it is

reasonable to assume that this receptor is also involved in ovary development in larvae and adult workers in queenless situations.

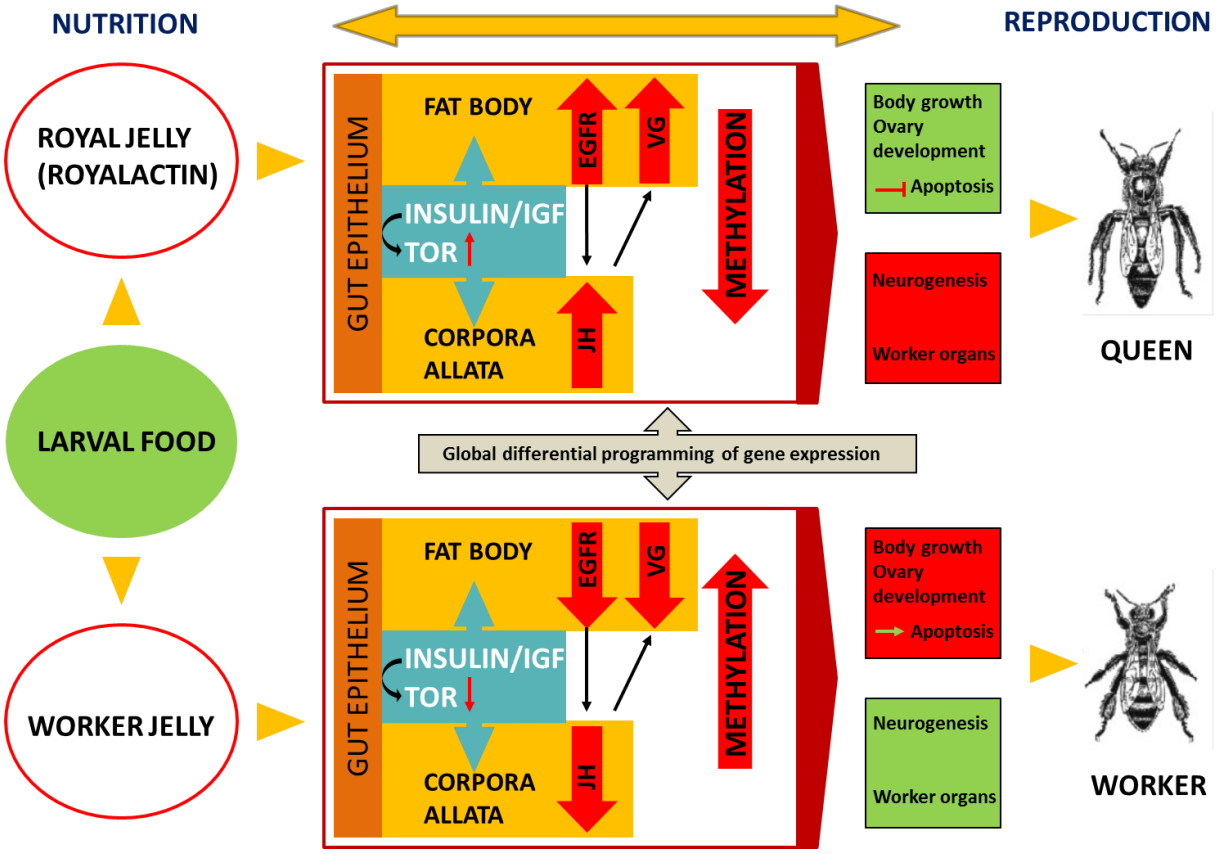


Figure 1.9: Model of involved molecular pathways and determinants in caste differentiation during *Apis mellifera* development. Nutritional input determines the developmental fate of the honeybee larvae and involves insulin/IGF and TOR signaling. This results in high juvenile hormone (JH) levels in queen destined larvae and low JH levels in worker larvae. Epidermal growth factor (EGFR) signaling is suggested to work upstream of JH. Juvenile hormone mediates vitellogenin (Vg) synthesis, which in turn controls the egg production. Phenotypical changes are manifested through contrasting anatomical and physiological features in both castes. Red box: inhibition of characteristics; green box: activation of characteristics. Modified from Malezka, 2008; Barchuk *et al.,* 2007; Corona *et al.,* 2007.

2.1.2.3. Epigenetic regulation of caste differentiation in honeybees

Alterations in diet are sensed by the IIS and TOR pathways and also affects EGFR signaling. These pathways are able to modify the developmental trajectory of an organism, despite having the same genetic background, resulting in different activated genes between a honeybee queen and worker larvae (Grozinger *et al.,* 2007; Evans and Wheeler, 1999; Hepperle and Hartfelder, 2001). However, in honeybees epigenetic regulation was also found to be involved in caste differentiation (Kucharski *et al.,* 2008). Although CpG (cytosine-phosphate-guanosine) DNA methylation in the honeybee genome is present at low levels, it is a common strategy to induce epigenetic changes, which results in preventing gene

expression. Cytosine methylation occurs at CpG dinucleotides within the transcription units and is modulated by highly conserved DNA cytosine-5-methyltransferases (Dnmts). Honeybees possess Dnmts of three main subfamilies, Dnmt1, Dnmt2 and Dnmt3 (Schaefer and Lyko, 2007). The latter was found to play a key role in developmental fate of honeybee larvae. High methylation levels were found in the heads (73%) and whole bodies (58%) of worker larvae, compared to 63% and 48% in queen larvae respectively. Furthermore, high expression levels of Dnmt3 are present in the *corpora allata* of workers, which is the gland known to produce JH. Interestingly, these methylation levels are also associated with the nutritional input the larvae receive during their development, because larvae fed with royal jelly displayed lower Dnmt3 activities. Silencing of Dnmt3 in first instar larvae mimicked the effects of royal jelly during larval development and resulted in decreased methylation levels and an increased frequency of adult queens with fully developed ovaries. However, brood cell size was also found to be involved in methylation, since larvae reared in queen cells displayed lower Dnmt3 activity and methylation levels, despite acquiring the same diet (Kucharski *et al.,* 2008; Shi *et al.,* 2011).

Genomewide analysis confirmed that queen larvae have lower DNA methylation levels compared to workers and that these levels increase with age in the latter. Many downmethylated genes in queens are involved in development, reproduction, metabolic regulation and caste differentiation. Although, methylation of caste differentiation related genes did not differ between two day old queen and worker larvae, it became different after the fourth day. Previous studies already revealed that the developmental fate of larvae is fixed at a certain age since worker larvae of 3 days old are totipotent and can still develop into queens, however, at later ages only intercastes develop. Interestingly, genes involved in IIS, MAPK and Wnt signaling are all downmethylated in queen larvae (Shi *et al.,* 2013).

The ability of royal jelly to control epigenetic pathways is linked to its histone deacetylase inhibitor (HDACi) activity, performed by the fatty acid, (E)-10-hydroxy-2-decenoic acid (10HDA). Histone deacetylases are also involved in the epigenetic regulation of pathways. Since 10HDA does not inhibit DNA methylation, it may act synergistically with agents that inhibit parallel pathways involved in transcription repression. An analogue of 10HDA, (E)-9-oxodec-2-enoic acid (90DA) with similar HDACi activity is produced by the queen and may be involved in maintaining the queen phenotype (Plettner et al, 1996; Spannhoff *et al.,* 2011).

2.1.3. Suppression of worker reproduction

In queenright colonies only the queen acquires the monopoly to reproduce. In order to maintain this hierarchy and inform workers that a healthy queen and brood are present in the colony, a variety of pheromones are used. Both queen and brood pheromones are used to inhibit ovary development in workers, since task performance of reproductive workers is not efficiently as normal workers (Katzav-Gozansky *et al.,* 2001). Honeybee queens produce a complex mixture of pheromones that attract workers, resulting in the formation of a retinue around the queen. Consequently, during these queen-worker interactions, the latter are exposed to several pheromones. Subsequently, worker-worker interactions, including antennation and trophallaxis, ensures the dispersion of these pheromones which enables the inhibition of ovary activation (Kaminsky *et al.,* 1990; Hoover *et al.,* 2003). In a natural bee hive, pheromones work in concert to regulate behavior, task differentiation and reproduction. However, if workers are able to activate their ovaries, several levels of activation can be observed (Fig. 1.10). Furthermore, queen and brood pheromones are less effective in inhibiting ovary activation in anarchistic *A. mellifera* bees, implying an altered response, compared to wild type workers (Oldroyd and Osborne 1999; Hoover *et al.,* 2005). Four quantitative trait loci (QTL) were found to play a possible role in this impaired pheromone response, which one QTL contains an odorant-binding protein 9 (Obp9) and a putative odorant receptor 13a (Oxley *et al.,* 2008).

Figure 1.10: Ovary activation levels of honeybee workers. No sign of ovary activation or swelling (**A**), ovarioles contain visible, round oocytes (**B**), oocytes are sausage-shaped (**C**), ovaries containing at least one fully developed egg (arrow head) with its characteristic, non-transparent color (**D**) (cf. legend, scale bar = 1 mm, asterisk: ovariole), intact ovaries of a queen (**E**). Adapted and modified from Cardoen *et al.,* 2012 and Jackson *et al.,* 2011.

2.1.3.1. Queen and brood pheromones

Queen mandibular pheromones (QMP) are known to inhibit worker ovary activation and consist of large amounts of 9-keto-(E)-2-decenoic acid (9-ODA), together with other

compounds that attract the workers (Maisonnasse *et al.,* 2010). The major compound found in workers is (2E)-10-hydroxydecenoic acid (10-HDA) which is also present in royal jelly (Moritz *et al.,* 2000, 2004; Winston and Slessor, 1992; Plettner et al. 1995, 1996; Boch *et al.,* 1979). Furthermore, in queenless environments, queen pheromones are absent, including the attractive 9-ODA pheromone. Long term absence of this compound will eventually result in ovary development in some workers (de Groot and Voogd, 1954; Butler and Fairey, 1963). Workers with activated ovaries also have an altered pheromone composition, which is more queen like. In both *A. cerana* and *A. mellifera*, reproductive workers have higher amounts of 9-ODA compared to sterile workers (Tan *et al.,* 2012). In *A. mellifera capensis*, reproductive workers that produce these queen-like pheromones like 9-ODA, also received more trophallaxis by nurse-age bees (Moritz and Hillesheim 1985). Nutrient uptake by trophallaxis was found to be necessary to promote oogenesis in reproductive *A. mellifera* workers (Schäfer *et al.,* 2006). In addition, workers that receive their food by trophallaxis from other workers also had elevated Vg levels compared to bees that have direct access to food (Lin *et al.,* 1999). Nutrition directly influences caste determination and hence affects ovary development in honeybees. Workers receiving a low-protein diet cannot sustain oogenesis, while a protein rich diet of pollen and royal jelly is able to promote oogenesis in both adult queens and workers (Lin and Winston 1998; Hoover *et al.,* 2006). Nurse-age workers that digest pollen are involved in trophallaxis and may mediate ovary development through differentiation of worker dominance status and hence the related food exchange (Lin *et al.,* 1999). In addition, tergal gland and Dufour gland pheromones are also involved in regulating worker ovary development (Wossler and Crewe, 1999; Hoover *et al.,* 2003). Dufour's gland pheromones of queens are also inducing retinue behavior and are composed of n-alkanes in workers, while queens have additional wax-type esters including tetradecyl hexadecanoate and hexadecyl tetradecanoate (Katzav-Gozansky *et al.,* 2001). However, upon the irreversible loss of the queen, the pheromone composition becomes more diverse in reproductive workers, compared to workers without developed ovaries. The observed increase of queen-like esters in reproductive workers is tightly linked with ovary development and hence the Dufour's gland pheromones are considered as a fertility signal among workers (Dor *et al.,* 2005). Moreover, brood pheromones are also involved in inhibiting worker ovary activation. Ethyl palmitate (also produced by the queen)

and methyl linolenate are two esters released by the larvae, which inhibit partly ovary development in workers (Arnold *et al.,* 1994; Mohammedi *et al.,* 1998).

2.1.3.2. Policing

Although worker reproduction in queenright colonies is rare, theoretically 7% of the total male eggs may come from these workers (Visscher, 1996). However, only 0.1 % of the males in a colony are actual sons of workers. This is the result of a social control system called worker policing, performed by workers that actively remove worker-laid eggs. Since workers cannot discriminate between queen and worker derived larvae, the eggs are targeted through policing. Discrimination between queen-laid and worker-laid eggs is assumed to be directed by unknown egg-marking pheromones on the surface, which are not derived from the Dufour gland, resulting in the selective oophagy of the eggs (Ratnieks 1995, Katzav-Gozansky *et al.,* 2001). Furthermore, policing is also manifested through interactions between workers to suppress worker reproduction, including aggressive behavior towards workers with activated ovaries. This suggests that workers can detect bees with activated ovaries through pheromone changes (Barron *et al.,* 2001; Vissher and Dukas 1995). Aggressive behavior towards reproductive bees can occur by attacking these workers, which may result in the loss of food by trophallaxis. Since nutrition is also involved in ovary development, the loss of food may arrest further development of the ovaries (Hoover *et al.,* 2006).

2.1.4. Actions of Juvenile hormone and vitellogenin in adult honeybees

In contrast to larval development, JH titers follow an opposite pattern in adult bees and are found to be age dependent in both adult queens and workers. Newly emerged virgin queens possess elevated JH (±75 ng/ml) and Vg hemolymph titers. These JH titers drop during the following days and stay low in older queens (below 40 ng/ml) whereas Vg titers are elevated (Fahrbach *et al.,* 1995). The importance of JH on *Vg* gene expression was confirmed in studies using methoprene (a JH analog) and JH III (an endogenous honey bee hormone) treatments in both castes. However, young nursing bees display low JH and higher Vg levels compared to foragers, while higher JH levels are found in foragers and approximate the levels found in virgin queens. However, Vg protein levels are much higher in queens than in workers, which is also reflected in the *Vg* mRNA levels of queens. In workers the *Vg* levels

are just above background. Interestingly, methoprene was found to decrease *Vg* mRNA levels in queens whereas no differences could be detected in workers because of the low *Vg* mRNA levels. In addition an increase in *AmILP-1* expression was observed in both castes. These findings suggest that JH may affect vitellogenesis in adult bees. However, Vg also acts as an antioxidant and is involved in queen longevity. This may clarify the shorter lifespan and undeveloped ovaries of queenright workers, whereas in queenless workers vitellogenin is strongly upregulated and developed ovaries are present (Corona *et al.,* 2007; Koywiwattrakul and Sittipraneed, 2009). Since low JH titers are involved in specific patterns of apoptosis during worker ovary development in the larval stages, this hormone may also play a key role in the ovary activation observed in adult workers (Tanaka and Hartfelder, 2004; Cardoen *et al.,* 2012; Capella and Hartfelder, 1998).

2.1.5. Caste differentiation and honeybee reproduction: overlapping pathways

Since honeybee workers are able to reproduce under queenless conditions, many studies were undertaken to find the molecular pathways involved in this process. Large-scale screenings of the genome (Linksvayer *et al.,* 2009; Oxley *et al.,* 2008) transcriptome (Cardoen *et al.,* 2011b; Grozinger *et al.,* 2007; Thompson *et al.,* 2006, 2008) and the proteome (Cardoen *et al.,* 2012; 2011a) delivered many candidate genes and proteins that might underlie this shift in the reproductive capacity of worker bees. These studies revealed that sterile workers display an inhibition of cell cycle and cell organization, while proteins related to protein degradation are upregulated in the ovaries of sterile workers. As a result, ovary activation might be suppressed through a constant interplay between primordial oogenesis and subsequent degradation. Furthermore, proteins involved in the 20-hydroxyecdysone (20E) receptor complex were found to be upregulated in activated ovaries while treatment with QMP resulted in a downregulation (Cardoen *et al.,* 2011a; Grozinger *et al.,* 2003). Interestingly, large-scale microarray studies and knockdown experiments revealed that there is indeed a significant overlap in the genes that are involved in regulating worker reproduction, behavior and queen-worker caste determination. Several genes involved in TOR and insulin signaling pathways, steroid biosynthesis and EGFR signaling were found in these studies (Grozinger *et al.,* 2007; Cardoen *et al.,* 2011b; Mutti *et al.,* 2011a). These data suggest that the same pathways are involved in both caste determination and the regulation of reproduction in adult honeybee workers.

PART II

RESULTS

Chapter 2

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

Contributions

The study design was developed by D. de Graaf, E. Danneels and E. Formesyn. E. Danneels was responsible for the communication between the Microarray Facility (VIB), the coauthors and the follow-up of the manuscript until it was published, as well as the materials and method section, parts of the results and discussion, including the topics concerning development, genetic information processing, immunity and the sensory system. E. Formesyn was responsible for the following topics: programmed cell death, detoxification, metabolism and transporters. Both insect cultures were maintained equally by E. Danneels and E. Formesyn. The optimization of the parasitation conditions was performed by E. Formesyn. The optimization of the screening method to detect parasitized pupae was performed by L. Baillon. The parasitation experiments, sample preparation and PCR were performed equally by E. Danneels and E. Formesyn. E. Danneels performed the selection of the reference genes, while E. Formesyn was responsible for the validation of the microarray by qRT-PCR and the statistical processing afterwards. KOG analyses were performed by Dieter De Koker (°24/04/1987 †30/05/2012) and Werner De Koker. Both E. Danneels and E. Formesyn performed the BLAST2GO analyses, including the enrichment analyses and the manual clustering of the data.

This chapter is redrafted from:

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1. Introduction

Endoparasitoids, wasps that deposit their eggs inside the body of their arthropod hosts, have evolved sophisticated mechanisms to disable the host's defensive responses. An array of virulence factors including polydnaviruses, venoms, virus-like particles, ovarian fluids and teratocytes are used to combat the host's defense system, thus enabling the wasp larvae to freely manipulate and devour the host (Glatz *et al.,* 2004; Labrosse *et al.,* 2003; Pennacchio and 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 parasitation, 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, the jewel wasp *Nasonia vitripennis*, on one of its favored hosts (Whiting, 1967), the flesh fly *Sarcophaga crassipalpis.*

N. vitripennis seeks newly pupariated fly larvae, inserts its ovipositor through the puparium, envenomating the fly pupa and then depositing its eggs on the surface of the pupal body. The egg is lodged within the space between the puparium and the pupal cuticle, thus it is encased within the puparium but remains on the external surface of the fly pupa. The wasp's venom arrests development in the host (Rivers and Denlinger, 1994a), alters host metabolism (Rivers and Denlinger, 1994b), and apoptosis (Rivers *et al.,* 1999), thus ensuring a suitable environment for growth and development of the parasitoid. Although *N. vitripennis* lacks the virulence factors commonly documented in endoparasitoids, like

teratocytes or polydnaviruses, the venom proteome shows high similarity to the venoms of endoparasitoids (Werren *et al.,* 2010; de Graaf *et al.,* 2010). This means that the affected processes in the host solely are the result of the venomous mixture injected by *N. vitripennis* and no other virulence factors, that on the other hand are necessary in endoparasitoids to have surviving offspring, are needed.

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.* Firstly, a screening method was developed by targetting two maternal derived factors, histone-lysine N-methyltransferase NSD2-like and nanos (*nos*) in *N. vitripennis* oocytes (Olesnicky and Desplan, 2007; Lynch and Desplan, 2010; Clough *et al.,* 2007). This avoids the need to open the pupae to search for parasitoid eggs which is an undesired manipulation when performing transcriptome studies. Using microarrays we examine transcript expression in the host 3 h and 25 h 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 h after envenomation, but by 25 h after envenomation expression patterns of key immune, developmental, and metabolic pathways have clearly been altered by envenomation. We contrast responses elicited by this ectoparasitoid with what has previously been observed in hosts attacked by endoparasitoids.

2. Material and methods

2.1. Preparation of parasitized and nonparasitized flesh fly pupae

2.1.1. Insect strains

2.1.1.1. *Nasonia vitripennis*

The laboratory strain *Nasonia 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 and Jachmann, 1999). This infection causes reproductive incompatibility between related species (Werren, 1997).

To maintain the *N. vitripennis* culture approximately 15 to 20 *S. crassipalpis* pupae and 10 to 15 female wasps of at least 1 day old were deposited in a tube which was closed with a ceapren stop. Subsequently, the culture was placed in an incubator at 25 °C under a 15:9 light: dark cycle. Under these conditions, the wasps emerge after 14 days.

2.1.1.2. *Sarcophaga crassipalpis*

Sarcophaga crassipalpis flesh flies were provided from a culture maintained by Dr. Hahn, University of Florida, and cultured in the laboratory as described by Denlinger, (1972b). In order to start the cultures, a box with approximately 1000 pupae was placed in a specialized insect tent (Bugdorm, MegaView Science Co). When the flies start to emerge, water and sugar were provided. Three to four days after hatching, they were fed on small pieces of beef liver in order to start egg development. Fresh beef liver was provided every 2 days, until the flies were 7 or 8 days old. From this point on, they start to lay eggs or larvae, and fresh beef liver was given every day, until no more larvae were deposited. Liver with larvae was removed from the culture and placed in a large bowl provided with a large piece of liver and peat and placed in an incubator. All flesh fly stadia were kept at 25 °C and exposed to a 15:9 light: dark cycle. After pupariation, pupae were dived over several boxes, which contained approximately 1000 pupae. According to amount of flies needed for the culture, boxes were placed back in the insect tents, while others were stored in the cold room at 4 °C until further use.

2.1.2. Collection of parasitized and control hosts

2.1.2.1. *Nv_nos* and *Nv_Setdb1* detection

Pupae of 5 days old were placed in tubes with their posterior side upwards and exposed to an excess of *N. vitripennis* females in a 3:1 ratio, during several time points ranging from 3 h to 24 h. *N. vitripennis* females of approximately 4 to 5 days old were used for all experiments.

2.1.2.2. Microarray experiment

Before the start of the experiment, female wasps were exposed to flesh fly puparia (4 days after puparium formation) for 6 hours to condition them to oviposit. Only the posterior region of the puparium was accessible to the parasitoids. The experienced *N. vitripennis*

females and fresh puparia, 5 days after pupariation, were placed together in a tube in a 3:1 ratio, to promote parasitation. After 2 hours, females of *N. vitripennis* were removed. Flies were sampled 2 or 24 h 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-parasitation. Control pupae were treated identically except that they were not exposed to the parasitoid.

2.2. Sample preparation

2.2.1. 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 individual pupae were analysed, meaning that 4 individual pupa were used as replicates. 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 one 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 µg/µl) and was carried out according to the RevertAid H Minus First strand cDNA Synthesis kit protocol (Fermentas).

2.2.2. Screening of parasitized pupae for the two maternal factors

To ensure that the pupae were parasitized, a prior experiment was performed to screen samples for the presence of *N. vitripennis nanos* (*nos*) (NM_001134922.1) and histone-lysine N-methyltransferase NSD2-like (*Nv_Setdb1*; XM_001603648.2) (Olesnicky and Desplan, 2007; Clough *et al.,* 2007). Subsequently, the maternal factors that were successfully detected in parasitized pupae were used for further experiments. The following primer sets were used to screen for *Nv_nos*: 5'-TGGCAAGATTCTTTGTCCTAT-3'; 3'-AGAAACAGGTTAACTGTCCGC-5' and *Nv_Setdb1*: 5'-CCCGAAATAGAACCACCA-3'; 3'-CCTTCCGATTTGCGTTGT-5'. The obtained amplicon had a length of respectively 264 and 354

base pairs. Primers were purchased at Intergrated DNA Technologies and tested for their specificity in the abdomen of *N.vitripennis* females, control pupae and parasitized pupae. The RT-PCR was executed using an Eppendorf Mastercycler with the following conditions: 95 °C 15 min; 93 °C 1 min; 55 °C 30 s; 72 °C 1.5 min. Step 2 to 4 were repeated 35 times and finishes the program by heating the mixtures for 10 min at 72 °C. PCR products were loaded on 0.8%, 1.4% or 2% agarose gels and visualized by ethidium bromide staining.

2.3. Microarray study of *S. crassipalpis* pupae transcriptional response to parasitation by *N. vitripennis*

2.3.1. 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.

2.3.2. 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). 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 S2.1; supplemental materials can be obtained by contacting Prof. de Graaf). Per sample, an amount of 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-cytidine triphosphate (Cy3-CTP) or Cyanine 5-cytidine triphosphate (Cy5-CTP) 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.

2.3.3. 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, in conjunction with packages developed within the Bioconductor project (http://www.bioconductor.org; Gentleman *et al.,* 2004). Differential expression between the conditions 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 and 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.

2.4. Validation experiment

2.4.1. Reference gene selection and primer design

Since no reference genes for qRT-PCR were available for *S. crassipalpis*, genes homologous or related to proposed reference genes from other insect species (i.e., *D. melanogaster* and *Apis mellifera*) after a bacterial challenge were selected from the *S. crassipalpis* EST database (Ling and 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 S2.2).

Table 2.1. 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]	$R^{2,b}$
gi|296333647|gb|EZ596832.1|	actin	actin(1)	GGTATGTGCAAGGCTGGTTT	107	102.6	0.998
			TTTTGACCCATACCGACCAT			
gi|296336747|gb|EZ599932.1|	tubulin alpha chain	tubulin (1)	CAATTATGCTCGAGGCCATT	142	102.9	0.992
			ACCACTTCCAGTTCCACCAC			
gi|296336001|gb|EZ599186.1|	glutathione-s-transferase	GST	TTGAGAAAACGAGCCGAAGT	114	110.4	0.990
			GGAGCAATCAGTGGAACCAT			
gi|296334900|gb|EZ598085.1|	glyceraldehyde 3 phosphate dehydrogenase 2	GAPDH	AGCAGGAGCCTGAGCATTTA	112	107.4	0.995
			CCATGAGGTGGATTTTGCTT			
gi|296334383|gb|EZ597568.1|	ribosomal protein 49	RP49	CGCACAAATGGAGAAAACCT	104	112.4	0.987
			GGTACGCTTGTTGGAACCAT			
gi|296347152|gb|EZ610337.1|	ribosomal protein l13	RPl13	GGAATTGAAGGGTGCTGGTA	106	102.1	0.993
			GATATTGCGTTGACGGGATT			
»» gi|296337299|gb|EZ600484.1|	ubiquitin-conjugating enzyme	Ubq	AAAATGGCGAATCAATCCAC	127	104.0	0.993
			TGTCGTCGTCATTTTCAAGG			
gi|296339815|gb|EZ603000.1|	elongation factor isoform a	EF	CTCAAGCTGATTGTGCCGTA	121	94.7	0.993
			TGTTTGACACCCAAGGTGAA			
»» gi|296334587|gb|EZ597772.1|	eukaryotic translation initiation factor 1a	EIF	CAAGATTCAAAGGCCGATGT	124	99.9	0.984
			AACCGTCTTCCACAAAGGTG			
gi|296340585|gb|EZ603770.1|	sodium potassium-dependent atpase beta-2 subunit	NaK-ATPase(2)	GTTTTCGCCCCATTAGTGAA	139	103.5	0.995
			TTGCCACTGTGAGTGCTTTC			

[a]Measure of the real-time PCR reaction efficiency (calculated by the standard curve method)
[b]Reproducibility of the real-time PCR reaction
»»Reference genes used to normalize the results of the qRT-PCR experiment for microarray data validation.

2.4.2. qRT-PCR reaction mixture and cycling program

qRT-PCR 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 min; 95 °C 2 min; and 40 cycles of a combined denaturation (95 °C 20 s) and annealing (60 °C 40 s) 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 sec over a range from 65 °C to 95 °C.

2.4.3. 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.

2.5. Microarray data analysis

Sarcophaga crassipalpis genes were functionally annotated with Blast2GO (Conesa *et al.,* 2005; Gotz *et al.,* 2008). After GO term annotation, an enrichment analysis (two-tailed Fisher's exact test with default settings) within the Blast2GO environment was undertaken (table S2.2). 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.

3. Results and Discussion

3.1. *Nv_nos* is detected in female wasps and parasitized pupae

Both *Nv_nos* and *Nv_Setdb1* were detected in the abdomen of female wasps, using reverse transcriptase PCR (Fig. 2.1A-B). When infected and uninfected pupae were tested for the presence of *Nv_Setdb1*, no amplicon was generated and only a smear was visible. However, *Nv_nos* could be detected in parasitized pupae at all selected sampling points (Fig. 2.1C-D). Since uninfected pupae, that were not exposed to female wasps, did not generate the *Nv_nos* amplicon, its presence is considered as a specific characteristic for successful parasitation of pupae. This technique ensures us that pupae selected for future experiments are certainly 100% parasitized and can be extended to other parasitoids as well.

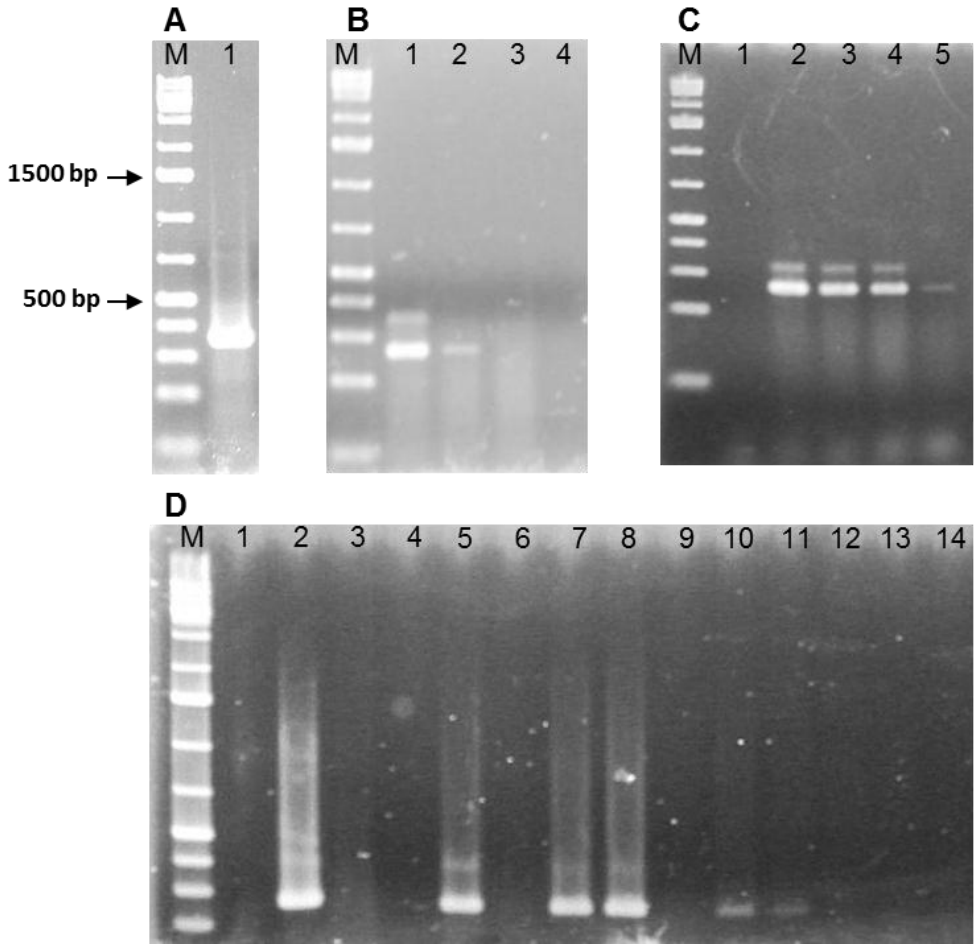

Figure 2.1: Agarose gel electrophoresis on both *N. vitripennis* females and parasitized *S. crassipalpis* pupae in order to detect the presence of *Nv_nos* and *Nv_Setdb1*, using a 1 kb marker (M). **A**) Detection of *Nv_Setdb1* in *N. vitripennis* females (1). **B**) Detection of *Nv_nos* in *N. vitripennis* females (1), parasitized pupa (2), control pupa (3) and negative control (4). **C**) *Nv_nos* detection in negative control (1), *N. vitripennis* females (2) and parasitized pupae at 3 h (3), 6 h (4) and 9 h (5) after parasitation. **D**) Screening for *Nv_nos* presence in parasitized pupae 4 h (3-8) and 24 h (9-14) after parasitation, negative control (1), positive control (2).

3.2. Verifying parasitation of the microarray samples

Before conducting the microarray experiment, we verified whether individual flesh flies were parasitized by screening for transcripts of *nanos* (*nos*), a developmental patterning factor 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 the presence of *N. vitripennis nos* were used for further experiments (Fig. 2.2A-B).

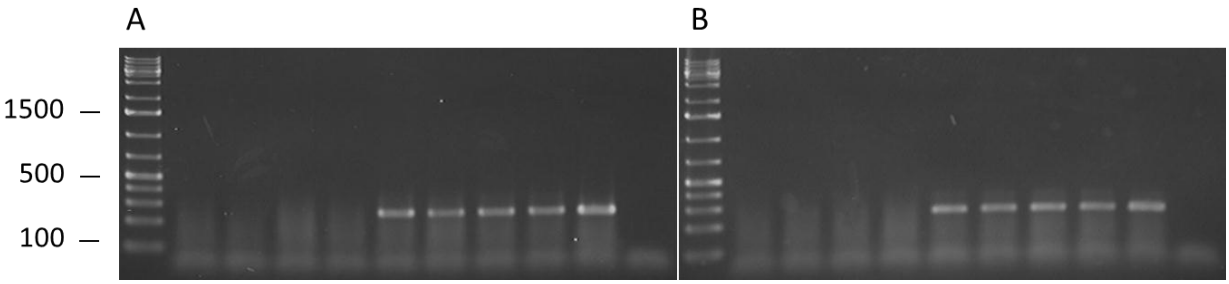

Figure 2.2: Gel electrophoresis analysis for the presence of nos in test samples for microarray and validation experiment. (**A**) samples at 3 hours parasitation, (**B**): samples at 25 hours parasitation, from left to right: 1 kb ladder (Fermentas), 4 control samples, 4 parasitised samples, positive control (abdomen from *N. vitripennis* female), negative control

3.3. Overview of differential gene expression

In our analyses, we considered a transcript to be differentially expressed between envenomated and control samples if both the FDR-adjusted p-value was < 0.05 and the fold change was > 1.25, which is an accepted value to use (Ha *et al.,* 2012). Furthermore, validation of the microarray results was performed by selecting a wide range of differential expressed genes (20) with different fold changes. 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 h and 25 h samples in both control and parasitized samples (Fig. 2.3). 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 and Zdarek, 1994, Ragland *et al.,* 2010). Interestingly, the numbers of differentially expressed transcripts in the 3 to 25 hours parasitized pupae are larger than the amount of differentially expressed transcripts in the 3 to 25 hours control pupae. Besides the transition these pupae make from 3 to 25 hours, the excess amount of differentially expressed transcripts in the parasitized pupae

possibly represent the onset of alteration in host processes caused by the envenomation. When comparing solely the time-matched parasitized and control samples, only 1 transcript was differentially expressed 3 h after envenomation and 128 transcripts were differentially expressed 25 h after envenomation. The 1 differentially regulated transcript at 3 h after envenomation was a leucine rich repeat protein that was slightly down-regulated (-1.67 fold change). By 25 h after envenomation, 19 transcripts were down-regulated and 109 were up-regulated (Fig. 2.3). 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, our results represent only the earliest stages of the host response to envenomation.

A

	Downregulated		**Upregulated**	
	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

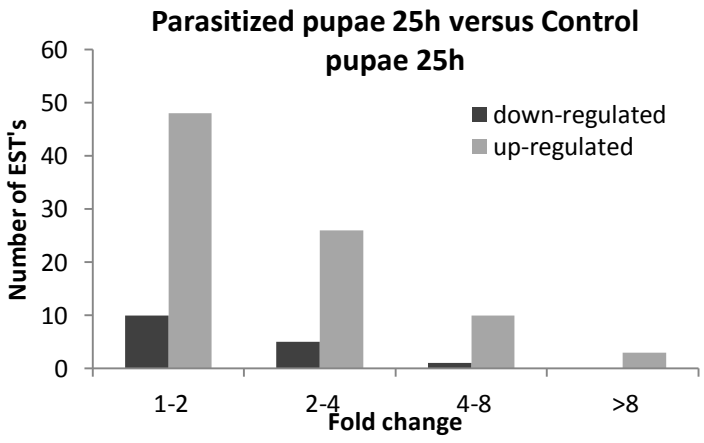

Figure 2.3: General statistics on the differentially regulated EST's in response to parasitation. **A**) The four comparisons with their respective amounts of up- and down-regulated genes that were identified by microarray hybridization according to the following selection criteria: p-value < 0.05 and 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.

3.4. 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 h vs. 25 h samples showed enrichment of GO terms associated with energy metabolism (Table S2.3). As with the time comparisons in our 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 and Zdarek, 1994; Ragland *et al.,* 2010).

To explore the effects of envenomation at levels above single ESTs, we tested for enrichment across categories in the COG (Clusters of Orthologous Groups from eukaryotic genomes) functional classification using Blast2GO (Fig. 2.4). We observed enrichment that suggested differential regulation in categories associated with growth (including replication, transcription, and translation), cell signaling, 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 h 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 2.2 and Fig. 2.5).

Table 2.2: 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		
Down-regulated genes		
EUA37Q302I4V57	leucine rich repeat protein	-1.67
Control_25h vs Paras_25h		
Down-regulated genes		
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
Up-regulated genes		
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
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|	GI18822 [Drosophila mojavensis]	1.89
gi|296346814|gb|EZ609999.1|	tyrosyl-trna synthetase	1.90
EUA37Q302GBMZS	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

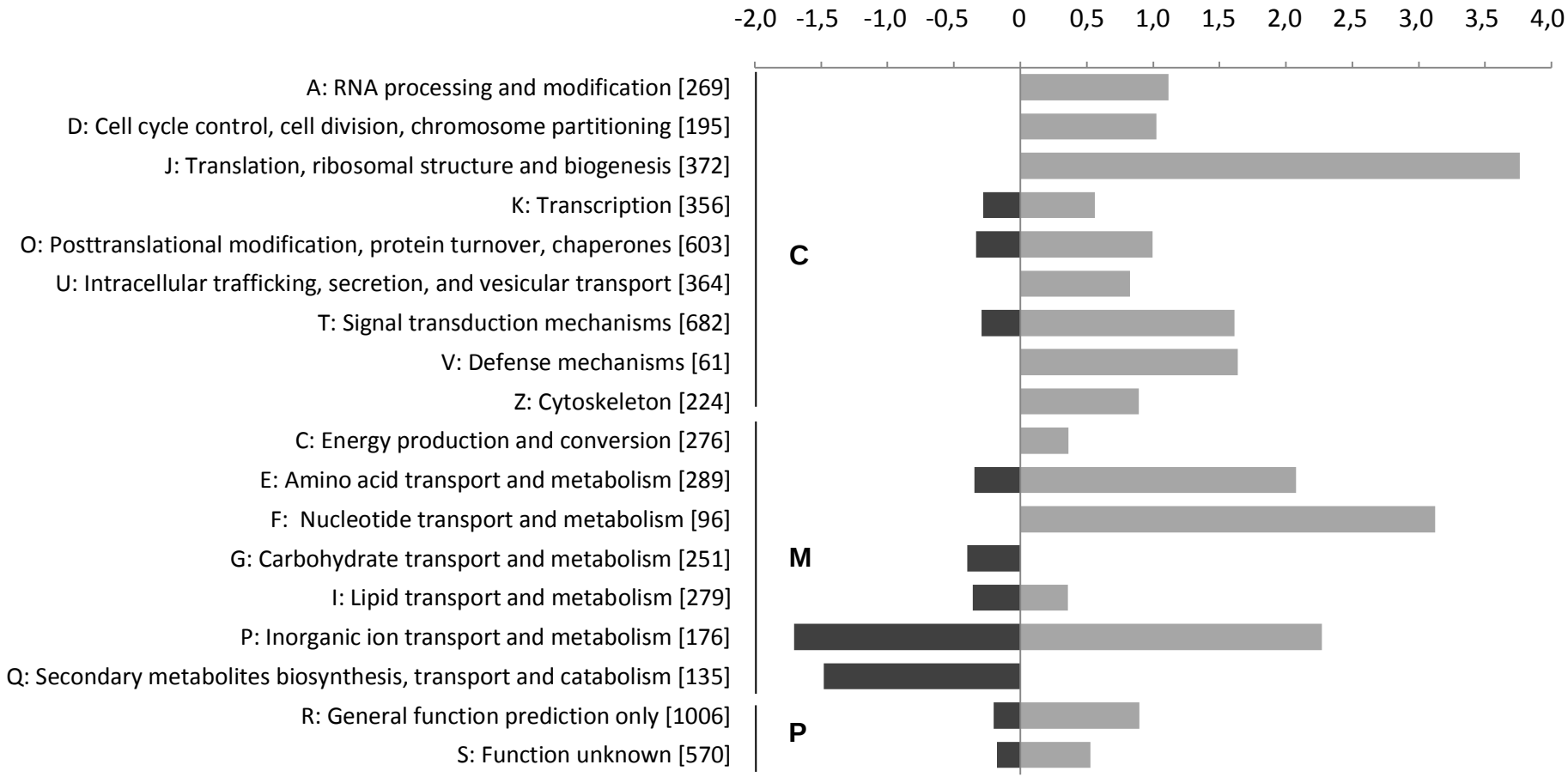

Figure 2.4. Square brackets: Total number of EST's within the *S. crassipalpis* EST database. Capital letters: KOG functional category label. C: cellular processes and signaling. M: metabolism. P: poorly characterized. Dark bars: down-regulation. Grey bars: up-regulation.

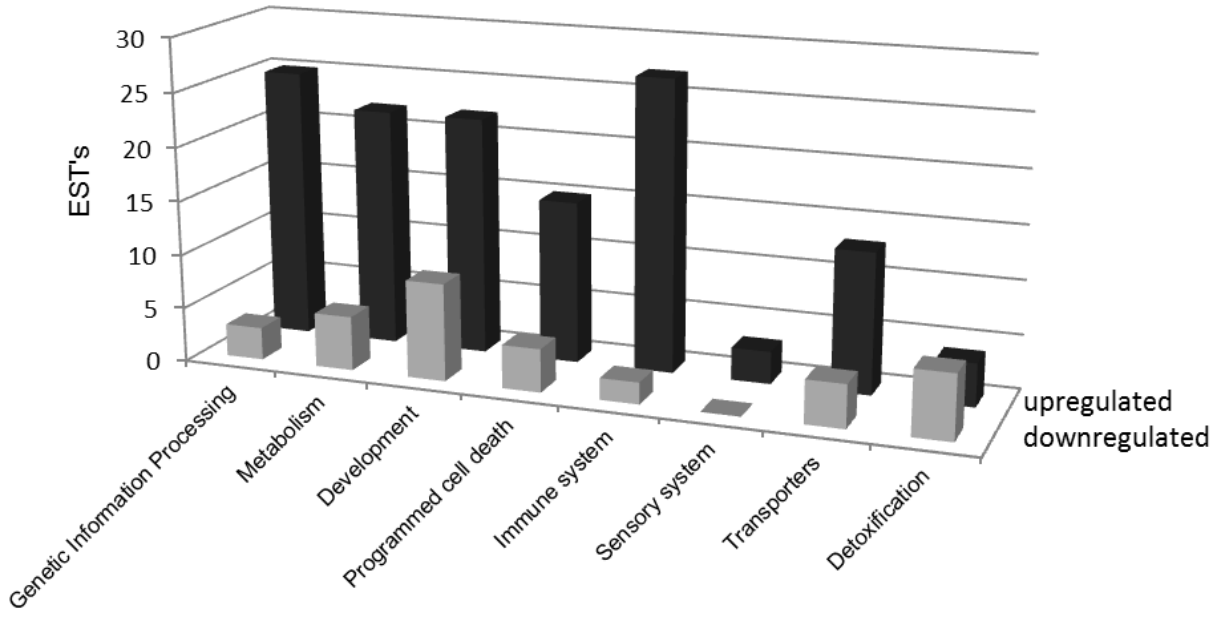

Figure 2.5: Differentially expressed EST's for comparisons Contr_25h vs Paras_25h, manually clustered into 8 different classes. Schematically presented.

One challenge for transcriptomic studies like ours 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 and Denlinger, 1994a, 1994b, 1995; Rivers *et al.,* 2002, 2010, 2011) 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 favor parasitoid development. Below, we interpret our transcriptomic results in the context of these three host manipulations. Many patterns of transcript abundance are products of host manipulation (e.g., changes in many downstream players in genetic information

processing), but here we consciously focus our discussion on regulatory processes that we believe may be important in modulating host immunity, development, and metabolism. We acknowledge that other, more subtle interactions between parasitoid venom and host physiology, beyond those observed in our data or discussed here, are undoubtedly occurring. We hope that our data, the first to our knowledge on 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.

3.5. 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 and 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). In a study of hemocyte dynamics, Rivers *et al.,* (2002) showed that envenomation of *Sarcophaga bullata* pupae by *N. vitripennis* resulted in a substantial drop in hemocytes within 24 h. Envenomation induced the rapid death of plasmatocytes and inhibited proliferation and differentiation of pro-hemocytes into plasmatocytes. While numbers of granulocytes were not affected by envenomation, the ability of granulocytes to spread was greatly diminished (Rivers *et al.,* 2002). Envenomation also diminishes hemolymph clotting and melanization in *Sarcophaga* species, but melanization and clotting responses do occur when *N. vitripennis* larvae feed on a non-preferred host, like the house fly *Musca domestica* (Rivers and Denlinger, 1995).

3.5.1. Programmed cell death in hemocytes

A series of *in vivo* studies in *Sarcophaga* hosts and *in vitro* work on lepidopteran cell lines suggests that venom-induced hemocyte cell death occurs by an apoptotic process that includes signaling by phospholipase A_2 (PLA_2) that alters cellular Ca^{2+} signaling 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 and Denlinger, 1994a, 1994b; Rivers *et al.,* 2002, 2010; Formesyn *et al.,* 2013). Consistent with these cellular-level observations our transcriptional analyses show differential regulation of apoptotic transcripts, including substantial up-regulation (2.15-fold) of a secretory PLA_2 transcript that appears to facilitate immune modulation by promoting apoptosis through phospholipid signaling cascades that activate caspase activity. Consistent with the previous observations that PLA_2 activity is associated with changes in cellular Na^+ and Ca^{2+} homeostasis, we found a putative small mitochondrial calcium-binding protein transcriptionally up-regulated 25 h after envenomation (1.83-fold), as well as a putative calcyphosine that was also up-regulated (1.44-fold). Imbalances in Ca^{2+} homeostasis in the mitochondria are well known to induce mitochondrial breakdown that induces apoptosis. Up-regulation of a putative cytochrome oxidase III subunit (3.81-fold) may also be related to mitochondrial dysfunction induced by PLA_2 signaling. Cytochrome III and IV members of the electron transport chain interact with the heme-protein cytochrome C. 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 and Borutaite, 2008; Wu *et al.,* 2009).

Among the transcripts most highly up-regulated 25 h after envenomation were three annotated to the Rho family of GTP-binding proteins and a guanine nucleotide exchange factor (13.2, 9.7, 1.38, and 1.3-fold upregulation respectively, Table 2.2). Rho GTPases belong to the Ras superfamily and are known to play diverse roles in intracellular signaling (Rossman *et al.,* 2005; Cox and Der, 2003; Hall *et al*., 2006). Considering the rapid death of *Sarcophaga* hemocytes following envenomation, we propose that Rho GTP-binding proteins and the observed guanine nucleotide exchange factor modulate Rho signaling and promote apoptosis by activating the RASSF1/Nore1/Mst1 signaling pathway that leads eventually to caspase activation. We did not detect differential regulation of any members of the downstream RASSF1/Nore1/Mst1, but this cellular signaling pathway is largely post-translationally activated by phosphorylation (Rossman *et al.,* 2005; Cox and Der, 2003). Rho signaling 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 cell-biology interrogation of the potential roles of Rho signaling 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, as described below (see 3.6.1.).

Rho GTPases are also known for their involvement in actin reorganization in the cytoskeleton (Coleman and Olson, 2002; Fiorentini *et al.,* 2003). Blebbing, wherein 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 signaling has been shown to promote blebbing by actin cytoskeleton reorganization in cultured mammalian cells (Aznar and Lacal, 2001). Consistent with this view, several cytoskeleton-related transcripts were up-regulated by envenomation in our study (Table 2.2). 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 (Strand, 2008; Asgari and Rivers, 2011; Richards ann Edwards, 2002a). In support of this central role for cytoskeletal organization, differential regulation of cytoskeletal transcripts has been observed in numerous other transcriptional studies of parasitism (Wertheim *et al.,* 2005; Schlenke *et al.,* 2007; Mahadav *et al.,* 2008; Fang *et al.,* 2010; Bitra *et al.,* 2011; Fang *et al.,* 2010; Provost *et al.,* 2011; Wertheim *et al.,* 2011), suggesting further conservation of the cellular mode of action between endoparasitoid and ectoparasitoid venoms.

3.5.2. Cellular and humoral immune responses

Although *N. vitripennis* venom can dramatically suppress *Sarcophaga* immunity by rapidly killing hemocytes, hosts still do mount an immune response characterized by both cellular and humoral mechanisms (Rivers *et al.,* 2002). 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 when activated translocate into the nucleus to regulate transcriptional activity. The p38K and JNK

signaling 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 clearly distinguish whether our putative MAPK 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 that could be clearly assigned to either cascade. However, both of these stress-responsive signaling cascades are well known to participate in immune system function and down-regulating this arm of MAPK signaling 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 signaling are important in immune modulation at envenomation will involve further biochemical work at the level of immune cells. Previous studies using commercially available phosphorylation-specific antibodies for each of the three main MAPKs (p38K, JNK, and ERK) have shown that activation of p38K was associated with rapid acclimation to thermal stress and activation of ERK was associated with the termination of diapause-induced developmental arrest and the resumption of development in the flesh fly *S. crassipalpis* (Fujiwara and Denlinger, 2007a, 2007b). Functional studies that would distinguish what parts of the MAPK signaling cascade are modified by envenomation in hemocytes or other tissues (see 3.6.1.) are quite feasible and should follow.

Parasitoid detection and immune-system activation may also be modulated by the NF-κB pathway (Strand, 2008). A transcript for a putative tumor necrosis factor (TNF) receptor associated factor (TRAF) was up-regulated 25 h after envenomation (1.7-fold). In *Drosophila*, this protein is known to interact with *Pelle* and to regulate NF-κB activity (Zapata *et al.,* 2000). Additionally, a protein tyrosine phosphatase involved in intracellular immune signaling 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 h after envenomation (1.36 and 1.35-fold respectively). In addition to their known roles in antimicrobial immunity in insects (Fullaondo and Lee, 2012; Asgari, 2013), microRNAs have also recently been implicated in the response of a lepidopteran host, *P. xyloste*lla, 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). 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 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 melanization of *Sarcophaga* host hemolymph (Rivers and Denlinger, 1994; 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 crucial role for melanization in host immunity, alterations of this process in hosts likely represents an ideal strategy for successful parasitism. 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 melanization response (de Graaf *et al.,* 2010). We found substantial up-regulation of transcript abundance for two serine proteases that we hypothesize may compete with venom-derived protease inhibitors in an effort to disrupt the parasitoids immune-suppressive strategy, similar to the pattern of enhanced expression of transcripts for enzymes in the melanization cascade observed when *D. melanogaster* was parasitized by the virulent parasitoid wasp *Leptopilina boulardi* (Schenkle *et al.,* 2007).

Associated with the melanization response is the targeted oxidative burst that is facilitated by both cellular and humoral elements of immunity (Strand, 2008). For hosts, this targeted burst of free radicals and pro-oxidants must be delivered precisely to cause oxidative stress to the invader while protecting host tissues. Concomitantly, parasitoids must try to reduce the effects of oxidative damage on both their own bodies and critical host tissues like the fat body. Consistent with this view of a dynamic interplay of host and parasitoid control of free radical damage, we find substantial enrichment of detoxification proteins. Many of these

detoxification proteins control redox events and could be playing roles directly in the immune oxidative burst reactions including down-regulation of putative homologues for a metallothionein family 5 protein (-2.01-fold), cytochrome p450-28a1 (-1.91-fold), a copper uptake protein (-1.65-fold), and up-regulation of transcripts for putative homologues of myoinositol oxygenase (1.83-fold) and ribonucleoside-diphosphate reductase (1.46-fold). These redox proteins may be important in detoxification responses that facilitate immunity because transcripts of other detoxification proteins also appear to be differentially regulated, like the glutathione metabolism enzyme alanyl aminopeptidase (-2.93-fold), and transcripts for an apparent ABC transporter are up-regulated (2.75-fold). However, proteins in the detoxification pathways can participate in many functions besides immunity and more work, starting with studies of tissue localization would be useful to determine whether they may play roles in modulating the metabolism or development of *Sarcophaga* hosts.

For the other major component of humoral immunity, we see no change in the abundances of antimicrobial peptide transcripts. This observation is consistent with other studies on host-parasitoid responses that show minimal up-regulation of antimicrobial peptides in responses to parasitoids relative to exposure to microorganisms (Ross and Dunn, 1989; Nicolas *et al.,* 1996; Masova *et al.,* 2010; Wertheim *et al.,* 2005). Further work on the immune signaling pathways and effectors that we have observed to be up-regulated will be needed to determine which components are modulated as part of the direct response of *Sarcophaga* hosts to *N. vitripennis* venom and which components are not employed directly against the parasitoids, but may modulate the internal milieu of the host to suppress the proliferation of microorganisms that may cause a decline in host quality.

3.6. 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 and Rivers, 2011). As an ectoparasitoid without obvious viral symbionts, *N. vitripennis* relies on venom components to initiate developmental arrest in hosts until the external parasitoid eggs can hatch and the larvae can begin to 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 eventually dies (Rivers and Denlinger, 1994b; Rivers *et al.,* 2011). Envenomation-induced developmental arrest in *Sarcophaga* pupae superficially resembles the developmental arrest induced at pupal diapause in this species. However, ultra structural studies suggest that the developmental arrest induced by envenomation manipulates host physiology to maximally benefit parasitoid larval development. Specifically, within 24h 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 likely contributes to envenomation-induced developmental arrest because the release of ecdysteroids from the prothoracic glands or the ring gland in higher dipterans is needed to coordinate the pupal-adult metamorphosis. However, unlike diapausing pupae that can be caused to resume development with exogenous ecdysteroid exposure, exogenous ecdysteroids cannot restart development in envenomated pupae (Rivers and Denlinger, 1994). This observation suggests that the developmental arrest in envenomated *Sarcophaga* pupae is regulated differentially than pupal diapause. Envenomation-induced developmental arrest must somehow disrupt ecdysteroid signaling 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 accumulating greater fat reserves (Rivers and Denlinger, 1995). This clear contrast in the cellular viability responses amongst the three *Sarcophaga* tissues that have been intensively studied is consistent with venom manipulating the host environment to favor developing 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?

3.6.1. Developmental signaling pathways

Almost 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 2.2 and S2.4). Many genes classified as having biological functions in promoting development by cellular proliferation and growth also have functions in apoptosis. Understanding the molecular basis of selective maintenance of some tissues during developmental arrest while others are destroyed will require carefully picking apart the activity of candidate genes and signaling pathways at the level of individual tissues.

The multifunctional mitogen-activated protein kinase (MAPK) pathway is one of the most promising candidates for regulating developmental arrest suggested by our transcriptomic study. Within the broader umbrella of MAPK signaling, the ERK pathway promotes cellular proliferation and morphogenesis whereas the p38K and JNK signaling cascades are associated with modulating development and immune function in the face of stress including apoptosis and cell cycle arrest responses (Concannon *et al.,* 2003; Rane *et al.,* 2003). As mentioned above (see 3.5.2.), 25 h after envenomation we observed up-regulation in transcripts of a putative MAPK 3 or MAPK 4-phosphatase (1.86-fold) that could inhibit MAPK signaling 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 signaling (-1.46-fold).

The MAPK-ERK signaling cascade in insects has been shown to regulate growth and metamorphosis. Because envenomation initiates a developmental arrest in pupae that prevents further pharate adult metamorphosis we expected that the ERK-signaling cascade would be down-regulated after envenomation. Interestingly, Torso, a peptide-hormone receptor that activates ERK signaling was up-regulated 25 h after envenomation (2.17-fold). Torso is the receptor for prothoracicotropic hormone (PTTH), a peptide that signals the prothoracic glands to produce ecdysone to precipitate molting and morphogenesis via ERK signaling (Rewitz *et al.,* 2009). It may seem counterintuitive for transcripts of the PTTH receptor to be in greater abundance in pupae in developmentally arrested pupae compared to control animals that are already undergoing pupal-adult metamorphosis by 25 h after envenomation. However, by the time of metamorphosis developing flies have already

completed PTTH signaling and released ecdysteroids so PTTH reception may not be necessary. In contrast, the developmentally arrested pupae may still be expressing the PTTH receptor even though exogenous ecdysteroids cannot trigger the resumption of development in envenomated pupae (Rivers and Denlinger, 1994a), indicating that these pupae are stuck perpetually in a state of molecular stasis that prevents further development. A putative Torso/PTTH-receptor transcript was also clearly up-regulated in larvae of *P. xylostella* that fail to successfully pupate in response to parasitism by *D. semiclausm* (Etebari *et al.,* 2011), again suggesting that developmental arrest may occur up-stream of ecdysone reception. Another transcript that could be involved in developmental signaling is a putative cytochrome p450-28a1 that is down-regulated (-1.91-fold). This p450 is similar to *C. elegans* daf-9, an enzyme that has been implicated in regulating the dauer developmental arrest in these worms by producing the steroid hormone dafchronic acid that acts through a nuclear steroid hormone receptor, daf-12, appearing superficially to have parallels with ecdysteroid signaling (Gerisch and Antebi, 2004). Further detailed investigations of both steroid hormone production and signaling downstream of the receptor are needed to tease apart their regulatory roles in developmental arrest in these host-parasitoid interactions. Specifically, we expect that investigation of sensitivity to the action of PTTH and ecdysteroids through the ERK signaling cascade relative to p38K and JNK signaling holds promise for understanding the regulation of envenomation-induced arrest of host development.

Rho signaling, mentioned above in the context of immunity (see 3.5.1.), may also play a critical role in envenomation-induced developmental arrest as suggested by the 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 2.2). The Rho signaling cascade affects cytoskeletal structure and appears to play key regulatory roles in both embryonic and pupal morphogenesis in *Drosophila* (Chen *et al.,* 2004). Rho signaling is GTP-mediated and pathway members have been identified as oncogenes because mutations that lead to enhanced signaling or transgenic increases in Rho signaling lead to increased cellular proliferation and tissue overgrowth (Clark *et al.,* 2000). Thus, the up-regulation of Rho family GTP-binding proteins and a guanine nuclear exchange factor that regulates cyclic GMP levels may help to induce developmental arrest by sequestering and decreasing GTP to inhibit the Rho signaling that would normally lead to pupal-adult metamorphosis (Chang *et*

al., 1998). Rho signaling can also interact with signaling in another pathway that affects 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 signaling, a Prl protein-tyrosine phosphatase (Pagaragain *et al.,* 2013), are up-regulated 25 hours after envenomation (2.17-fold), suggesting inhibition of Src signaling may contribute to the envenomation-induced developmental arrest. Testing whether Src is inhibited during developmental interactions with Rho signaling or through other upstream inhibitors should prove fruitful for understanding developmental arrest in the *Sarcophaga-N. vitripennis* interaction and other parasitoid systems as well.

3.6.2. Other regulators of development

The only transcript to be detectably differentially expressed 3 h after envenomation was a putative leucine rich-repeat protein that was down-regulated (-1.67-fold). Putative homologues of this transcript are highly expressed during the pupal-adult transition in *D. melanogaster* where they are thought to regulate programmed cell death of larval-pupal structures as the animal undergoes adult morphogenesis (Berry and Baehrecke, 2007). Down-regulation of this protein may contribute to an early arrest in development and morphogenesis upon envenomation, preventing the pupal host from further development that may make the host less suitable for parasitoid larval development (Rivers and Denlinger, 1995). Other possible regulators of developmental arrest include the down-regulation (-2.87-fold) of a putative alkyldihydroxy-acetonephosphate synthase (ADHAPS) that is required for normal development in humans and *C. elegans* (Motley *et al.,* 2000) and the action of miRNAs. Besides their possible involvement in immune responses (see 3.5.2.), miRNA processing in particular has emerged as important for regulating development and morphogenesis in insects (Asgari, 2013) because they can modulate major transcriptional programs and RNA processing. The up-regulation of transcripts for two putative argonaute-2 proteins (1.35 and 1.16-fold respectively) in our data combined with a previous observation 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 in the *N. vitripennis-Sarcophaga* interaction and more generally across parasitoids and their hosts.

3.7. Metabolism

Many parasitoids are capable of manipulating host intermediary metabolism to improve the nutritional milieu for larval development. Alterations in host metabolism are typically initiated by venom and symbiotic viruses, followed by an increasingly direct role of the developing parasitoid and accessory cells such as teratocytes (Dahlman *et al.,* 2003; Nakamatsu and Tanaka, 2003, 2004b; Nurullahoglu *et al.,* 2004, Formesyn *et al.,* 2011). As an ectoparasitoid with no apparent viral symbionts, *N. vitripennis* venom plays an important role early in manipulating host metabolism to favor larval development, followed by direct manipulation of the host as the parasitoid larvae grow and begin to actively feed on host tissues. Over the 25 h timeframe of our study the egg is external to the host body and would not have yet hatched (Whiting, 1967). Thus all effects we observe must be due solely to envenomation. A series of prior physiological studies have shown that envenomation causes a precipitous drop in the metabolic rates of hosts (decreased O_2 consumption), even without an egg or larva present (Rivers and Denlinger, 1994a, 1995). Although the decrease in host metabolism upon envenomation may be partly due to arresting host development, components of *N. vitripennis* venom are also clearly manipulating host intermediary metabolism, because just after envenomation hosts show increased levels of alanine and pyruvate, but decreased oxaloacetate (Rivers and 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 and Denlinger, 1994b, 1995). Here we use our observations of transcript abundance after envenomation to construct hypotheses for the regulation of increased lipid storage and metabolic depression. Because our data represent whole-body transcript abundance, we interpret these data in the context of intermediary metabolism being increased in some tissues and decreased in others.

3.7.1. 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 lipids synthesized by their hosts to provide the fatty acids necessary for juvenile growth and adult reproduction (Visser *et al.,* 2010, 2013). Consistent with this view, previous work on several different systems have shown

parasitoid manipulation of host lipid metabolism from physiological (Rivers and Denlinger, 1994a; Rivers and Denlinger, 1995; Dahlman *et al.,* 2003; Nakamatsu and Tanaka, 2003, 2004b; 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 cannot feed and are thus a nutritionally-sealed system, *N. vitripennis* venom must trigger a tissue-specific starvation response wherein 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 tightly 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 and Mitchell-Foster, 2011; Kroemer and Levine, 2008). Autophagy is a critical component of both starvation responses and responses to mild cellular damage. Mild starvation or cellular damage that leads 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 *et al.,* 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 via intermediary metabolism. 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.

Physiological studies have shown that the lipid content of host fat bodies increases 3-4 fold within 6 days of envenomation (Rivers and Denlinger, 1994a, 1995). We expect that the raw nutrients for producing this substantial increase in fat body lipids must come from materials trafficked from peripheral tissues undergoing some combination of slower-acting autophagy that has just begun to increase 25 h after envenomation and more-rapid apoptosis triggered just after envenomation (see 3.5.1.). We observe enrichment in several GO categories that are consistent with our 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 (Fig. 2.4).

When assessing our gene-by-gene analysis (Table S2.4) several candidate transcripts for autophagy were differentially expressed. A candidate for autophagy regulation is a putative damage-related autophagy modulator (DRAM), shown to actively control autophagy in *D. melanogaster* (O'Prey *et al.,* 2009), that was highly up-regulated 25 h after envenomation (1.47-fold). Mitochondria play an important role in either promoting or inhibiting autophagy (Cooper and Mitchell-Foster, 2011; Kroemer and Levine, 2008). Specifically, damaged or energy-stressed alterations in the mitochondrial trans-membrane potential that are regulated by mitochondrial Ca^{2+} signaling can trigger recruitment of individual mitochondria to the autolysosome (e.g., mitophagy). Transcripts for two possible regulators of mitochondrial Ca^{2+} homeostasis that have been previously associated with autophagy were up-regulated 25 h after envenomation (Cardenas and Foskett, 2012; Lin *et al.,* 2012), a putative calcyphosine (1.44-fold) and a putative small mitochondrial calcium-binding protein (1.83-fold). One of the most highly up-regulated transcripts after envenomation (13.2-fold) is a putative DGP-1 homologue. As mentioned above, autophagy is intimately linked to the cell cycle and DGP-1 is an elongation factor that has been shown to play an important regulatory role in taking damaged cells out of the cell cycle and inducing autophagic repair (Gruenewald *et al.,* 2009; Blanco *et al.,* 2010). A final candidate is a putative lysosomal aspartic protease that was up-regulated (1.74-fold) 25 h after envenomation. This particular transcript has substantial similarities to cathepsin D, an important lysosomal protease that has been found to be 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 rapid apoptotic response of host hemocytes and brain cells in the first 24 h after envenomation in the *Sarcophaga-Nasonia* interaction (see 3.6.; Rivers *et al.,* 2002, 2011) as well as in other parasitoid-host manipulation systems (Song *et al.,* 2008; Fang *et al.,* 2010; Etebari *et al.,* 2011). We expect that apoptotic pathways will be rapidly initiated as part of the 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 will be needed across multiple tissue types to test our hypotheses about the relative contributions of autophagy and apoptosis to the three major axes of host manipulation: immunity, development, and nutrition.

Beyond breaking down cellular components in peripheral tissues into trafficable units by autophagy and apoptosis, recycled nutrients must be processed through intermediary metabolism to support lipid accumulation in the fat body. 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 eventual anabolic synthesis of lipids in the fat body. As mentioned above, GO 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 h after envenomation (Fig. 2.3). When considering specific genes and biochemical pathways that may favor lipid synthesis from amino acids produced by autophagy, there is a clear up-regulation of transporters including a cationic amino acid transporter (2.13-fold) and an oligopeptide transporter (YIN) (2.87-fold) that has been associated with transport of small peptides (especially alanylalanine) in *D. melanogaster* (Charriere *et al.,* 2010). Of particular note for fat synthesis from amino acids is the up-regulation (1.63-fold) of 2-amino-3-ketobutyrate coenzyme a ligase (Edgar and Polak, 2000), an enzyme that plays a critical role in the metabolism of serine, threonine, and glycine into acetyl-CoA for use in fatty acid synthesis. Similar transport of amino acid catabolism intermediates is suggested by up-regulation (2.05-fold) of transcripts for a putative sodium-associated monocarboxylate transporter 25 h after envenomation. Sodium-associated monocarboxylate transporters function to traffic numerous short-chain fatty acid metabolites including: lactate, pyruvate, acetate,

proprionate, and some ketones. In this context monocarboxylate transporters may be important for moving metabolic intermediates of catabolism of peripheral tissue protein into the fat body for subsequent lipid synthesis. Furthermore, we also observed very substantial up-regulation (7.43-fold) of a putative hydroxy-acid oxidase transcript. Hydroxy-acid oxidase plays a critical role in glyoxylate and dicarboxylate metabolism following serine, threonine, and glycine metabolism. Envenomation could trigger glyoxylate and dicarboxylate metabolism in the host's peripheral tissues, wherein two-carbon precursors could be used for gluconeogenesis so that simple carbohydrates or Krebs-cycle intermediates could be trafficked to the host fat body to support increased fatty-acid synthesis (Voet and Voet, 2011). Taken together these data suggest that envenomation of *Sarcophaga* host pupae by *N. vitripennis* causes a substantial shift in protein and amino-acid metabolism that supports mobilization of 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.,* 2011; Visser *et al.,* 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 with careful tracking of labeled amino acid substrates through their metabolic intermediates (Zera, 2011; Visser *et al.,* 2012).

3.7.2. Metabolic depression

Consistent with the view that venom helps to reduce host use of catabolic and anabolic precursors so more will be available for parasitoid larval growth, upon envenomation by *N. vitripennis* the metabolic rates of *Sarcophaga* pupae decline precipitously (Rivers and Denlinger, 1994a). Some portion of the observed metabolic depression may simply be a byproduct of host developmental arrest, however previous biochemical and physiological studies have shown that envenomation dramatically shifts host intermediary metabolism. Within 24 hours after envenomation by *N. vitripennis, Sarcophaga* pupae show a rapid increase in alanine and pyruvate levels, but not the Krebs-cycle intermediate oxaloacetate (Rivers and 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 and Denlinger, 1994b).

As in other parasitoid-host interactions that have been studied from a transcriptomic or proteomic perspective (Etebari *et al.,* 2011; Song *et al.,* 2008; Nguyen *et al.,* 2008; Wertheim *et al.,* 2005, 2011; Zhu *et al.,* 2009), genes associated with energy metabolism were differentially expressed in our study (Fig. 2.4, Table 2.2). Across a wide range of taxa from turtles to insects, metabolic depression is often associated with inducing hypoxic-like states that include greater anaerobic metabolism through glycolytic/gluconeogenic pathways and decreased reliance on the Krebs cycle (Guppy and 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 were kept normoxic (Michaud and Denlinger, 2006; Ragland *et al.,* 2010), and several transcriptional observations in our data suggest that envenomation may also encourage a shift towards increased anaerobic metabolism. Lactate dehydrogenase regenerates NAD^+ by converting pyruvate to lactate under anaerobic conditions (Voet and Voet, 2011), and a putative lactate dehydrogenase transcript was substantially up-regulated 25 h after envenomation (3.83-fold). The elevated pyruvate levels observed in *Sarcophaga* pupae just after envenomation (Rivers and Denlinger, 1994b) are consistent with the hypothesis that envenomation increases glycolysis relative to aerobic Krebs-cycle activity and lactate dehydrogenase may play an important role in maintaining the load of NAD^+ available to facilitate glycolysis. While lactate levels have not yet been assessed in envenomated individuals alanine can also be an anaerobic end-product of regenerating NAD^+ from pyruvate that does not enter the Krebs cycle, so the high levels of alanine previously observed upon envenomation are also consistent with a shift to anaerobic metabolism (Rivers and Denlinger, 1994b). Another transcript suggesting greater glycolysis in envenomated hosts is up-regulation of a putative myoinositol oxygenase (1.83-fold). Myoinositol is a six-carbon sugar that can be processed into glycolysis or used to produce the lipid-precursor inositol, and myoinositol oxygenases have been implicated in sugar balance and diabetic pathology in vertebrates (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 must be caused

by components of *N. vitripennis* venom altering pathways that regulate metabolic responses to hypoxia. In diapausing *Sarcophaga* pupae that also appear to be acting anoxic even though they were kept in normal-oxygen atmospheres there was a clear up-regulation of genes associated with hypoxia signalling via the hypoxia-inducible factor (HIF/Sima, Ragland *et al.,* 2010). In the current study, a putative hypoxia-inducible domain family 1 protein was up-regulated 25 h 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 our hypothesis that envenomated host pupae are induced into a hypoxia-like state despite being in a normal-oxygen atmosphere. 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. Detailed understanding of the reorganization of intermediary metabolism may provide important insights for novel control methods for insect pests and even insights into the basis of metabolic regulation more generally, an important goal given a societal focus on metabolic dysfunction in disease states like diabetes and obesity.

3.8. Validation by qRT-PCR

A sample of 20 ESTs were selected for validation with qRT-PCR, including the single transcript found in the 3 h group (lrr-pr) (Table S2.2). 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 2.1, Fig. 2.6). EIF or eukaryotic translation initiation factor 1 has a 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, qRT-PCR 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 (Fig. 2.7).

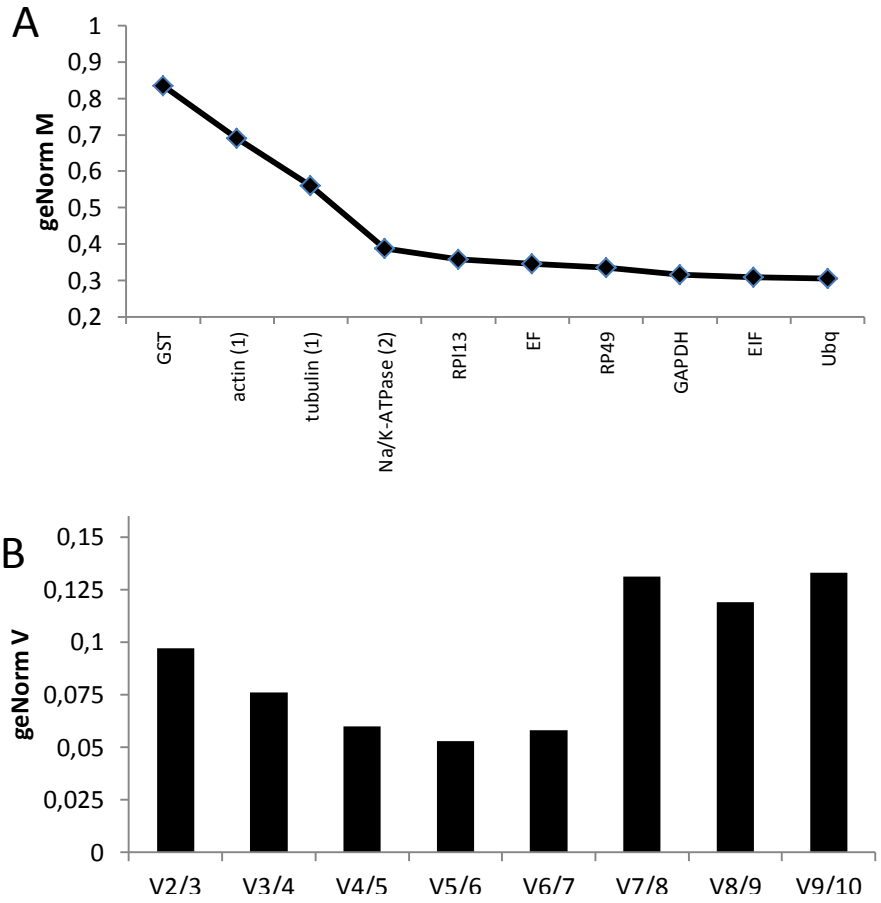

Figure 2.6: A) Average expression stability of remaining reference targets using a stepwise exclusion of the least stable expressed reference gene which ends with the two most stable genes on the right. **B**) Determination of the optimal number of reference targets with geNormPLUS, using pairwise variations by adding extra reference genes to the equation, starting with the two most stably expressed genes on the left (a value of 0.15 is used as cut-off).

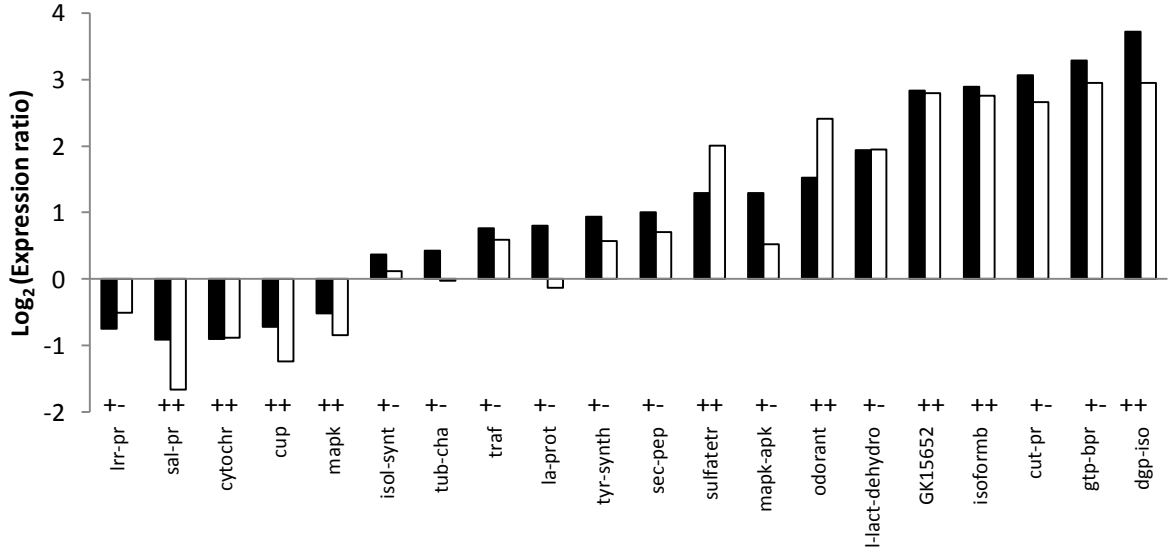

Figure 2.7: Validation of microarray data with qRT-PCR. Log2-transformed expression ratio of Paras_3h compared to Contr_3h (lrr-pr) and of Paras_25h compared to Contr_25h. White bars: qRT-PCR experiment. Black bars: microarray experiment. +: significant differential expression ($p < 0.05$). -: no significant expression ($p \geq 0.05$).

3.9. Conclusions

This paper supports earlier studies demonstrating that *N. vitripennis* venom influences diverse physiological processes in its preferred host organism *S. crassipalpis*. Our molecular assay to confirm parasitation *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 parasitation 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 our 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.

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Chapter 3

Localization of cell death in *Sarcophaga crassipalpis* pupae parasitized by *Nasonia vitripennis*

Contributions

D. de Graaf and A. Gregorc assisted with the study design. E. Formesyn was responsible for the insect cultures, executed all parasitation experiments and did the dissections of the pupae. E. Formesyn executed the paraffin embedding of the fly pupae. A. Gregorc performed the stainings of the samples and assisted with their analysis. E. Formesyn analyzed the samples after their staining, took the majority of the photographs and wrote the manuscript.

1. Introduction

The ectoparasitoid wasp *Nasonia vitripennis*, also known as the jewel wasp, prefers to parasitize Sarcophagidae and Calliphoridae hosts. Pupae and pharate adults of these fly families are defenseless when they encounter a *N. vitripennis* female. Upon detection of a suitable host, the wasp inserts its ovipositor through the puparium, injecting venom and depositing eggs on the exterior side of the pupa. However, unlike several endoparasitoid wasps, successful parasitation is accomplished solely by the venom, without the additional compounds such as polydnaviruses and teratocytes (Formesyn *et al.,* 2011; Asgari, 2011).

In the past, the venom composition of *N. vitripennis* was extensively studied, leading to the identification of 80 venom proteins, which may have a defined role in several processes upon host parasitation (Rivers *et al.,* 2006; de Graaf *et al.,* 2010; Ye *et al.,* 2010; Danneels *et al.,* 2010). This proteinacious mixture is able to subdue the host and alter its physiology in order to maximize progeny development. The first step to ensure successful parasitation is to avoid that hosts can mount an immune response which may destroy the eggs or prevent larval feeding. Many parasitoids are known to target the immune system of their hosts by eliciting cell death in the hemocytes, as observed with the parasitoid wasps *Pimpla turionellae*, *Meteorus pulchricornis* and *Cotesia kariyai* (Er *et al.,* 2010; 2011; Suzuki et al. 2008; Teramoto and Tanaka, 2004). *Nasonia* venom is capable of suppressing the immune system by targeting the granulocytes and plasmatocytes of its host (Rivers *et al.,* 2002). However, cell death is not limited to hemocytes as the venom induces programmed cell death in Lepidopteran cell lines derived from the ovaries of *Trichoplusia ni* (BTI-TN-5B1) and *Spodoptera frugiperda* (Sf21) (Rivers *et al.,* 2010; Formesyn *et al.,* 2013). Furthermore, envenomated hosts are hampered in their development and will never complete their metamorphosis. This developmental arrest elicited by *N. vitripennis* venom is accompanied by a reduction in host respiratory metabolism and was found to involve apoptosis in brain tissues of *Sarcophaga bullata* (Rivers and Denlinger, 1994 a, b; Rivers *et al.,* 2011). In addition, host metabolism is altered in such a way that it is synchronized with the needs of the developing parasitoids which results in elevated lipid levels in the fat body (Rivers *et al.,* 1995).

The physiological and biochemical alterations observed in envenomated hosts seem to involve signaling pathways that impair calcium homeostasis which cease in cell death (Rivers *et al.,* 2005). A microarray study examining the pupal transcriptome of *S. crassipalpis* 3 and 25 h after parasitation, revealed differential regulation of several transcripts involved in apoptosis at 25 h but none in pupae 3 h post parasitation (Chapter 3).

To localize the occurrence of cell death during parasitation, *S. crassipalpis* pupae were parasitized by *N. vitripennis* and histologically analyzed after 4, 26 and 50 h. Two terminal deoxynucletidyl transferase-mediated dUTP nick end labeling (TUNEL) procedures were used in order to detect DNA breakdown in the nucleus and differentiate between cell death types. To our knowledge, this is the first study that localizes cell death in whole pupae after exposure with parasitoid wasps.

2. Materials and methods

2.1. Insect rearing

N.vitripennis Asym C, were reared on pupae of the flesh fly *Sarcophaga crassipalpis* as described in Van den Assem and Jachmann, 1999. In brief, approximately 15 to 20 *S. crassipalpis* pupae and 10 to 15 female *N. vitripennis* wasps of at least 1 day old were deposited in a culture tube and closed with a ceapren stop. They were reared under a light-dark cycle (LD 15:9 h) at 25 °C. Under these conditions, the wasps emerge after 14 days.

Flesh flies (*S. crassipalpis*) were provided by Dr. Hahn, University of Florida, and cultured in the laboratory as described by (Denlinger *et al.,* 1972a). During the first days after hatching, the adult flies were fed ad libitum on sugar and water, after 3 to 4 days, additional beef liver was provided in order to start egg development. All flesh fly stadia were kept at 25 °C with a photoperiodic cycle of LD 15:9.

2.2. Exposure of hosts to parasitoids

Prior to the experiment, female wasps were allowed to feed on flesh fly pupae for 6 hours. The next day, *S. crassipalpis* pupae were placed in a cultivation tube and parasitized as described in Chapter 2. In brief, holes with the size of a flesh fly puparium were made into the ceapren stop, so only the posterior tip of the puparium was accessible to the parasitoids. The experienced *N. vitripennis* females and fresh flesh fly pupae (5 days after pupariation)

were placed together in a cultivation tube in a 3:1 ratio, so parasitation was ensured. Two hours after exposure, female wasps were removed and the pupae were maintained at 25 °C and sampled after 4, 26 and 50 additional hours. Since the *N. vitripennis* females had 2 hours to parasitize the pupae and the dissection procedure took approximately 2 h per group, the sampling points correspond on average to 4, 26 and 50 hours post-parasitation. In the 50 h group, half of the pupae were opened 24 h post-parasitation at their posterior side only, in order to remove the wasp eggs before they start hatching. The other part of the 50 h group was left unopened to investigate the additional influence of the feeding wasp larvae. Control pupae were treated identically except that they were not exposed to the parasitoids. In the 50 h control group, half of the pupae were opened at their posterior side only. All insects were maintained at 25 °C, LD 15:9.

2.3. Paraffin embedding and sample preparation of flesh fly pupae

In order to properly expose the tissues to several solutions, fly pupae were dissected and the puparium was removed completely. Only parasitized pupae that had both melanization spots and parasitoid eggs were included in the study. Flesh fly samples were processed as described by Gregorc and Ellis (2011). The developing flesh flies were fixed in a 10% formaldehyde solution for 24 h and dehydrated in an ascending series of ethanol (50%, 70% and 2 x 100%) and xylene (25%, 50%, 75% and 100%) both diluted in distilled water and incubated at room temperature for 24 h each. Prior to embedding, samples were infiltrated with several changes of paraffin wax for approximately 14 days. Subsequently, the samples were embedded in paraffin wax and sections of 5 µm were cut on a Leica microtome, floated on distilled water (42 °C), collected on cleaned slides and placed in an oven at 60 °C for approximately 4 h. Afterwards, the sections were stored at room temperature until further analyses.

Removal of the paraffin wax was performed in three consecutive changes of xylene for 5 min each and in three changes of ethanol (100%) for 2 min each. Sections were then washed in phosphate buffer solution (PBS, 0.01 M, pH 7.1) and prepared for the different staining procedures.

2.4. In situ cell death detection kit, AP (ISCDDK)

After paraffin wax removal and rehydration, sections were prepared according to the manufacturer's protocol (ISCDDK, AP, Roche Cat. No. 11 684 809 910). The kit relies on the principle that apoptotic cells undergo genomic DNA cleavage yielding double-stranded as well as single strand breaks which can be identified by labeling the free 3'-OH termini, according to the terminal deoxynucleotidyl transferease (TdT)-mediated dUTP nick end labeling (TUNEL) principle. Prior to labeling, tissue sections were treated with proteinase K (20 µg/ml in 10 mM Tris/HCl, pH 7.4) for 15 min at 21 °C. Before labeling, tissue sections were rinsed twice with PBS. Labeling was performed by terminal deoxynucleotidyl transferase (TdT) that incorporates the fluorescein labeled dUTP's to the strand breaks. Subsequently, the samples are incubated for 60 min at 37 °C. dUTPs incorporated with fluorescein were detected by anti-fluorescein antibodies from sheep, conjugated with alkaline phosphatase (AP). After an incubation period of 30 min at 37 °C, sections were rinsed with PBS, incubated with the substrate solution (Fast Red) using the EnVision System alkaline phosphatase kit (Dako) and subsequently washed in tap water for 5 min. The sections were counterstained with Mayer's hematoxylin (Dako) and afterwards rinsed with tap water according to the protocol. Negative control labeling was performed by substituting the TdT enzyme with PBS as described in Gregorc and Smodis Skerl (2007). TUNEL-positive cells contain a red nucleus as a result of the localized reaction product which is indicative for cell death. Healthy cells have TUNEL-negative nuclei which are colored blue.

2.5. ApopTag peroxidase in situ Apoptosis detection kit

Sections were processed according to the ApopTag protocol (Millipore). The principle of the kit is also based on the TUNEL assay. The TdT enzyme catalyzes the addition of digoxigenin-conjugated nucleotides to the 3'-OH ends of DNA breaks, and allows binding to an anti-digoxigenin antibody conjugated with a peroxidase reporter molecule. Dewaxed sections were treated with proteinase K and quenched in 3% endogenous peroxidase in PBS at room temperature for 5 min. After 2 consecutive wash steps with PBS and subsequent treatment with equilibration buffer, the working strength TdT enzyme was applied to the tissue sections and incubated for 1 h at 37 °C. Next, sections were treated with stop/wash buffer and after rinsing with PBS, Anti-Digoxygenin-Peroxidase conjugate was administered to the

sections and incubated for 30 min. Sections were washed in PBS and covered with peroxidase substrate (3,3'-diaminobenzidine tetrahydrochloride, DAB) and washed in dH_2O once color development was achieved. Counterstaining was performed with Mayer's hematoxylin. Apoptotic cells display brown nuclei, while healthy cells are colored blue.

Tissues from 2-3 hosts at each time point were examined using a Zeiss light microscope. For each pupa, at least 3 consecutive sections were examined.

3. Results

3.1. Dissection of the pupae

Since *N. vitripennis* was only allowed to parasitize the posterior side of their hosts, these pupae displayed several dark melanization spots at this side of the abdomen. These spots are indicative for the place where *N. vitripennis* females inject venom or construct a feeding tube. Furthermore, the abdomen displayed a brownish color and the turgor decreased in parasitized pupae, even without feeding larvae, compared to control pupae (Fig 3.1A, C). Several wasp eggs were found on or around these melanization spots (Fig. 3.1B, C). In all of the analyzed pupae, parasitoid eggs and 1-day-old larvae were located at the abdomen in which the majority could be found under or in the vicinity of the legs.

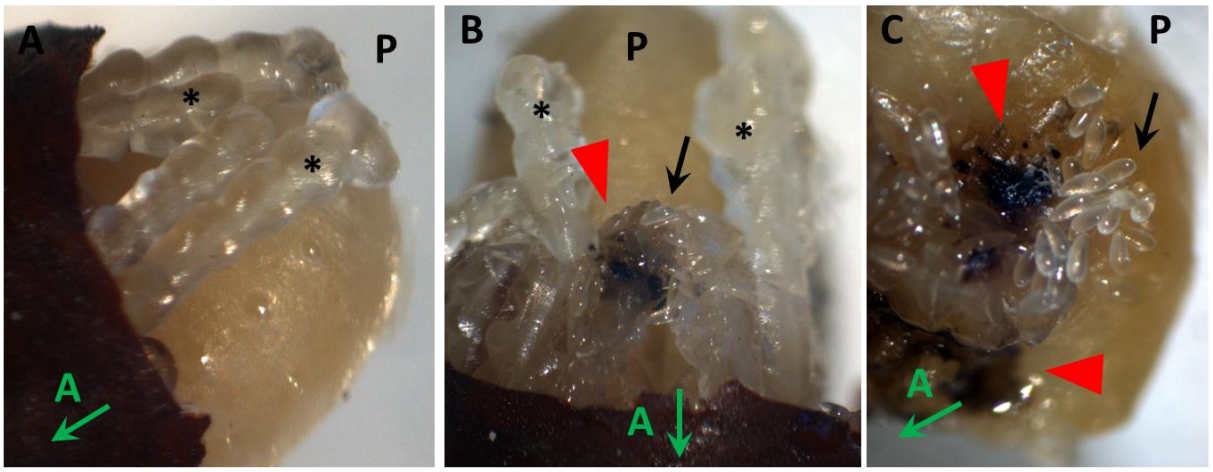

Figure 3.1: Detail of posterior side of the abdomen of a parasitized pupa. Asterisks indicate the legs of the pupa, red arrow heads indicate the melanization spots, the black arrow shows the deposited parasitoid eggs. P: Posterior side, the green A and arrow indicate the direction of the anterior side of the pupa. **A**) Unparasitized pupa, **B**) parasitized pupa with eggs and melanization spots. **C**) Detail of a parasitized pupa with eggs and melanization.

3.2. Four hours after parasitation

In order to localize apoptosis in tissues of whole *S. crassipalpis* pupae, the TUNEL reaction was used. Approximately 4 h after parasitation with *N. vitripennis*, flesh fly pupae displayed a red azo-dye reaction product with ISCDDK and a brown staining with the ApopTag kit in several nuclei clustered in areas, peripheral in the abdomen (Fig. 3.2B, C). Furthermore, in the proximity of these areas, some trophocytes were TUNEL positive (Fig. 3.2E, F) while the reaction products in the majority of the other tissues, including Malpighian tubules were absent. Diffusion of the reaction product was only visible in the abdomen of the parasitized pupae using the ISCDDK and ApopTag kit. The reaction product was found sporadically in trophocytes and hemocytes throughout the abdomen of control pupae. In addition, control pupae displayed no TUNEL-reaction marked nuclei in clustered areas, peripheral in the abdomen (Fig. 3.2A, D).

3.3. Twenty six hours after parasitation

Parasitized pupae stained with both ApopTag and ISCDDK displayed red or brown reaction products in nuclei which are clustered in areas, peripheral in the abdomen. In the proximity of these areas, nuclei of trophocytes were marked with TUNEL reaction products indicating the occurrence of cell death (Fig. 3.3B, D). However, in contrast to pupae 4 h after parasitation, parasitized pupa of the 26 h group, displayed a stronger extracellular diffusion of the reaction product in the abdomen using both kits (Fig. 3.3B, D). Malpighian tubules were unaffected in parasitized pupae (Fig. 3.3B, C). Parasitized pupae sometimes displayed TUNEL negative nuclei in the gonads using the ApopTag kit (Fig. 3.3D), while in other pupae stained with the ISCDDK kit some nuclei of interstitial cells were TUNEL positive. Cell lysis in combination with positively stained nuclei in the peripheral parts of the abdomen was also observed in parasitized pupae using both kits (Fig. 3.3E). Interestingly, in one pupa this cell lysis was located around an unknown structure which seems to be inserted from the exterior (Fig. 3.3E).

Similar peripheral areas in the abdomen were negatively stained in control pupae as well as Malpighian tubules and ovaries, with the exception of some sporadic positive nuclei dispersed in the whole body (Fig. 3.3A, Fig. S3.1A,B; supplemental materials can be obtained by contacting Prof. de Graaf).

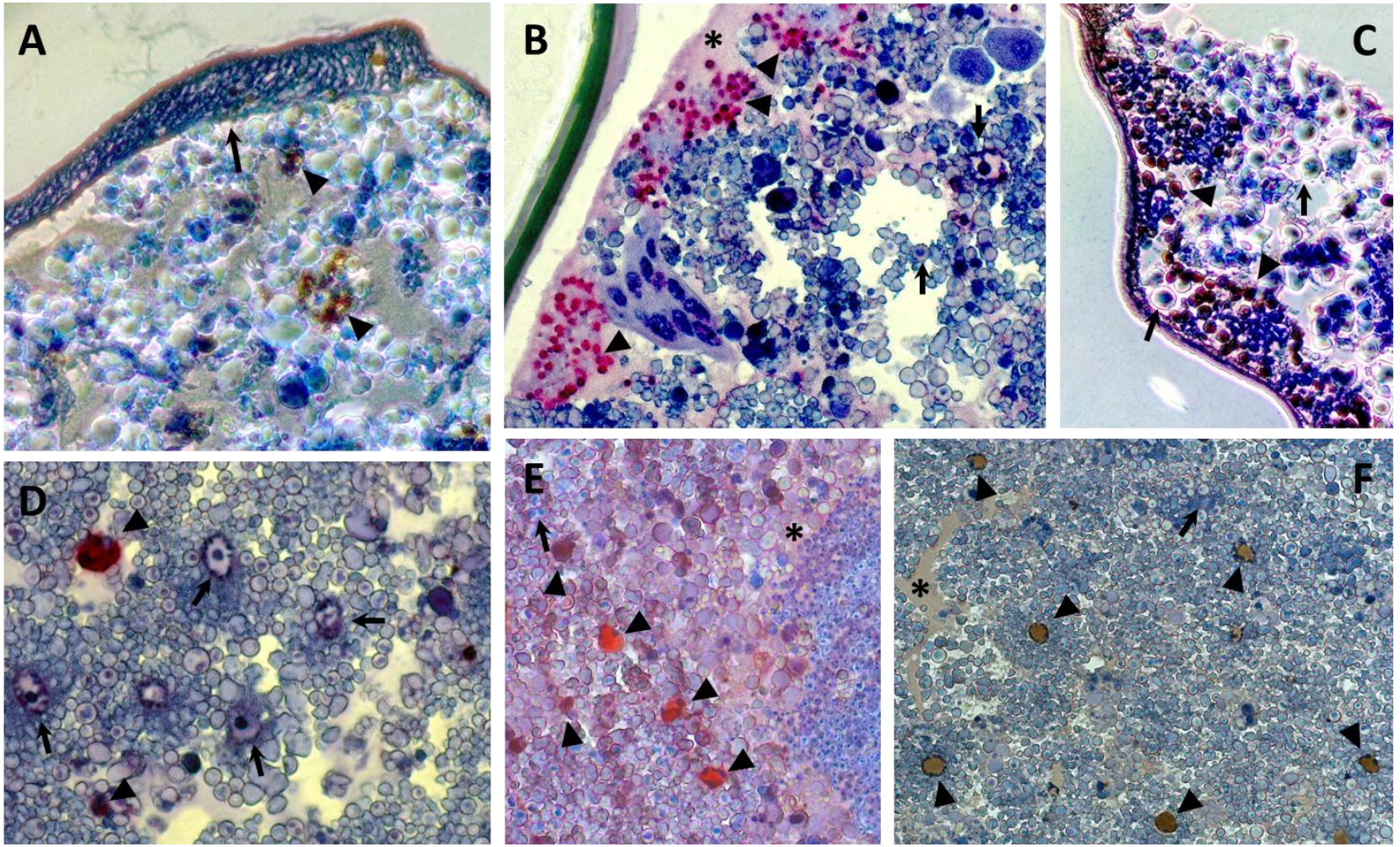

Figure 3.2: Abdominal tissues, located in the peripheral regions of 5-day-old fly pupae parasitized with *N. vitripennis* for ~ 4 h (**B**,**C**,**E**,**F**) and control pupae of the same age (**A**,**D**). Diffused reaction product in the abdomen in parasitized pupae is indicated by an asterisk. Reaction product localization is indicated by arrowheads, absence of reaction product is indicated by an arrow. **A**) Abdomen of a control pupa, stained with the ApopTag kit. Magnification: 100 x. **B**) Parasitized pupa stained by the ISCDDK assay. Magnification: 400 x. **C**) Parasitized pupa stained with the ApopTag kit in. Magnification: 200 x. **D**) Control pupa with ISCDDK. Magnification: 400 x. **E**) ISCDDK staining of trophocytes and hemocytes in a parasitized pupa with. Magnification: 100 x. **F**) Trophocytes in a parasitized pupa using the ApopTag kit. Magnification: 100 x.

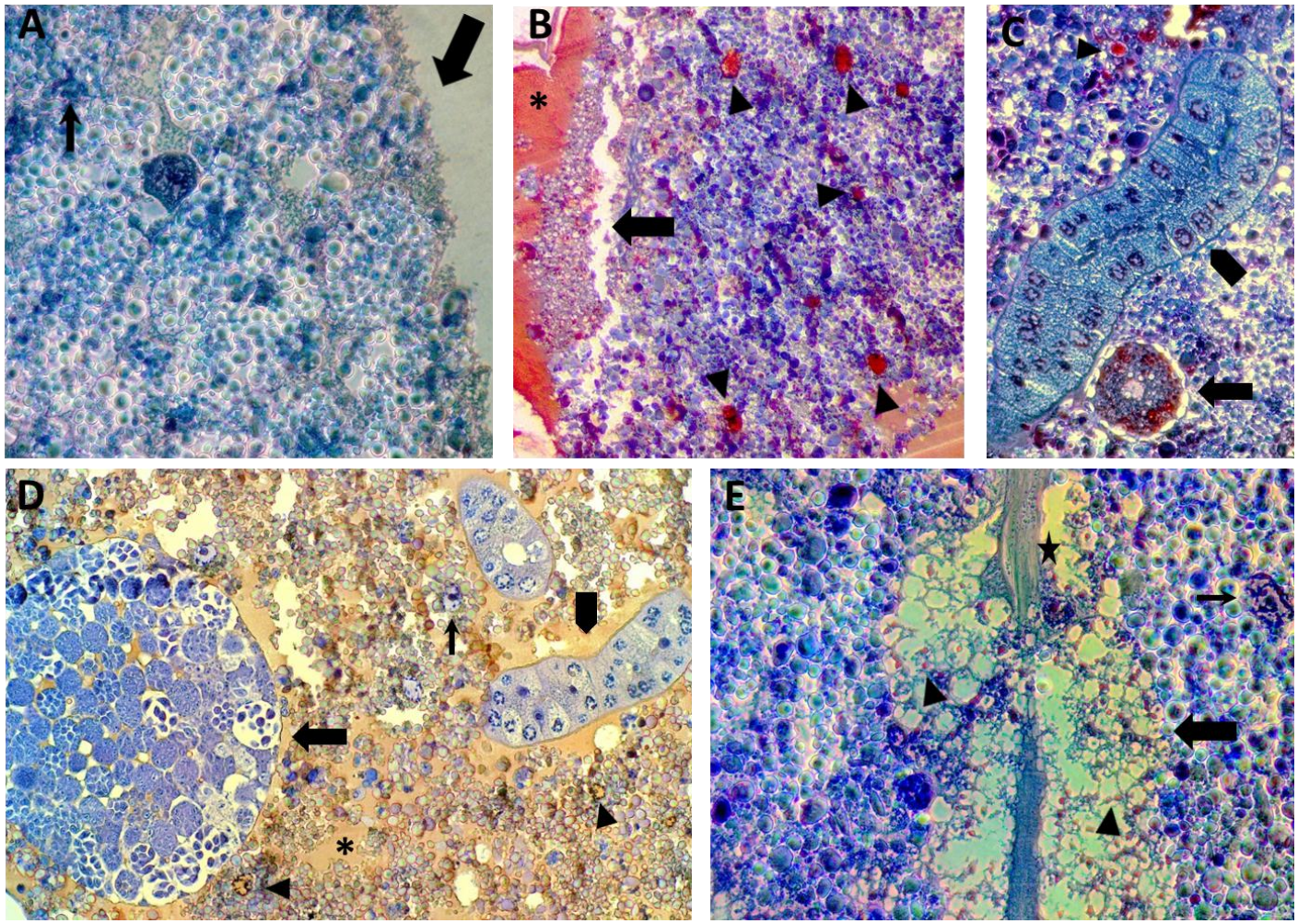

Figure 3.3: Abdominal tissues, located in the peripheral regions of 6-day-old fly pupae ~ 26 h after parasitation with *N. vitripennis* (**B-E**) and control pupae of the same age (**A**). Diffused reaction product in the abdomen of parasitized pupae is indicated by an asterisk. Presence of reaction product in death cells is indicated with black arrow heads, while the absence of the reaction product is indicated with small arrows. **A**) Periferal side (wide arrow) of the abdomen of control pupa stained with ISCDDK. **B**) Peripheral side (wide arrow) of the abdomen of a parasitized pupa with trophocytes (arrow head) and clusters of nuclei (ISCDDK). **C**) Malpighian tubules (block arrow) and hemocytes in parasitized pupa stained with ISCDDK. **D**) Reproductive tissues (wide arrow) and Malpigian tubules (block arrow) in the abdomen of a parasitized pupa (ApopTag). **E**) Unknown structure (black star) peripheral in the abdomen (ISCDDK). Magnification of panels **B** and **D**: 100 x, all other panels 200 x.

3.4. Fifty hours after parasitation

In contrast to parasitized pupae with or without wasp larvae, control pupae of the corresponding groups, displayed no extensive staining of the internal tissues, except of some sporadic TUNEL positive nuclei dispersed throughout the whole body using the ISCDDK kit. However, fifty hours after parasitation by female *N. vitripennis*, the red azo-dye reaction product was found in increased levels in the abdomen of both infected pupae with or without wasp larvae. In both parasitized groups, the red reaction product was observed at the peripheral sides of the abdomen, between the cells and underneath the cuticle of parasitized pupae, while it was absent in control pupae (Fig. 3.4A, D, Fig. S3.2A). Trophocytes at the peripheral side of the abdomen were also affected in both parasitized groups (Fig. S3.2B and Fig. 3.5A). Reproductive tissues remained TUNEL negative in two of the three pupae analyzed in both groups during parasitation. However, TUNEL positive nuclei and extensive extracellular diffusion of the reaction product was observed around these tissues (Fig. 3.4C, F). In two of the three parasitized pupae that contained wasp larvae, several nuclei in Malpighian tubules were found to contain the red reaction product (Fig. S3.3A, B), while Malpighian tubules in pupae devoid of wasp larvae seemed to be less affected. Cell death was also observed in the thorax of both parasitized groups, with many red stained nuclei located at the anterior side near the head (Fig. S3.2A; Fig. S3.5). Groups of TUNEL positive nuclei were also present near the wings in the flight muscles which are absent in control pupae (Fig. 3.4F). Interestingly, pupae that contain parasitoid larvae, displayed a strong red staining of the legs, including a diffusion of the reaction product, whereas the legs of control pupae contained viable cells (Fig. 3.5B, D, Fig. S3.3C, D).

In general, negative control sections were devoid of the reaction product in all observed time points.

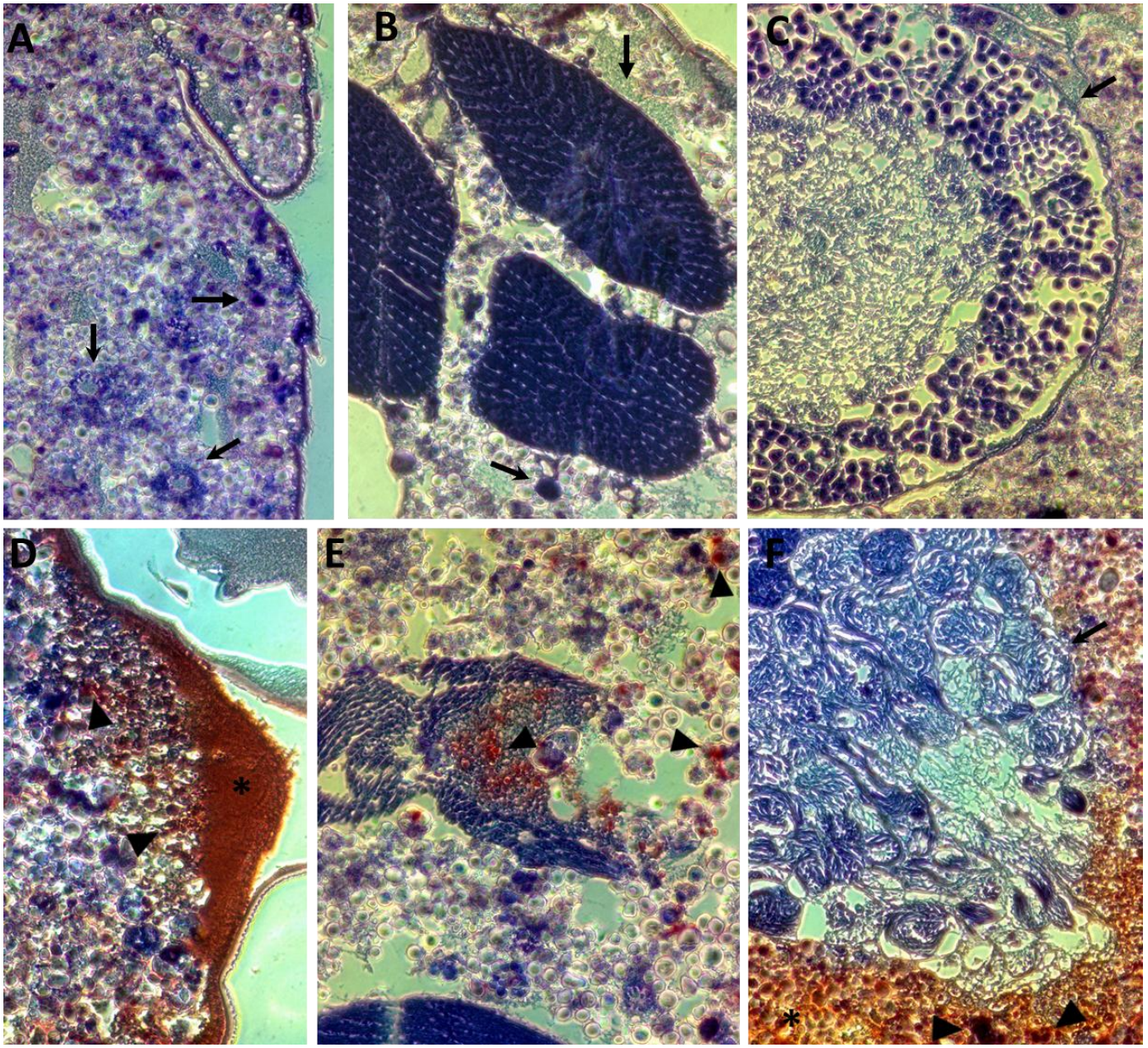

Figure 3.4: Immunohistochemical localization of TUNEL reaction product in 7-day-old fly pupae ~ 50 h after parasitation in the absence of wasp larvae (**D**-**E**) and in control pupae of the same age (**A**-**C**) using the ISCDDK kit. Reaction product is diffused throughout the abdomen in parasitized pupae is indicated by an asterisk. Presence of reaction product in death cells is indicated with an arrowhead, while the absence of the reaction product is indicated with the black arrow. **A**) Abdomen of a control pupa. **B**) Flight muscles of a control pupa. **C**) Reproductive tissues in a control pupa. **D**) Peripheral side of the abdomen of a parasitized pupa. **E**) Flight muscles of a parasitized pupa. **F**) Reproductive tissues in a parsitized pupa. Magnification of all panels: 200 x.

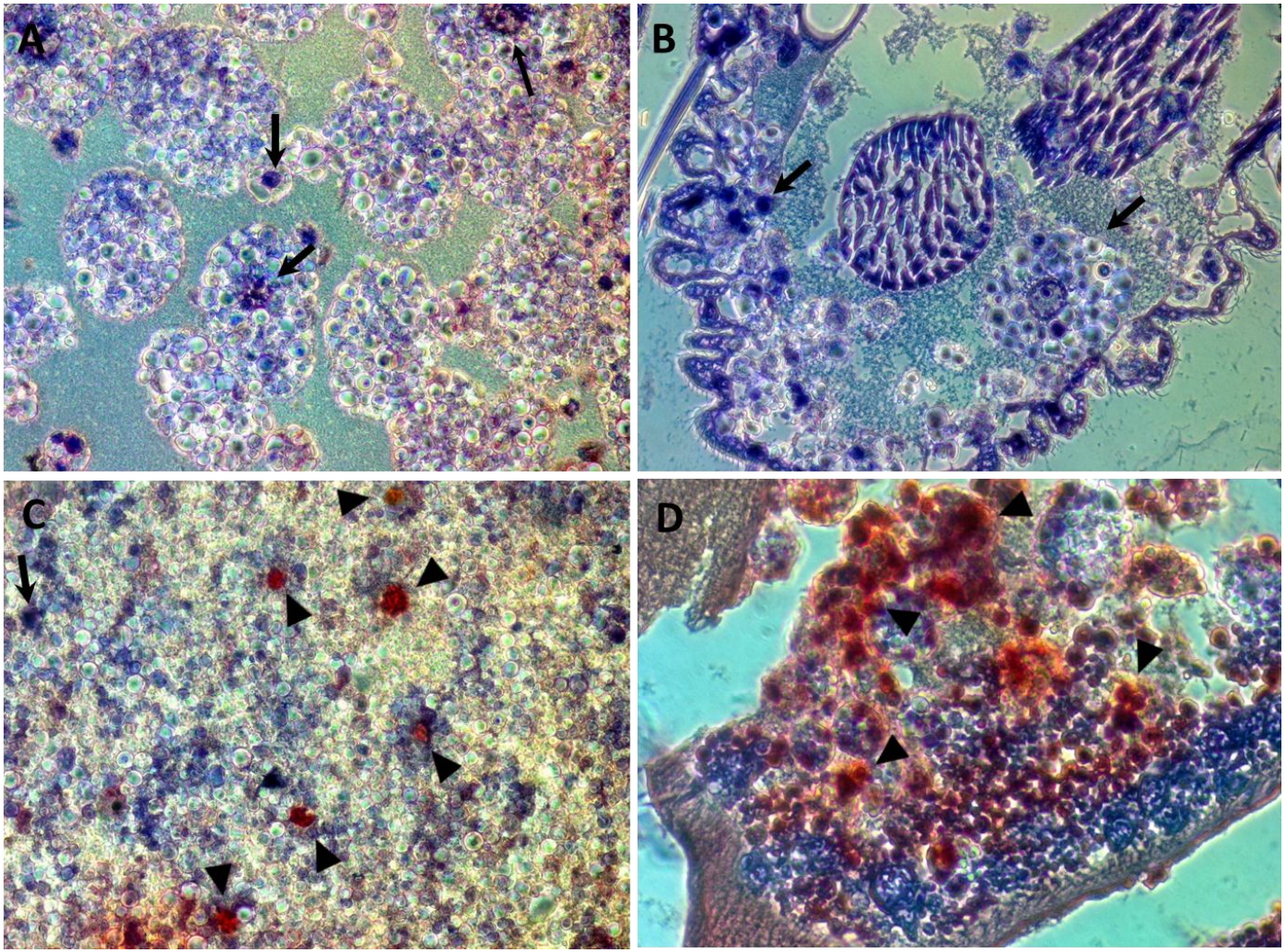

Figure 3.5: Localization of cell death in 7-day-old fly pupae ~ 50 h after parasitation in the presence of wasp larvae (C-D) and in control pupae of the same age (A-B) using the ISCDDK kit. Presence of reaction product in death cells is indicated with arrow heads, while the absence of the reaction product is indicated with a small arrow. **A**) Trophocytes in the abdomen of a control pupa. **B**) Detail of a leg in control pupa. **C**) Stained trophocytes in the abdomen of a parasitized pupa. **D**) Detail of a leg in a parasitized pupa. Magnification of all panels: 200 x, except for panel D, 400 x.

4. Discussion and future perspectives

Parasitoid wasp venoms are well known for their ability to subdue hosts, in order to maximize progeny development. Several studies on *N. vitripennis* were performed to gain better insights into the functionality of its venom (Rivers and Denlinger, 1995; Rivers *et al.,* 2005; Rivers and Brogan, 2008). Like many other parasitoids (Suzuki and Tanaka, 2006; Teramoto and Tanaka, 2004), the venom of *N. vitripennis* suppresses host immune responses by inducing cell death in hemocytes. Programmed cell death was also detected in the brain tissues of hosts, which may result in the developmental arrest (Rivers *et al.,* 1994; 2002; 2010; 2011). However, detailed information is lacking about the exact localization of cell death in the whole body of parasitized pupae and which other tissues are targeted. Programmed cell death is essential during development and maintenance of normal tissue turnover. In many pathological processes both apoptotic and necrotic cell death often occur simultaneously. Although necrosis can be caused upon tissue destruction, secondary necrosis is considered as the end stage of the complete apoptotic program (Silva, 2010). Both types of cell death display DNA fragmentation. However, only during apoptosis oligonucleosomal DNA cleavage occurs, resulting in the typical DNA ladder, while necrosis is characterized by the production of large random DNA fragments.

Cell lysis was observed in the abdomen of parasitized pupae 4 and 26 h after envenomation. Interestingly, an area of lysed cells with TUNEL positive nuclei was found in a pupa 26 h post parasitation, in the vicinity of an unknown, inserted structure. It is most likely that this structure is a remnant of the wound made by the ovipositor, as adult females feed on host hemolymph. The tube is constructed using the ovipositor and enables access to the hemolymph. Although areas of lysed cells contain positive nuclei using ISCDDK, this kit was previously shown to be more sensitive and detects both apoptotic and necrotic cells, compared to the ApopTag kit which is more specific to apoptotic cell death (Matylevitch et al.,1998; Orita *et al.,* 1999).

Melanization is a prominent immune response in insects and includes the activation of phenol oxidase (PO) which in turn is a key enzyme in melanin synthesis. This process is activated upon microbial infection, but also during wound healing and involves the aggregation of hemocytes which results in clotting (Lavine and Strand, 2002; Jiravanichpaisal

et al., 2006). The latter is accomplished by attracting hemocytes, subsequent degranulation and the establishment of extracellular aggregates (Tang, 2009; Nam *et al.,* 2012). Furthermore, melanization is readily manifested upon cuticular injury (Nappi *et al.,* 2005). Although venom of parasitoid wasps suppresses the immune system of their hosts, melanization does occur at the wound site upon envenomation by *N. vitripennis*, which indicates the injection place. In addition, parasitized pupae analyzed with both TUNEL kits at every time point, displayed several TUNEL positive nuclei at the peripheral sides of the abdomen, but only in restricted areas. It is most likely that these positive nuclei originate from hemocytes that aggregate around the wound site and are involved in immune responses. These cells probably underwent cell death as a response of the venom injection, since this area received the highest venom dose which subsequently diffuses further into the abdomen. The expansion of TUNEL positive nuclei, particularly hemocytes, in the thoracic regions of pupae 50 h post parasitation may denote the more advanced alterations of host physiology, including metabolism and the immune system. Furthermore, fly muscles also seemed to be affected upon envenomation. This suggests the occurrence of nutrient mobilization from peripheral tissues to the more internal fat body cells (trophocytes).

Several hemocytes near the outer edges of the legs of pupae with larvae were found to contain the red azo-dye. Parasitoid larvae were found near the legs, suggesting that this response is caused by the feeding behavior, as pupal legs are very fragile and provide an easy way to get access to the hemolymph. This observation is specific for parasitation, since no positive nuclei were found in the legs of control pupae which had the same treatment. Pupae without larvae, also displayed several positive nuclei in the legs, compared to control pupae. During removal of the parasitoid eggs, it is possible that some parts of the legs were damaged, resulting in cell death. Nevertheless, no extracellular diffused reaction product was observed around these legs.

Furthermore, the outer edges of the abdomen, displayed the red azo-dye reaction product which may indicate the location where clotting occurred in parasitized pupae. The reaction product was also found to be diffused between the abdominal cells using both TUNEL kits. This diffusion increased in the later stages (26 and 50 h) of parasitized pupae and may originate from excessive cellular degradation. One of the cell death types induced by *N. vitripennis* venom is oncosis/necrosis and involves cytolysis, which subsequently results in

releasing the cellular contents into the hemocoel (Rivers *et al.,* 1999; Rivers *et al.,* 2005). These cellular contents may contain DNA fragments, which in turn can be detected by TUNEL staining. However, when certain cell populations are targeted by the venom, numerous cells may undergo apoptosis which cannot be cleared by an affected immune system. Subsequently, cells may undergo secondary necrosis and release their cellular contents extracellular. These cellular contents may originate from apoptotic cells, since the ApopTag kit was also able to stain the extracellular environment at 26 h post parasitation. However, samples of the 50 h groups were only tested with ISCDDK and need to be analyzed with the Apoptag kit to confirm these observations.

Nasonia vitripennis is completely dependent on the lipids of its host and unable to synthesize lipids *de novo* (Rivero *et al.,* 2002, Visser *et al.,* 2012). Feeding behavior of the female wasps can influence the viability of these cells, however the venom itself is also known for its ability to raise host lipid levels in order to create an appropriate environment for the wasp larvae (Rivers *et al.,* 1994; 1995). Lipid levels in posteriorly-parasitized flies were found to be elevated the most (Rivers and Yoder, 1996). During metamorphosis of higher Diptera, the fat body undergoes a major transformation resulting in the redistribution of individual fat body cells or trophocytes throughout the whole body of the pupa (Nelliot *et al.,* 2006). At the time of parasitation, these pupae were already 5 days old and comprise mainly of individual trophocytes, containing lipids, proteins and sugars. These nutrient stores are essential for the developing pupa in order to complete metamorphosis, which may explain the preference for pupal hosts by *N. vitripennis*. Although, no cell death was detected in fat body cells in the head and anterior thoracic regions of *S. bullata* upon parasitation with *N. vitripennis*, until now, information was lacking about how tissues in the abdomen and thorax are affected (Rivers *et al.,* 2011). In this study, cell death was observed in trophocytes located in the abdomen. In all parasitized groups, trophocytes in the peripheral regions and some cells inside the abdomen were affected upon envenomation and contained TUNEL positive nuclei. These trophocytes and other cell types possibly underwent cell death as a result of the high venom dose present near the injection spot. This suggests that the venom induces cell death which subsequently can liberate the accumulated lipids in the abdomen, near the surface were they can be absorbed by the adults or larvae. The fact that not all trophocytes undergo cell death after 50 h, even in the

presence of the parasitoid larvae, may imply that this process is tightly regulated in order to synchronize with the nutritional needs of the parasitoid larvae. Indeed, parasitation of *S. bullata* by *N. vitripennis* resulted in high hemolymph and fat body lipid levels, but only after 3 days and before this period, lipid contents remained constant (Rivers *et al.,* 1994; 1995). This phenomenon is not only observed upon *N. vitripennis* parasitation, other parasitoid wasps such as *Cotesia kariyai* and *Meteorus pulchricornis* are able to liberate the accumulated lipids in their hosts by inducing cell death (Nakamatsu *et al.,* 2007; Suzuki and Tanaka, 2006). Hydrolases and lipases identified in *N. vitripennis* venom are predicted to be involved in fat body digestion and in addition, other proteases were also found to be cytotoxic for insect cells and can play a role in the induction of cell death in trophocytes (Danneels *et al.,* 2010; Formesyn *et al.,* 2013).

Although, cell death was observed in brain tissues of parasitized *S. bullata*, no substantial levels of cell death are observed in brain tissues of pupae analyzed at several time points. At first sight, gonadal tissues and Malpighian tubules also seemed to be unaffected by the venom in most of the pupae at all sampling times. However, some pupae displayed cell death in these tissues, especially in pupae with larvae 50 h post parasitation. Since we cannot exactly determine the injected amount of venom it seems reasonable to assume that some pupae received a larger venom dose than others. It is also possible that the presence of the feeding parasitoid larvae (e.g. larval secretions) accelerates the progress and spreading of cell death in host tissues. However more sections need to be screened to make clear conclusions, as some tissues such as the brains were not always present in the sections of all pupae, due to the orientation of the histological sections. In addition, sections of both parasitized groups 50 h post parasitation, need to be analyzed with the ApopTag kit to confirm the presence of apoptotic nuclei.

5. Conclusions

This study shows that parasitation of host pupae by *N. vitripennis* causes cell death such as apoptosis in the peripheral tissues of the abdomen, 4 and 26 hours after parasitation. Mostly trophocytes and hemocytes were targeted by the venom, which does not induce a wide spread response concerning apoptosis after the first 26 hours. This observation may be linked with the metabolic changes and the nutrient mobilization from peripheral tissues to

the more internal trophocytes, in order to maximize nutrient availability for the developing larvae. When examining pupae 50 hours after parasitation, cell death in the tissues starts to spread to the thorax, including the flight muscles. Pupae that contain the parasasitoid larvae displayed much more cell death in the legs after 50 h, compared to pupae without the parasitoid larvae. Overall, more sections need to be analyzed with both kits in order to test for the occurrence of cell death in the head.

Acknowledgements

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Chapter 4

The role of serine- and metalloproteases in *Nasonia vitripennis* venom in cell death related processes towards a *Spodoptera frugiperda* Sf21 cell line

Contributions

D. de Graaf and K. Heyninck assisted with the study design. E. Formesyn was responsible for the venom dessections, the insect and Sf21 cultures and execution of all experiments and data analyses. K. Heyninck provided technical assistance for the flow cytometer experiments. E. Formesyn wrote the manuscript.

This chapter is redrafted from:

Formesyn, E.M., Heyninck, K., de Graaf, D., 2013. The role of serine- and metalloproteases in *Nasonia vitripennis* venom in cell death related processes towards a *Spodoptera frugiperda* Sf21 cell line. Journal of Insect Physiology 59, 795–803.

1. Introduction

Parasitoid wasps are known to subdue their hosts by venom injection, enabling successful progeny development which eventually ends in killing the host. The ectoparasitoid wasp *Nasonia vitripennis* parasitizes pupae and pharate adults belonging to the Sarcophagidae and Calliphoridae families. The venom of *N. vitripennis* females is able to elicit several responses in the host. The most profound consequence for an envenomated host is the appearance of a developmental arrest which might be the result of several metabolic changes combined with apoptosis mediated cell death (Rivers and Denlinger, 1995, 1994; Rivers *et al.,* 1999, 2006). Furthermore, the venom also plays a major role in suppression of host innate immunity by inhibition of hemolymph prophenoloxidase activity and encapsulation processes. The latter is affected by targeting granulocytes and plasmatocytes during parasitation of *Sarcophaga bullata* pupae (Rivers *et al.,* 2002). The same responses were observed during parasitation of *Pseudaletia separata* by *Meteorus pulchricornis* (Suzuki *et al.,* 2008) and *Galleria mellonella* exposed to *Pimpla turionellae* (Er *et al.,* 2010, 2011). In many parasitoid-host relationships, the total number of hemocytes declined due to cell death, particularly by apoptosis, a process that is largely tolerogenic (Suzuki and Tanaka, 2006; Teramoto and Tanaka, 2004). This type of programmed cell death was also found to be one of the dominant mechanisms in BTI-TN-5B1 cells (Lepidoptera: Noctuidae) exposed to venom of *N. vitripennis* (Rivers *et al.,* 2010).

Venoms of parasitoid wasps have been extensively studied in the past, in order to unravel the venom composition and their functionality in parasitoid-host interactions (Formesyn *et al.,* 2011). Parasitoid venoms are known for their complex composition and may be accompanied with additional components like calyx fluid, polydnaviruses, virus-like particles and teratocytes to assure the successful development of the progeny (Asgari, 2010). Most of the attempts to unravel the venom composition of parasitoid wasps, were directed towards endoparasitoids. Previous analyses revealed the proteinacious character of *N. vitripennis* venom, which is known to lack those additional components such as polydnaviruses and virus-like particles (Rivers *et al.,* 2006). Nevertheless, *N. vitripennis* is the only ectoparasitoid wasp whose complete genome and venom composition are known (Werren *et al.,* 2010). The screening of its venom resulted in the identification of 79 venom proteins, of which proteases were the predominant venom components (de Graaf *et al.,* 2010). In addition,

another study identified a defensin protein in *N. vitripennis* venom, which brings the total count on 80 identified venom proteins (Ye et al., 2010). Sixteen serine proteases (± 20% of the protein ID's) and two metalloproteases were discovered in *N. vitripennis* venom. Members of these protease protein families were also identified in *Pteromalus puparum* (Zhu *et al.,* 2010), *Chelonus inanitus* (Vincent *et al.,* 2010) and *Pimpla hypochondriaca* (Parkinson *et al.,* 2002a, 2002b). In *Cotesia rubecula* a serine protease homolog venom protein was found to inhibit melanization in its host *Pieris rapae* (Asgari *et al.,* 2003). A reprolysin-like metalloprotease in *Eulophus pennicornis* venom showed toxic activity towards *Lacanobia oleracae* (Price *et al.,* 2009). These protease families in *N. vitripennis* venom may also play a profound role in the cytotoxic effects observed after envenomation.

In this study, the *Spodoptera frugiperda* 21 cell line (Lepidoptera: Noctuidae) was used for *in vitro* MTT assays, analyzing cytotoxicity induced by *N. vitripennis* venom. Previous research revealed the susceptibility of the Sf21 cell line to this venom (Rivers *et al.,* 1999). Although not all Lepidopteran species tested so far are equally susceptible, they are considered as a suitable model for wasp venom research (Rivers and Denlinger, 1995; Rivers *et al.,* 1999, 1993, 2005; Zhang *et al.,* 2006). The involvement of both serine-and metalloprotease protein families present in the venom towards cytotoxicity was determined in adherent and suspension cells by treating venom with protease inhibitors. The type of cell death caused by complete venom or venom treated with protease inhibitors was characterized using flow cytometry, which enables the distinction between early, late apoptosis and necrotic cell death combined with DNA fragmentation assays.

2. Materials and methods

2.1. Chemicals

Insect saline solution (ISB, 150mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid, pH 7) was prepared in distilled water (dH_2O). AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (Pefabloc),Roche), an irreversible serine protease inhibitor, was dissolved in ISB at a final concentration of 100 mM. Complete protease inhibitor tablets (Roche), an inhibitor for serine, cysteine and metalloproteases, were dissolved in dH_2O in order to obtain a 25 × stock solution (9.25 mg/ml). Ethylene glycol tetra acetic acid 99% (EGTA), a metalloproteases inhibitor (Acros

Organics) was dissolved in dH_2O to obtain a final concentration of 100 mM (pH 7.5). Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich USA) was dissolved in ISB to obtain a concentration of 5 mg/ml. Hank's buffered salt solution (HBSS, 5 mM D-(+)-Glucose monohydrate, 5.4 mM KCl, 0.137 mM NaCl, 0.44 mM KH_2PO_4, 0.338 mM Na_2HPO_4, 4.16 mM $NaHCO_3$, pH 7.2) was prepared in dH_2O. All solutions were sterilized using a 0.22 µm filter. The Pierce® BCA Protein Assay Kit was purchased at Thermo Scientific.

2.2. Insect cell line and cultures

Spodoptera frugiperda 21 cells (Sf21, Invitrogen) were cultured as adherent monolayers in 25 cm^2 flasks at 28 °C in BacVector® Insect Cell Medium (Novagen), supplemented with or without 10% fetal bovine serum (FBS, Biochrom AG). This cell line originates from immature ovaries of *S. frugiperda* pupae and was established by Vaughn et al. (1977). Suspension cultures of Sf21 (20 ml) were maintained in BacVector® Insect Cell Medium using Erlenmeyer flasks, supplemented with 10% fetal bovine serum (FBS), under continuous shaking at 100 rpm and 28 °C. All insect cell cultures were supplemented with 45 U/ml Penicillin and 0.045 mg/ml Streptomycin in 0.9% NaCl (Sigma-Aldrich USA).

2.3. Insect rearing

N.vitripennis Asym C, kindly provided by Prof. Dr. L. W. Beukeboom (Evolutionary Genetics, Centre for Ecological and Evolutionary Studies in The Netherlands), were reared on pupae of the flesh fly *Sarcophaga crassipalpis* under a light-dark cycle (LD 15:9 h) at 25 °C (Van den Assem and Jachmann, 1999). Flesh flies (*S. crassipalpis*) were provided by Dr. Hahn, University of Florida, and cultured in the laboratory as described by (Denlinger *et al.,* 1972a).

2.4. Venom collection and protein estimation

Crude venom from *N. vitripennis* was collected according to Formesyn et al. (2011). Briefly, *N. vitripennis* females (age: 2-3 days) were allowed to feed on a sugar solution (10%) for 24 hours at 25 °C under a long-day cycle (LD 15:9 h). Subsequently venom reservoirs were isolated by dissection at ISB and centrifuged to liberate the venom from the reservoirs. All venom samples were stored in -80 °C until further use. Before use, venom samples were pooled and the protein concentration of crude venom samples was estimated with the BCA assay using a BSA standard (Pierce).

2.5. Venom treatments

To test the protease families, venom was partially inactivated by pretreatment with protease inhibitors. Venom samples were independently treated with AEBSF (15 mM), EGTA (20 mM) and Complete protease inhibitor mix (5 × conc.) and incubated for 2 h at 28 °C. Furthermore, venom was treated to denaturate and inactivate venom proteins by heating the samples for 1 h at 100 °C, while other venom samples were depleted of larger proteins by filtering using a 10.000 MWCO filter (Sartorius Stedim Biotech). Subsequently, the filtered venom samples were examined on SDS-PAGE gels (15%) and stained with Coomassie Brilliant Blue R-250 to confirm depletion.

2.6. Viability assay with MTT

Effect of venom on the viability of Sf21 cells was determined by MTT bioassays. In this assay yellow tetrazolium bromide (MTT) is used as a substrate and is reduced into purple formazan by mitochondrial succinate-dehydrogenase in viable cells only. Adherent Sf21 cells (4×10^5 cells/ml) were seeded into 96-well plates (100 µl/well). After 24 h, spent medium was replaced by fresh medium and cells were incubated for another 24 h. Subsequently, cells were treated with venom in serial dilutions (0.31, 0.62, 1.25, 2.5, 5, 10 and 25[1] µg/ml) for 6, 12, 24, 36 and 48 h in order to determine the inhibitory concentration inducing 50% loss of cell viability (IC_{50}). Furthermore, adherent Sf21 cells were exposed to venom (10 µg/ml) that was pretreated with protease inhibitors (see 2.5). Additionally venom proteins (25 µg/ml) were inactivated by heating or depleted by filtration. All adherent cultures were incubated for 24 h at 28 °C. In order to test whether protease inhibitors were acting directly on the venom proteins and not the Sf21 cells and the influence of FBS, a supplementary MTT assay was performed using adherent cells with or without 10% FBS. Here, cells were immediately treated with venom and protease inhibitors without any pre-incubation period. Alternatively, suspension Sf21 cells (5×10^5 cells/ml to $1x10^6$ cells/ml) were seeded in 125 ml Erlenmeyer flasks (Corning) containing 20 ml cells and cultured for 24 h at 28 °C and continuous shaking at 100 rpm. Next, the Sf21 cell cultures were induced with venom (2.5 µg/ml), venom + protease inhibitor or ISB. In all experiments, ISB treated cells were included as the control group. After 24 h, samples were taken and diluted (1/5) with medium in 96-

[1] A concentration of 25 mg/ml venom was only used for the 24h measuring point.

well plates. Subsequently, 20 µl of Thiazolyl Blue Tetrazolium Bromide (MTT) solution (5 mg/ml) was added to the wells and cells were incubated for 5h. To solubilize the precipitate, 80 µl SDS/HCl (10% SDS, 0.01 M HCl) solution was added to each well and incubated over night at room temperature. Absorbances were measured at 595 nm with the Victor 3^{TM} 1420 Multilabel Counter plate reader (Perkin Elmer). Relative viability (%) was calculated as the ratio of MTT reduction between treated cells and control cells. All MTT tests were performed in triplicate and were repeated at least three times.

2.7. Flow cytometry

Sf21 suspension cells (5×10^5 cells/ml to $1x10^6$ cells/ml) were seeded in 125 ml Erlenmeyer flasks and cultured in 20 ml BacVector® Insect Cell Medium with 10% FBS. Twenty four hours later, cells were treated with a low venom concentration (0.625 µg/ml) and samples containing approximately 1×10^6 cells/ml were analyzed at 1, 4, 6 and 24 h.

Sf21 cells treated with a high concentration of venom (2.5 µg/ml), venom + protease inhibitor, ISB, or non-treated cells were analyzed after an incubation period of 24h. For time course experiments with AEBSF treated venom, Sf21 cells were induced with a venom concentration of 1.25 µg/ml and analyzed at several time points (1, 3, 6 and 24 h). The same concentrations of protease inhibitors were used as for the MTT assays (see 2.4.). Cells were collected by centrifugation at 130 × g for 2 min and washed once with ice-cold HBSS/2% FBS. Cells were stained according to the CELL LAB ApoScreen™ Annexin V-Fluorescein isothiocyanate (FITC) Apoptosis protocol (Beckmann Coulter). The kit relies on the principle that early apoptotic cells loses their membrane phospholipid asymmetry, caused by translocation of membrane phosphatidylserine (PS) to the cell surface. Annexin V-FITC is a protein used to detect PS-containing phospholipid bilayers. In conjunction with propidium iodide (PI), which accumulates in the cells when membrane integrity is lost, several subpopulations can be evaluated. Viables (AN-/PI-; lower left), early apoptosis (An+/PI-; lower right), late apoptosis or secondary necrosis (AN+/PI+; upper right) and necrosis (AN-/PI+; upper left) were defined and presented in density plots. Briefly, cells were resuspended to provide a concentration of 1×10^6 to 1×10^7 cells/ml in cold Annexin V Binding Buffer supplemented with 2% FBS. Cells (100µl) were stained with 0.7 µl Annexin V-FITC and incubated on ice for 5 min, protected from light. Subsequently, 380 µl of cold Annexin V

Binding Buffer/2% FBS and 5 µl PI (50 µg/ml) was added and cells were immediately processed in a Cell Lab Quanta SC MPL flow cytometer with an 488 nm laser (Beckman Coulter). FITC fluorescence (Fl-1) was measured by a 525 nm band pass filter, and PI fluorescence (Fl-3) by a 670 nm long pass filter. Approximately 1×10^4 cells were counted in each assay and analyzed with the Cell Lab Quanta SC MPL Analysis software (Beckman Coulter). All FACS experiments were repeated at least three times.

2.8. DNA fragmentation assay

The DNA fragmentation assay was used to detect apoptosis which results in a DNA ladder with bands separated by approximately 180 base pairs. Briefly, suspension cells were grown in cultures (50 ml) as described above and received a treatment with venom (1.25 µg/ml), venom + AEBSF (15 mM) or ISB. After 3, 5 and 24 h, cells (10 ml) were collected by centrifugation at 900 rpm and the supernatant (the venom solution that retains on the filter) thwas centrifuged again at 5000 rpm to collect the apoptotic bodies. Samples were stored at -80 °C until further use. Genomic DNA (gDNA) was extracted following the instructions of the Invisorb® Spin Tissue Mini Kit (Stratec Molecular GmbH, Germany), followed by an ethanol precipitation. DNA samples (5 µg) were run on a 0.8% agarose gel at 100 V and stained with ethidium bromide.

2.9. Statistical analyses

Respective IC_{50}'s with corresponding 95% confidence limits were calculated using nonlinear regression analysis and the accuracy of data fitting the sigmoid curve model was evaluated with R^2 values. Data from all experiments were presented as percentages (mean ± SEM). Data from flow cytometry experiments was arcsine transformed prior to analysis. Statistical significance between groups was evaluated using analysis of variance (ANOVA) followed by Student-Newman-Keul's (SNK) multiple comparisons tests. When data did not met the assumptions for ANOVA, Kruskal Wallis was used, combined with Dunn's post tests. Comparisons within the same treatment during time course experiments were analysed using one-and two-way repeated measures ANOVA followed by a Tuckey post test. A value of $p < 0.05$ was considered statistically significant. All statistical analysis were performed with Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. Effect of venom on the viability of Sf21 cells

Cytotoxic effects of *N. vitripennis* venom on the adherent Sf21 cell line was estimated with serial dilutions at different times. Results showed a dose and time dependent inhibition of cell viability at increasing concentrations, as shown in Fig. 4.1, which demonstrate the high cytotoxicity of *N. vitripennis* venom towards this non-host cell line. Generally, cytotoxicity of the venom was maximal at 48 h. Results indicated that after 24 h induction, significant cytotoxic effects of the venom became visible at all concentrations, leading to a sigmoid curve with an IC_{50} of 4.011 µg/ml (3.501-4.595, 95% CI), which differed slightly compared to 36 h (Table 4.1). Suspension cells treated with pure venom displayed the same pattern at 24 h (Fig. S4.1; supplemental materials can be obtained by contacting Prof. de Graaf). For this reason an induction time of 24 h was used for further experiments.

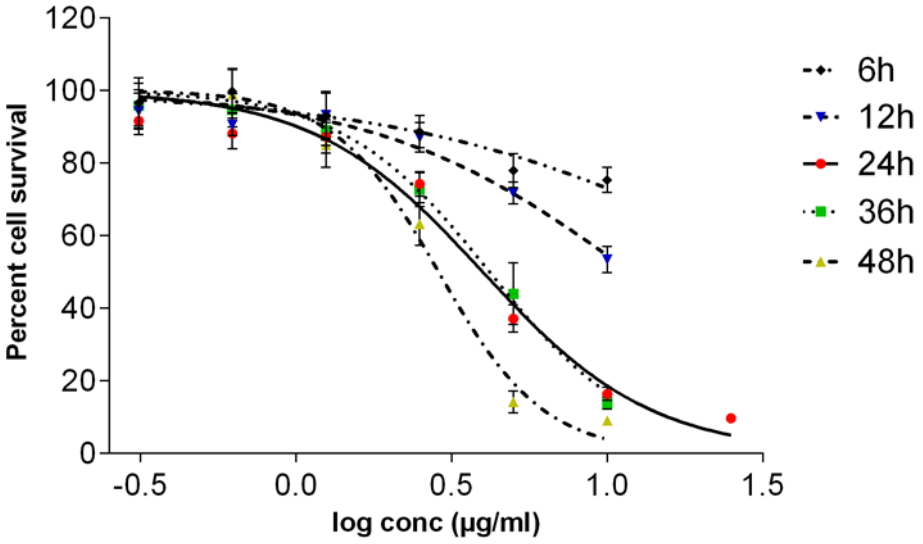

Figure 4.1: Dose-response curves after venom induction in adherent *Spodoptera frugiperda* 21 cells for cell viability in MTT assays (IC50) during 6, 12, 24, 36 and 48 h. Data are presented as viable cells (% of negative control, ISB) and given as mean response values together with the 95% confidence interval after sigmoid curve fitting in Prism v5.

Table 4.1: Time dependent tendencies of *N. Vitripennis* venom toxic activity on the viability of Sf21 cells in MTT assays when venom concentrations of 0.31, 0.62, 1.25, 2.5, 5, 10 and 25 µg/ml are used.

Time (h)	IC_{50} (µg/ml)	95% CI	R^2
24	4,01	3,501-4,595	0,97
36	4,25	3,806-4,744	0,88
48	2,85	2,337-3,474	0,95

IC50 values are given with corresponding 95% confidence intervals (CI) and R2 values.

3.2. The protein fraction in the venom is cytotoxic

To identify the cytotoxic compounds in the venom, venom proteins were inactivated by heating at 100 °C or filtered through a 10.000 MWCO filter. Both treatments increased cell survival after 24 h compared to venom treated cells (Fig. 4.2A). Filtered venom analyzed with SDS-PAGE, revealed no protein bands in the filtrate (Fig. S4.2).

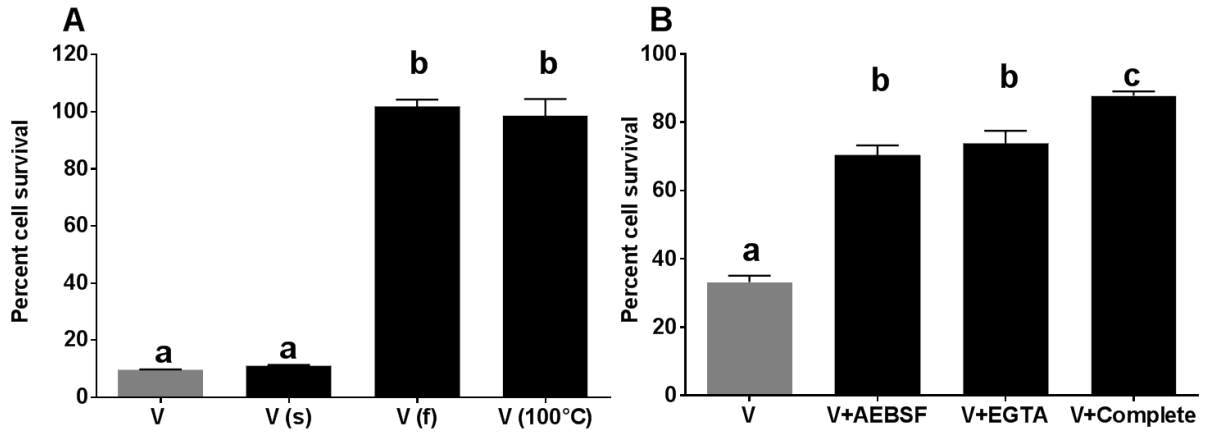

Figure 4.2: A) Viability assay on adherent Sf21 after venom degradation (V 100 °C) or filtering the venom V (f). 25 µg/ml venom (V) and the supernatant V(s) were included as positive controls. **B**) Viability assay of Sf21 after treatment of the venom (10 µg/ml, positive control) with AEBSF (V+AEBSF), EGTA (V+EGTA) and Complete (V+Complete). Viability was measured after 24 h. Letters indicate the significance between the different treatments. Data are presented as viable cells (% of negative control, ISB).

3.3. Serine- and metalloproteases are cytotoxic for adherent Sf21 cells

In order to determine which proteases in the venom are responsible for the cytotoxic effects in Sf21 cells, venom was treated with protease inhibitors using concentrations that are not cytotoxic for the cells (Fig. S4.3A, B). Venom samples treated with all tested protease inhibitors, were less cytotoxic towards the adherent Sf21 cell line, as shown in Fig. 4.2B. Inhibition of serine proteases or metalloproteases resulted in a twofold increased cell viability of respectively 70.35 ± 2.87% (mean ± SEM; $p < 0.0001$) with AEBSF, and 73.79 ± 4.27% (EGTA; $p < 0.0001$), compared to untreated venom samples [33.15 ± 2.53%]. However an inhibitory effect on cell viability of approximately 29.64 ± 2.87% and 25.33 ± 4.94% was still visible in the insect cells. Venom samples treated with Complete which inhibits both serine- and metalloproteases, was able to rescue the majority of the cells and resulted in a cell viability of 87.76 ± 1.23% ($p < 0.0001$). The same effect was observed when serine proteases were inhibited in suspension cells. However, inhibition of metalloproteases did not result in an increased cell viability at all (Fig. S4.4). When venom and protease inhibitors

were added directly to adherent cells, cytotoxicity of all treated venom samples did not differ significantly compared to untreated venom (Fig. S4.5).

3.4. *N. vitripennis* venom induces apoptosis

Sf21 suspension cells analyzed with flow cytometry for apoptosis detection demonstrated that approximately 10% of the cell population is early apoptotic when exposed to low venom concentrations (0.625 µg/ml). Early apoptotic cells were observed at each time point (1, 4, 6 and 24 h) and their number remained constant (Fig. 4.2A-F). The transition to late apoptosis or secondary necrosis was already observed after 1 h when treated with 0.625 µg/ml venom [30.86 ± 2.89%; $p < 0.0001$]. Furthermore, a small subset of Sf21 cells also underwent necrosis when treated with venom (Fig. 4.2F and 4.4D). Higher venom concentrations (2.5 µg/ml) only increased the number of late apoptotic or secondary necrotic cells in comparison to the control group (Fig. 4.3A-B).

3.5. Inhibition of serine proteases, but not metalloproteases decreases cytotoxic activity of the venom in suspension Sf21 cells

Inhibition of serine proteases in the venom (2.5 µg/ml) enhanced the viability of the suspension cells from 46.53 ± 8.09% (untreated venom) to 70.30 ± 2.83%, $p < 0.05$ (treated venom) when analyzed with flow cytometry after 24h. Furthermore, as shown in Fig. 4.3, cells treated with venom devoid of active serine proteases had much lower amounts of double positive cells [9.69 ± 3.49%; $p < 0.05$] compared to cells induced with fully active venom [38.68 ± 17.92%]. Inhibition of metalloproteases was not able to prevent cell death in suspension cells compared to venom treated samples and resulted in a viability of 43.33 ± 11.91% ($p > 0.05$), while 40.88 ± 13.67% ($p > 0.05$) of the cells were undergoing late apoptosis or secondary necrosis. Furthermore, no clear differences could be observed concerning early apoptotic cell populations between the different treatments (venom, venom + protease inhibitor, ISB) (Fig. 4.3A-D).

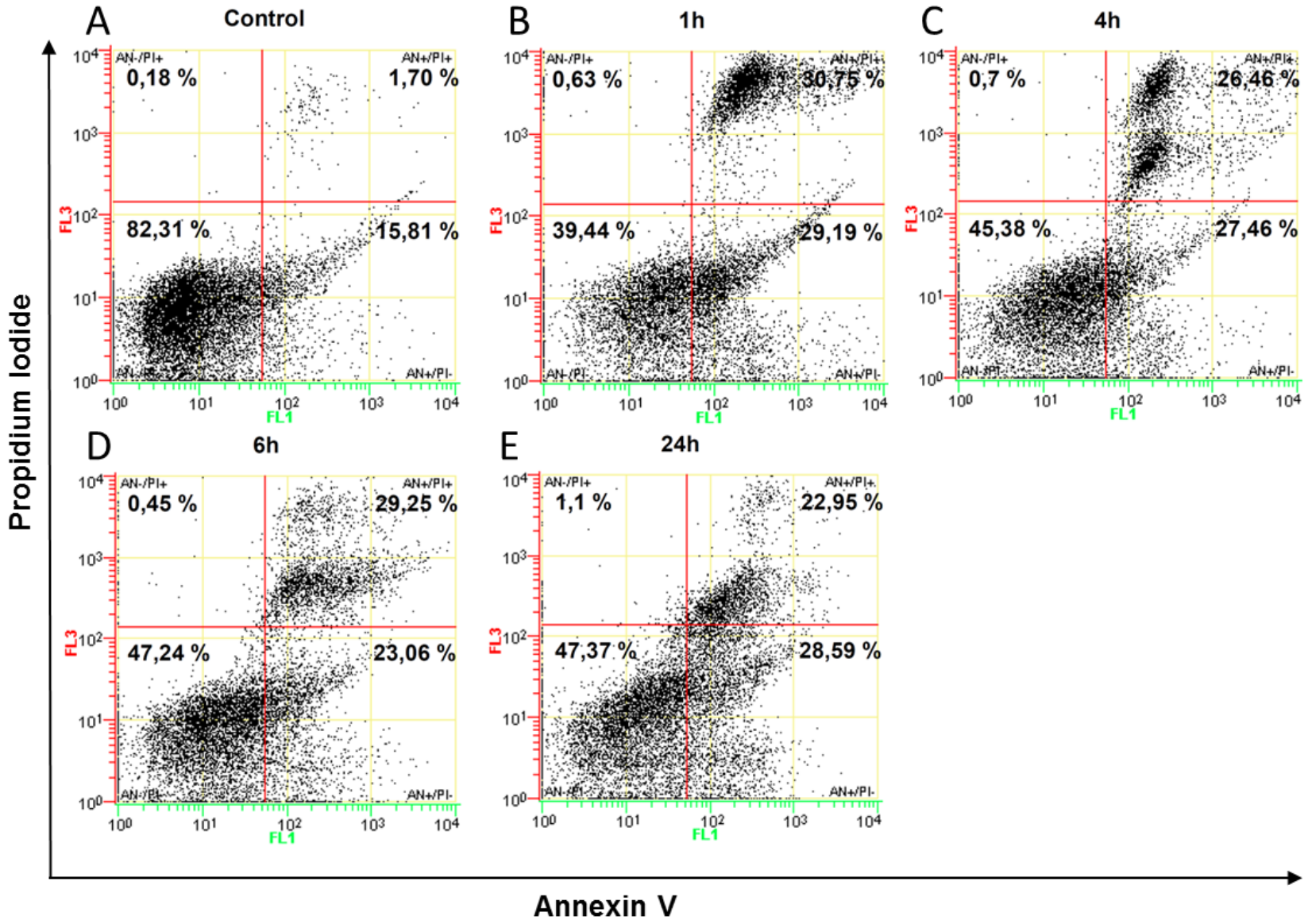

Figure 4.2. Legend, see next page.

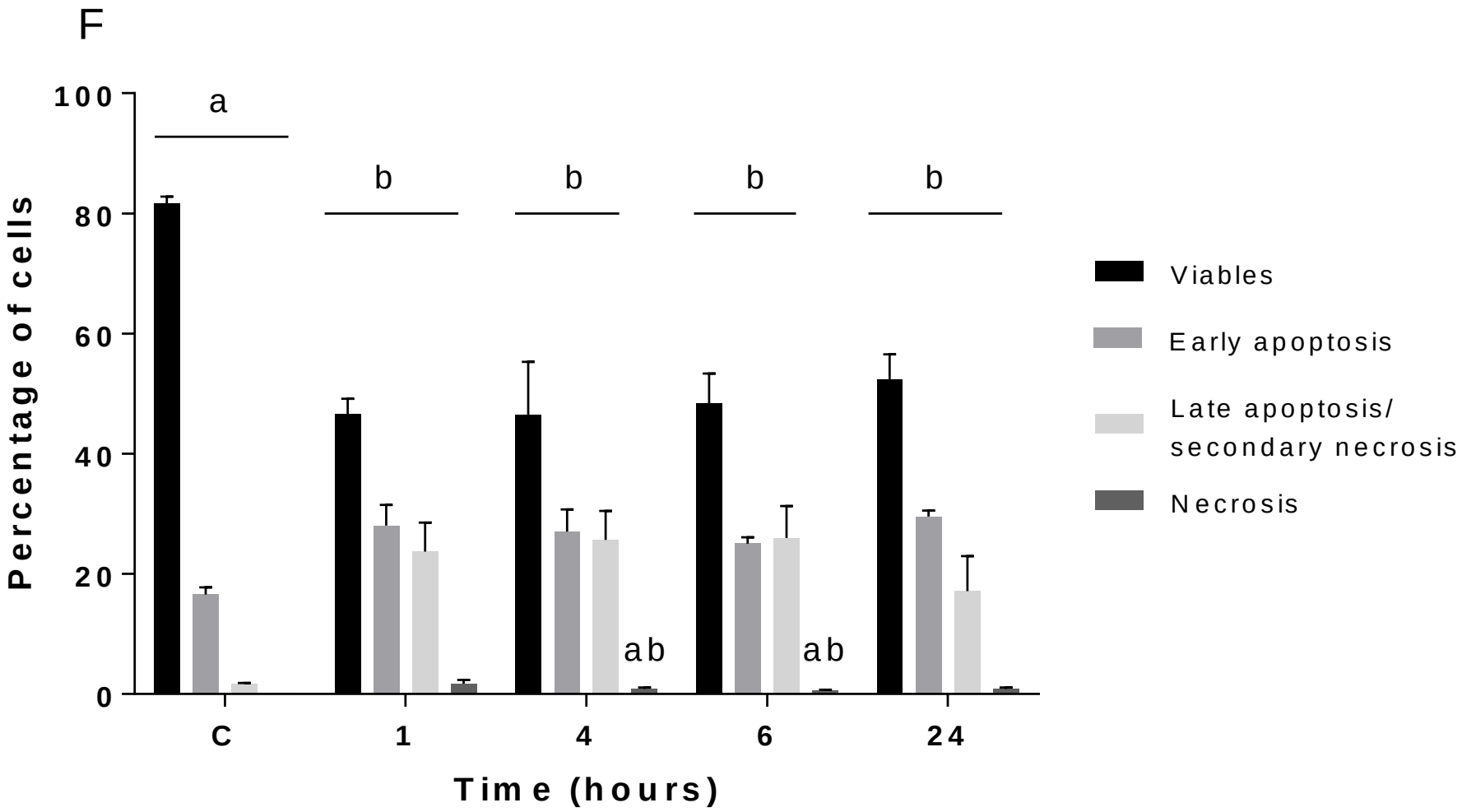

Figure 4.2: Annexin V-FITC (FL1) and PI (FL3) staining assay. Sf21 cells were treated with 0.625 µg/ml venom and examined during 1, 4, 6 and 24 h (**A**-**E**). **F**) Bar graph showing the proportion of Sf21 cells that were viable, early apoptotic, late apoptotic/secondary necrotic or necrotic after venom treatment during 1, 4, 6 and 24 h, compared to control values (c). Results are presented as the mean ± SEM of at least three independent experiments. Significance between the same cell populations over several time points are indicated with different letters.

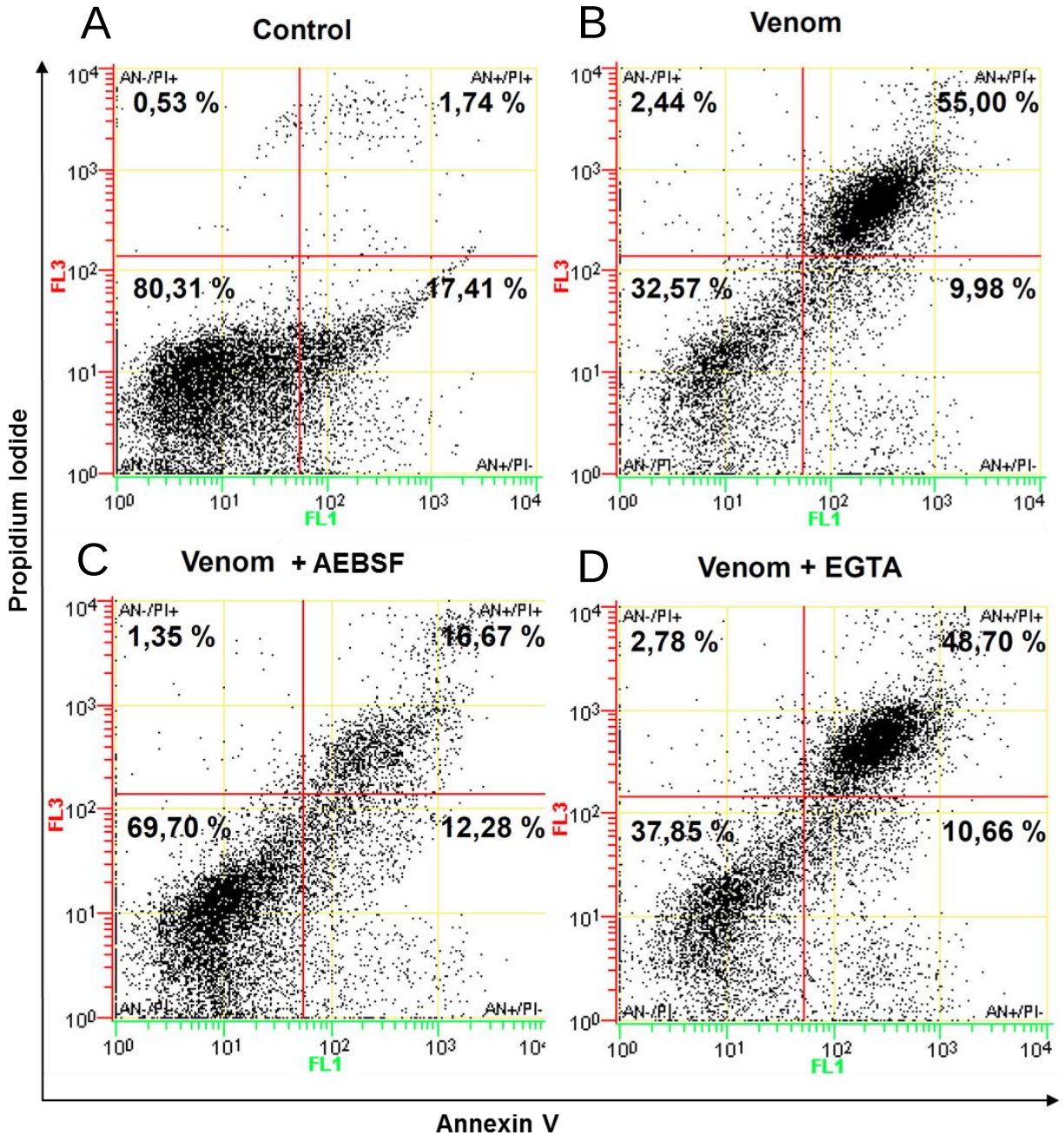

Figure 4.3: Annexin V-FITC (FL1) and PI (FL3) staining assay. Sf21 cells were treated with ISB (**A**), 2.5 µg/ml venom (**B**), 2.5 µg/ml venom + AEBSF (**C**) and 2.5 µg/ml venom + EGTA (**D**). The percentage of apoptotic cells was determined by Annexin V-FITC (FL1) and PI (FL3) staining. Dot-plot graphs show viable cells (AN−/PI−), early apoptotic cells (AN+/PI−), late apoptotic and secondary necrotic cells (AN+/PI+), and necrotic cells (AN−/PI+). Data are representatives of three independent experiments with similar results.

3.6. Serine proteases delay the onset of Sf21 cells to late apoptosis/necrosis

To investigate the involvement of venomous serine proteases in apoptosis during the transition to late apoptosis/secondary necrosis or necrosis, Sf21 cells were examined with venom (1.25 µg/ml) or venom without active serine proteases for different time points. Flow

cytometry assays revealed that inhibition of serine proteases in the venom lead to a higher viability of Sf21 compared to untreated venom during the first 6 h. However, the differences between the treatments became smaller at 24 h and the percentage of viable cells declined at 24 h, even with venom devoid of serine proteases (Fig. 4.4A-D). Inhibition of serine proteases in the venom could not prevent the onset of early apoptosis in a small part of the Sf21 culture, which did not differ compared to untreated venom. However, during the first 6h considerably more cells stained with both Annexin V-FITC and PI when they were treated with venom alone. After 24 h, the differences in both viable and late apoptotic or secondary necrotic cells declined between venom and AEBSF treated venom. Cultures treated with venom and AEBSF also had a lower percentage of necrotic cells compared to normal venom treatment.

3.7. Venom induced DNA fragmentation

To confirm that the previous observations of venom induced cytotoxicity in Sf21 cells were caused by apoptosis, gDNA of Sf21 cells was extracted and separated on agarose gels. As shown in Fig. 4.5, Sf21 cells exposed to low venom concentrations (1.25 µg/ml), demonstrate a clear 'ladder' pattern, which is the result of an activated endogenous endonuclease. However, this DNA fragmentation was only visible during the first 5 h after venom induction and could not be detected after 24 h. Furthermore, cells treated with venom pretreated with AEBSF, did not display any DNA fragmentation at the observed sampling points.

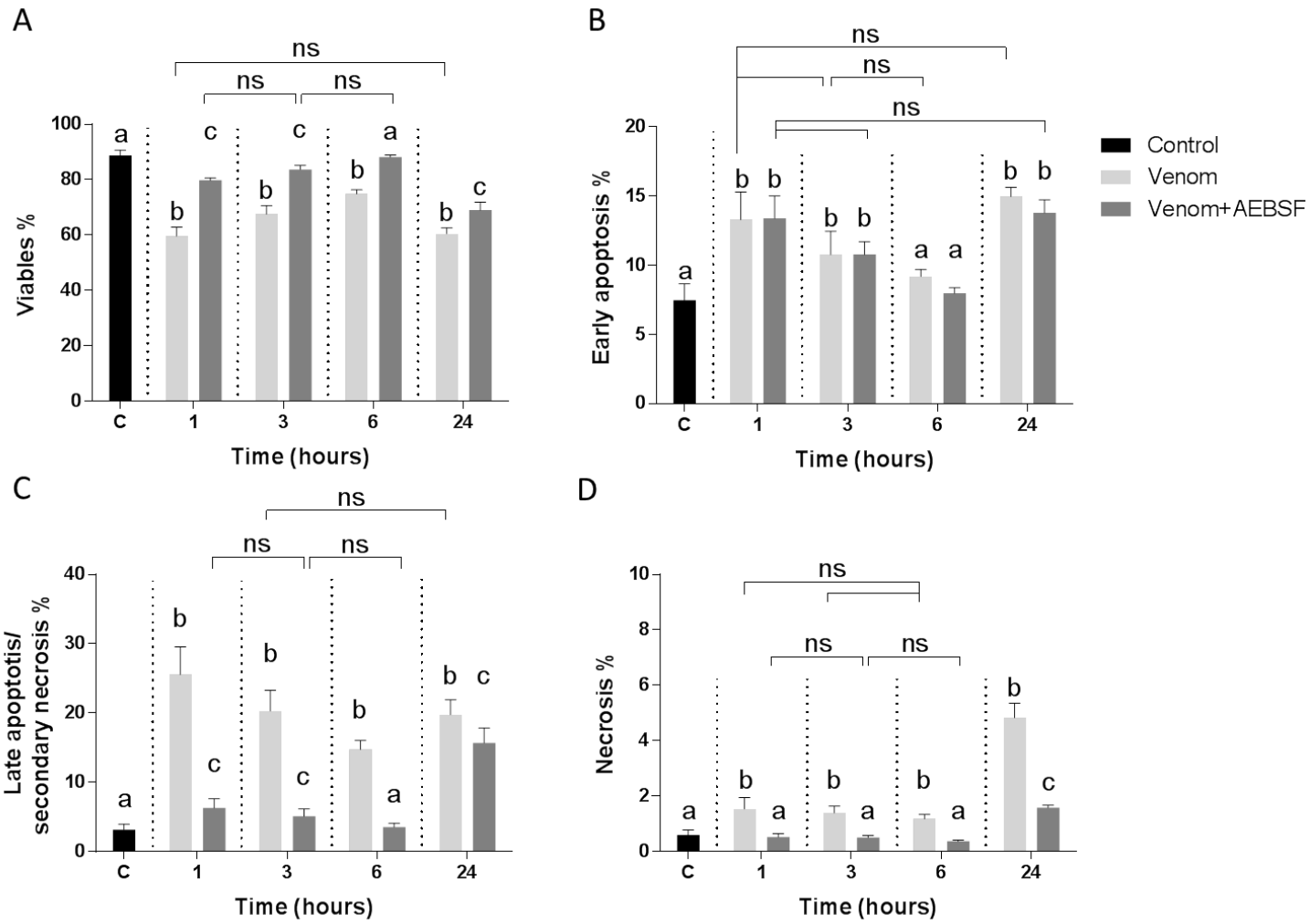

Figure 4.4: Bar graphs showing the four different cell populations of Sf21 cells during annexin V-FITC/PI staining after treatment with 1.25 µg/ml venom and AEBSF treated venom at 1, 3, 6 and 24h (A-D). Significance of different treatments are presented per time point in response to the control value (c). Brackets present the comparisons that were non-significant (ns) between the different time points, letters indicate the significance between the same cell populations over several time points.

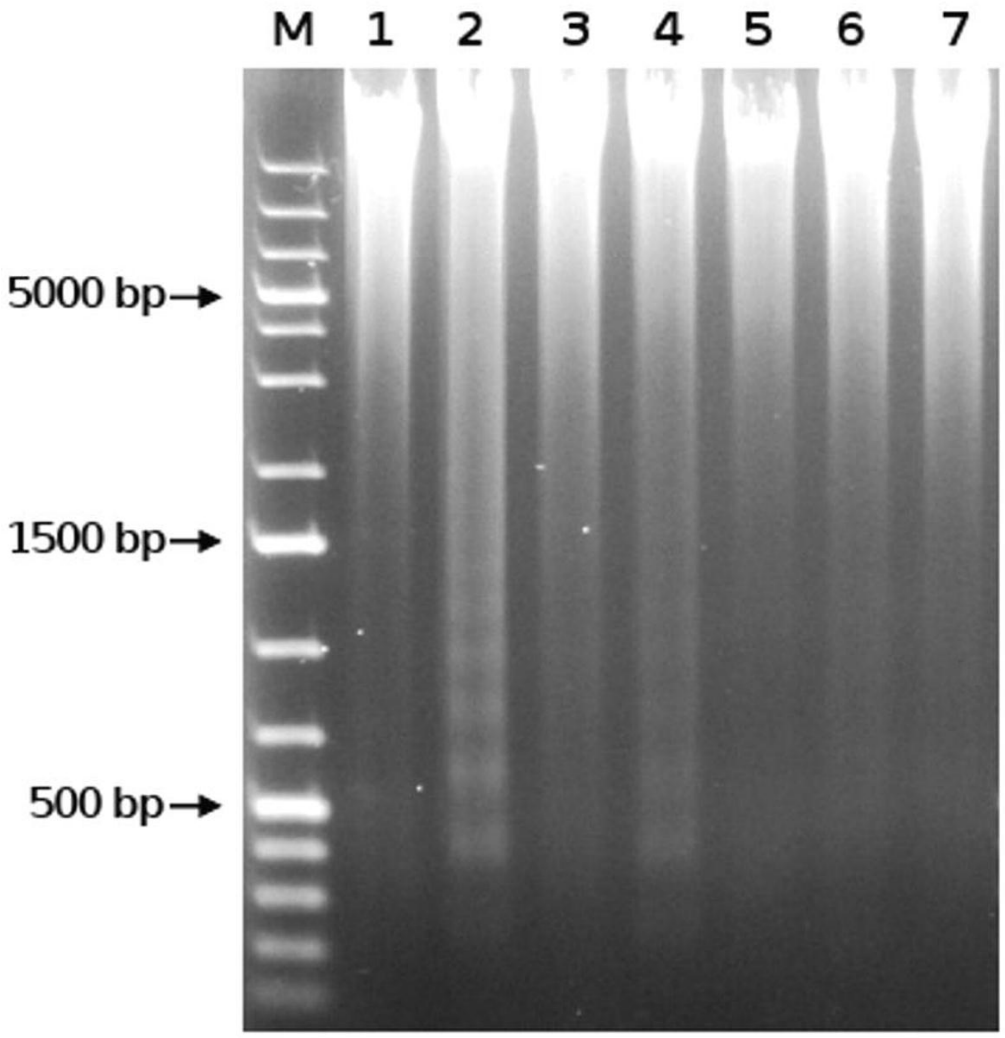

Figure 4.5: Agarose gel electrophoresis of DNA extracted from Sf21 cells. DNA was extracted from cells that were treated with ISB (lane 1), venom for 3 h (lane 2), venom + AEBSF for 3 h (lane 3), venom for 5 h (lane 4), venom + AEBSF for 5 h (lane 5), venom for 24 h (lane 6) and venom + AEBSF for 24 h (lane 7). Lane M is a 1 kb ladder marker.

4. Discussion

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 (Uckan *et al.,* 2004; Suzuki *et al.,* 2009; Parkinson and Weaver, 1999). Furthermore, this type of cell death may induce the developmental arrest seen in *N.vitripennis* hosts by targeting brain tissues (Rivers *et al.,* 2006; Rivers *et al.,* 2011). Under normal conditions, apoptotic cells are phagocytized, resulting in little or no immune

responses (Elmore, 2007). Consequently, apoptosis is a useful survival strategy applied by parasitoids that reside in/or attached to their host (Schmidt *et al.,* 2001).

Previous studies with BTI-TN-5B1 cells exposed to *N. vitripennis* venom already revealed the high susceptibility and the involvement of apoptotic and/or non-apoptotic programmed cell death in this cell line (Rivers *et al.,* 2005). In the current study, the cytotoxicity of *N. vitripennis* venom in Sf21 cells, a non-host Lepidopteran cell line was evaluated using MTT assays. These clearly indicate that this venom reduces viability of both suspension and adherent Sf21 cells in a time- and dose-dependent manner. Furthermore, flow cytometry of Sf21 suspension cells confirmed the susceptibility of these cells, even when exposed to low venom concentrations (0.625 µg/ml), resulting in a cell viability of 40.63 ± 2.87% after 1 h (Fig. 4.2A-F). However, during all examined time points the cell populations of the cultures that received a treatment other than ISB did not differ from each other. This may implay that only a part of the Sf21 population is susceptible for the venom concentrations used. Although some early apoptotic cells were detected after venom treatment, this phase is considered to be temporarily. Apoptotic cells can complete the programmed cell death program in approximately 2 to 3 hours and disappear very quickly (Elmore, 2007). One hour after venom treatment, already a large subset of the population was positive for both annexin V-FITC and PI staining. This indicates that membrane integrity is lost very rapidly during venom exposure, resulting in the accumulation of PI in these cells. Populations that stain for both annexin V and PI are referred in many studies as late apoptotic, necrotic or a combination of both, which makes it difficult to determine the exact type of cell death. However, in cell cultures, apoptotic cells cannot be cleared by phagocytes, which enables them to complete the apoptotic program. The final phase of the program ends in a late or post apoptotic state with a necrotic outcome, which is referred as secondary necrosis. The first phase of the complete apoptotic program comprises an apoptotic phase whereby plasma membrane integrity is maintained. The last part consists of a secondary necrotic phase with cytoplasmic swelling and a ruptured plasma membrane. Secondary necrosis often induces an immune response *in vivo* (Silva, 2010). Considering the short time needed for apoptotic cells to complete this process and the absence of clearance in these cell cultures, it is reasonable to assume that a subset of venom treated Sf21 cells may eventually undergo secondary necrosis as flow cytometry and DNA fragmentation assays confirmed the

occurrence of apoptotic cells. However, the characteristic ladder pattern was only visible at 3 h and 5 h after venom treatment. After 5 h venom treatment, the bands were already slightly fading away, while after 24 h no bands were visible. Nevertheless, this fragmentation takes place in the later stages of apoptosis, after the occurrence of membrane PS translocation and the loss of membrane integrity (Kroemer *et al.,* 2009; Elmore, 2007; Wlodkowic *et al.,* 2011). The absence of DNA fragmentation does not exclude the possibility that cells are apoptotic, since flow cytometry analysis of Sf21 cells demonstrated that a small subset of the population were still undergoing early apoptosis after 24 h when using the same venom concentrations.

In order to elucidate which components of the venom are cytotoxic, proteins present in the venom were inactivated by heating or depleted by filtration, resulting in a filtrate of proteins smaller than 10 kDa. These viability assays demonstrate that mainly proteins, larger than 10 kDa are responsible for cytotoxicity in Sf21 cells, which is in accordance with observations in BTI-TN-5B1-4 cells (Rivers *et al.,* 2006). Among these proteins, metalloproteases are cytotoxic for adherent Sf21 cultures, as venom pretreated with metalloproteases inhibitors, demonstrated increased cell viability compared to fully active venom. However, inhibition of metalloproteases in the venom did not improve the viability of suspension cells. The sensitivity between the different cultures of the same cell line may be caused by the differences in cell surface structures since the adherent cells differ in shape and have adhesive characteristics, while the latter is lacking in suspension cells. Two metalloproteases are present in the venom, one of them is an angiotensin-converting enzyme, which is found in other insects to have possible roles in the brain and insect reproduction (Schoofs *et al.,* 1998). The second is a member of a disintegrin and metalloproteinase with a thrombospondin motif 5-like (ADAMTSL-5) and a reprolysin domain. Reprolysin metalloprotease homologs EpMP1-3 are present in the venom of *Eulophus pennicornis*. Recombinant EpMP3 was found to be toxic in *Lacanobia oleracea* and may have a role in manipulation of host development (Price *et al.,* 2009; Asgari and Rivers, 2011). These metalloproteases are well represented in several snake and spider venoms, and play major roles in immunity and apoptosis related processes. Furthermore, metalloproteases of the ADAM family are known as potent inhibitors of both platelet aggregation and integrin-dependent cell adhesion. These enzymes are able to hydrolyze both extracellular matrix

(ECM) proteins and cell surface integrin receptors, which mediate cell attachment (You *et al.,* 2003; Takeda *et al.,* 2012). This could explain the difference in susceptibility of adherent and suspension cells when venomous metalloproteases were inhibited. A possible function of metalloproteases in parasitized hosts is to facilitate the distribution of the venom in the tissues.

Serine proteases were also found to be toxic for both adherent and suspension Sf21 cells. Flow cytometry analysis of AEBSF treated venom compared to untreated venom demonstrated a higher cell viability and a lower percentage of late apoptotic or secondary necrotic cells at all measured time points. However, 24 h after exposure to AEBSF treated venom, the differences between untreated venom declined for both viable and late apoptotic or secondary necrotic cells. Cells that only stained with PI were also less abundant in cultures subjected to AEBSF treated venom (Fig. 4.4D). These data suggest that inhibition of serine proteases results in a reduction of the cytotoxic activity of the venom and consequently alter the progress of the apoptotic pathway, during the first hours. However, since certain cells are positive for both annexin V-FITC/PI staining and no distinction can be made between cell death types in this state, there is a possibility that other cell death pathways may take over, when a specific type of cell death is inhibited. Furthermore, no ladder formation was observed in cells exposed to AEBSF treated venom at all examined time points, suggesting that serine proteases in the venom of *N. vitripennis* are indeed responsible for the induction of apoptosis. The serine protease proteins found in *N. vitripennis* venom all belong to the trypsin serine protease family (de Graaf *et al.,* 2010). Serine proteases have diverse functions and are involved in digestion, coagulation, moulting, larval development and ECM degradation. Furthermore, a secreted chymotrypsin-like serine protease Sc-SP-3 in the nematode *Steinernema carpocapsae* induces apoptosis in Sf9 cells, affects hemocyte spreading and suppresses prophenoloxidase in *Galleria mellonella* (Balasubramanian *et al.,* 2010; Toubarro *et al.,* 2009). It is reasonable that these proteases in *N. vitripennis* venom have several functions in the host and are involved in immunity and local tissue destruction.

The occurrence of cell death in both adherent and suspension cells exposed to protease inhibitor treated venom, could be the result of an incomplete inhibition of the proteases. Nevertheless, other venom compounds may also be involved in the cytotoxicity and

induction of apoptosis, since venom treated with Complete protease inhibitor cocktail could not protect all adherent cells and inhibition of serine proteases alone was also not sufficient to prevent cell death in suspension cells. Moreover, venom consists of a complex mixture of 80 venom proteins and some of them (γ-glutamyl transpeptidase-like venom protein 1 & 2 and a γ-glutamyl cyclotransferase-like venom protein) have assigned functions in the glutathione metabolism, a process that plays a role in apoptotic cell death (Franco and Cidlowski, 2009; Circu and Aw, 2008). *Nasonia vitripennis* venom also includes two laccases and an endonuclease-like venom protein with possible functions in programmed cell death (Danneels *et al.,* 2010). Calreticulin, another *N. vitripennis* venom protein was found to be involved in cytotoxic processes (de Graaf *et al.,* 2010; Asgari and Rivers, 2011; Rivers and Brogan, 2008) and is also present in the venom of *Microctonus spp.* (Crawford *et al.,* 2008) and *Pteromalus puparum* (Zhu *et al.,* 2010) and *C. rubecula* (Zhang *et al.,* 2006).

5. Conclusion

This study demonstrates that venom of *N.vitripennis* causes apoptosis in non-hosts and provides important insights into the functions of two protease families in *N. vitripennis* venom. Although, both serine proteases and metalloproteases are among the main cytotoxic compounds in *N. vitripennis* venom, their activity differ between adherent and suspension cells. Under physiological conditions, these venomous proteases may possibly function in immune related processes and tissue destruction enabling venom distribution. Further research of the separate serine and metalloproteases is needed to gain more insights in their functionality.

Acknowledgements

This work was funded by the Research Foundation-Flanders (FWO) (G041708N) and the Ghent University Bijzonder Onderzoeks Fonds (BOF12/DC1/006). We thank Prof. Van Craenenbroeck for the use of the flow cytometer.

Chapter 5

Activity of 45 human pathways in the HEK293T cell line upon treatment with *Nasonia vitripennis* venom

Contributions

D. de Graaf, K. Van Craenenbroeck and K. Heyninck assisted with the study design. E. Formesyn executed all experiments. Both E. Danneels and E. Formesyn performed the venom collection maintained the insect cultures. E. Danneels assisted with the reporter array experiments and data analysis (normalization). E. Formesyn performed the statistical analysis and wrote the manuscript.

1. Introduction

Hymenoptera venoms and other insect natural products have a wide range of functionalities and may have the potential to be used in the context of medical applications to treat infections, diseases and cancer like for instance breast and prostate cancer (Dkhil *et al.,* 2010; Heinen *et al.,* 2011; Son *et al.,* 2007). Several insect products, such as bee, wasp and ant venoms were found to possess anti-cancer properties by inhibition of NF-κB and the induction of apoptosis (Ratcliffe *et al.,* 2011; Park M. *et al.,* 2010; Park H. *et al.,* 2004). Until now, venoms of parasitoid wasps remained unexploited, regarding to this research area. These group of insect venoms may provide us a new source of pharmacologically active components. Although parasitoid venoms are mainly investigated in their natural hosts or in surrogate species within the same class of their usual hosts, their impact on mammalian cells, including human cell lines remains unknown. However, parasitoid venoms are known for their ability to modify host physiology such as immunity and development. Since many of their natural target pathways are conserved throughout evolution, especially those regarding to apoptosis and immune processes, it is possible that they may trigger similar responses in other organisms such as mammals (Belvin, 1996; Meier *et al.,* 2000a). Indeed, previous experiments indicated that *N. vitripennis* venom is able to inhibit the NF-κB pathway in mammalian cell lines (Danneels *et al.,* unpublished). Since several mammalian cell lines including, the human embryonic kidney cells 293T (HEK293T) are sensitive for *N. vitripennis* venom, a pilot study was conducted using Cignal Finder Multi-Pathway Reporter Arrays (SABiosciences). This straightforward approach enables to screen for the activity of 45 pathways at once and could provide us new information about the functionality of parasitoid venoms in human cell lines. Of the 45 pathways enclosed, 4 main research areas are targeted, including cancer, immunity, development and toxicology. Here, the activity of 45 pathways in HEK293T cells was screened upon *N. vitripennis* venom treatment, by reverse transfecting cells into multi-pathway reporter arrays. Transcriptional activity was monitored by comparing two reporter constructs, one is a pathway-focused transcription factor-responsive firefly luciferase reporter controlled by a basal promoter element (TATA box) joined to tandem repeats of a specific transcriptional response element (TRE; Fig. 5.1A). This construct monitors both increases and decreases in the activity of a key transcription factor, which is a downstream target of a specific signaling pathway. The other one is a

constitutively expressing *Renilla* luciferase construct (Fig. 5.1B) and acts as an internal control for normalizing transfection efficiencies and monitoring cell viability. The negative control contains the non-inducible firefly reporter construct and is under the control of a basal promoter element (TATA box) without any TREs (Fig. 5.1C) and enables the identification of specific effects and determination of background reporter activity. The positive control includes a constitutively expressed green fluorescent protein (GFP) construct (Fig. 5.1D), a constitutively expressing firefly (Fig. 5.1E) and a *Renilla* (Fig. 5.1B) construct. These constructs are used to monitor and confirm transfection efficiency. This dual luciferase technology allows for quantification of the degree of activation of each particular signaling pathway in a 96 well format. To our knowledge, this is the first time that venom of a parasitoid wasp is used in this context.

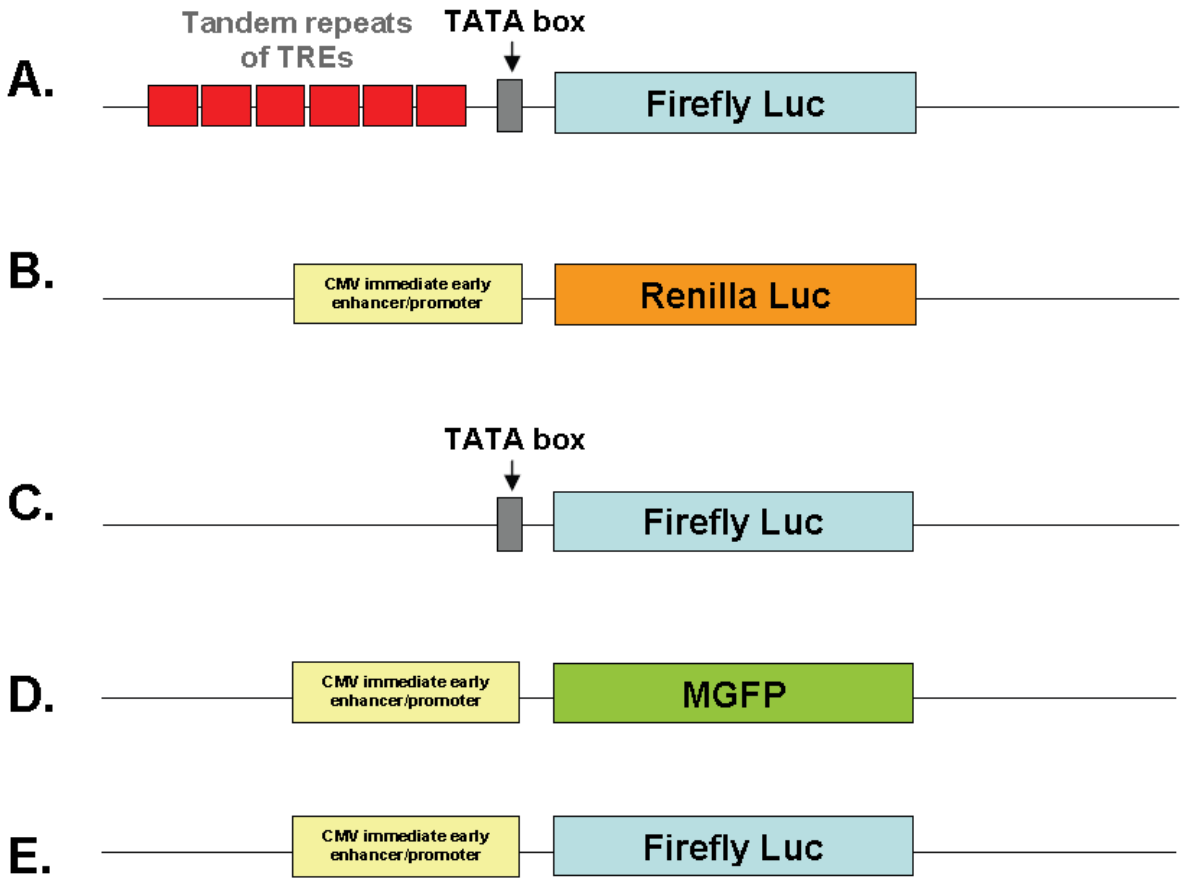

Figure 5.1: Overview of the constructs involved in the Cignal Reporter assay. **A**) Firefly luciferase construct controlled by TRE. **B**) Constitutively expressing *Renilla* luciferase construct, **C**) non-inducible firefly luciferase construct, **D**) Constitutively expressing GFP construct and **E**) constitutively expressing firefly luciferase construct (adopted from QIAGEN®).

2. Material and Methods

2.1. Insect rearing

Nasonia vitripennis Asym C, were reared according to Formesyn et al. (2013). In brief, 10 to 15 *N. vitripennis* females were placed together with approximately 15 *Sarcophaga crassipalpis* flesh fly pupae, under a light-dark cycle (LD 15:9 h) at 25 °C (Van den Assem and Jachmann, 1999). Flesh flies (*S. crassipalpis*) were cultured in the laboratory as described by (Denlinger *et al.,* 1972a). The first four days after hatching, adult flies were fed ad libitum on sugar and water. Starting from the fourth day, additional beef liver was provided in order to start egg development. All flesh fly stadia were kept at 25 °C with a photoperiodic cycle of LD 15:9 h.

2.2. Venom collection

Nasonia vitripennis venom was collected according to Formesyn et al. (2011). In brief, two to three days old *N. vitripennis* females were fed a 10% sugar solution for 24 hours at 25 °C under a long-day cycle (LD 15:9 h). Subsequently, venom reservoirs were dissected in Insect saline solution (ISB, 150mM NaCl, 10 mM KCl, 4 mM CaCl2, 2 mM MgCl2, 10 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid, pH 7) and centrifuged for 10 min. at 13500 rpm, in order to liberate the venom from the reservoirs. Venom samples were stored in -80 °C until further use. Before use, venom samples were pooled and the protein concentration was estimated with the BCA assay using a BSA standard (Pierce).

2.3. Cell culture

Human embryonic kidney cells 293T (HEK293T) were cultured as adherent monolayers in 25 cm^2 flasks in Opti-MEM reduced serum medium without phenol red (Invitrogen) supplemented with 5% FBS and 100 U/ml penicillin and 100 µg streptomycin. Cells were grown in an atmosphere with 5% CO_2 at 37 °C. Prior to the assays, cells were grown in 75 cm^2 flasks and cultured in such a way that they are sub confluent prior to collection.

2.4. Viability assay

Effect of venom on the viability of HEK293T cells was determined by MTT bioassays. This assay uses yellow tetrazolium bromide (MTT) as substrate which is reduced into purple

formazan by mitochondrial succinate-dehydrogenase in viable cells only. HEK293T cells (2 × 10^5 cells/ml) were seeded into 96-well plates (100 µl/well). After 24 h, spent medium was replaced by fresh Opti-MEM medium and cells were incubated with 2.5, 5 and 10 µg/ml venom for 8 h and 48 h. ISB treated cells were included as the control group. Subsequently, 20 µl of Thiazolyl Blue Tetrazolium Bromide (MTT) solution (5 mg/ml) was added to the wells and cells were incubated for 5 h. To solubilize the precipitate, 80 µl SDS/HCl solution was added to each well and incubated over night at room temperature. Absorbances were measured at 595 nm with the Victor 3TM 1420 Multilabel Counter plate reader (Perkin Elmer). All MTT tests were performed in triplicate.

2.5. Testing CMV promoter activity and Rluc degradation upon venom induction

The cytomegalovirus (CMV) immediate early enhancer/promoter controls the constitutive expression of *Renilla* luciferase, which acts as an internal control for normalizing transfection efficiencies and monitoring cell viability. In order to determine if *N. vitripennis* venom acts on the CMV promoter, 2.5×10^6 cells/ml HEK293T cells were seeded in a 10 cm^2 culture dish using Opti-MEM with 5% FBS and 100 U/ml penicillin and 100 µg streptomycin. After 24 h, media was replaced by fresh Opti-MEM medium containing 5% FBS and penicillin/streptomycin. Subsequently, all cells were transfected with 10 µg DNA mixture using a $CaPO_4$ transfection. A DNA mixture of 0.25 µg *Renilla* construct (pRL-CMV) containing the CMV promoter, 8.95 µg pcDNA3 empty vector and 0.8 µg βgal-construct (pAct βgal) was used to test the sensitivity of the CMV promoter. Another combination was included as control and contained 0.25 µg pRL-SV40 plasmid, 0.895 µg pcDNA3 empty vector and 0.8 µg βgal-construct (pUT651 βgal). The latter also includes a CMV promoter which controls the expression of β-galactosidase, while the pRL construct was included to test whether venom is able to degrade *Renilla* luciferase. Cells transfected with 10 µg pcDNA3 alone were used as a control for transfected cells. Green fluorescent protein (10 µg) was included to observe for the transfection efficiency with $CaPO_4$. Six hours after transfection, the medium was replaced by fresh Opti-MEM supplemented with 5% FBS and penicillin/streptomycin. Cells were incubated for another 18 h in a 5% CO_2 environment at 37 °C. Twenty four hours later, transfected cells were trypsinized, counted and seeded in poly-D-lysine (Sigma-Aldrich) coated 24 well plates at a density of 1.75×10^5 cells/well. For all construct combinations 4 replicate wells were used. The next day, medium was removed and fresh Opti-MEM medium

with 2% FBS and penicillin/streptomycin was added. After one hour, the transfected cells were induced during 8 h with 2.5 and 5 µg/ml venom. *Renilla*-luciferase reporter activity was determined using a dual-luciferase reporter assay system (Promega), following the manufacturer's instructions. βgal activity was measured with the Galacto-Star™ One-Step β-Galactosidase Reporter Gene Assay System (Invitrogen). For all measurements, a Victor 3TM 1420 Multilabel Counter plate reader was used. All experiments were performed twice.

2.6. Cell-based multi-pathway activity assays

To determine the signaling pathways induced upon venom treatment, transcription factor arrays (Cignal Finder™ 45-Pathway Reporter Arrays, SABiosciences), consisting of 45 dual-luciferase reporter assays, were used according to the manufacturer's instructions. Each of the 45 reporter assays encodes for an inducible transcription factor-responsive firefly luciferase reporter and a constitutively expressing *Renilla* construct in a 20:1 ratio (total DNA in each well = 0.2 µg). In brief, one hour before transfection, fresh Opti-MEM medium supplemented with 2% FBS was added to the cells and place back in the incubator. Meanwhile, DNA reporter constructs were first dissolved in 50 µl/ml Opti-MEM and incubated for 5 minutes at room temperature. Subsequently, polyethylenimine (PEI) transfection reagent was diluted in Opti-MEM without serum or antibiotics, and 50 µl/well was dispensed into 96-well white tissue-culture plates. For reverse transfection, freshly grown cells were counted and adjusted to 1.4 million cells/ml in Opti-MEM containing 2% FBS and 1% non-essential amino acids (NEAA, Invitrogen), without antibiotics. Cells (50 µl; 7×10^4 cells/well) were added to each well and incubated overnight at 37 °C with 5% CO2. Transfection media were removed 6-8 hours after transfection and replaced with 100 µl fresh Opti-MEM, supplemented with 5% FBS and 1% NEAA, and incubated again overnight at 37 °C. One hour prior to venom induction, media were removed and replaced with fresh Opti-MEM supplemented with 0.5% FBS and 1% NEAA. Cells (two array plates) were then induced with *N. vitripennis* venom at a concentration of 2.5 µg/ml for 8 h, the other 2 plates were treated with ISB and served as controls. Each plate contains a duplicate of the reporter. Dual-luciferase reporter activity was determined using a dual-luciferae reporter assay system (Promega), following the manufacturer's instructions using a Victor 3TM 1420 Multilabel Counter plate reader. Induced transcription factors were reported as luminescence ratios by

dividing the Firefly signal by the *Renilla* signal. Subsequently, normalization was performed using the ratio of control wells on every plate (negative and positive controls).

2.7. Statistical analysis

Data from all MTT assays were presented as percentages (mean ± SD). Statistical significance between groups was evaluated using Kruskal Wallis and two way analysis of variance (ANOVA) followed by Dunn's or Student-Newman-Keul's (SNK) multiple comparisons tests. Data from reporter arrays was evaluated by Student's t-tests and Mann-Whitney U tests. A value of $p < 0.05$ was considered statistically significant. All statistical analysis were performed with Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. The impact of *N. vitripennis* venom on HEK293T cells

The impact of *N. vitripennis* venom on HEK293Tcells was estimated by MTT assays. Results showed a time dependent inhibition of cell viability after venom treatment. After 8 h, no decrease in cell viability was detected with all venom concentrations (Fig. 5.2). However, 48 h after venom induction, HEK293T cells displayed reduced absorbancies of nearly 50 % caused by either a reduced cell viability or proliferation, which did not differ between the venom concentrations used (Fig. 5.2).

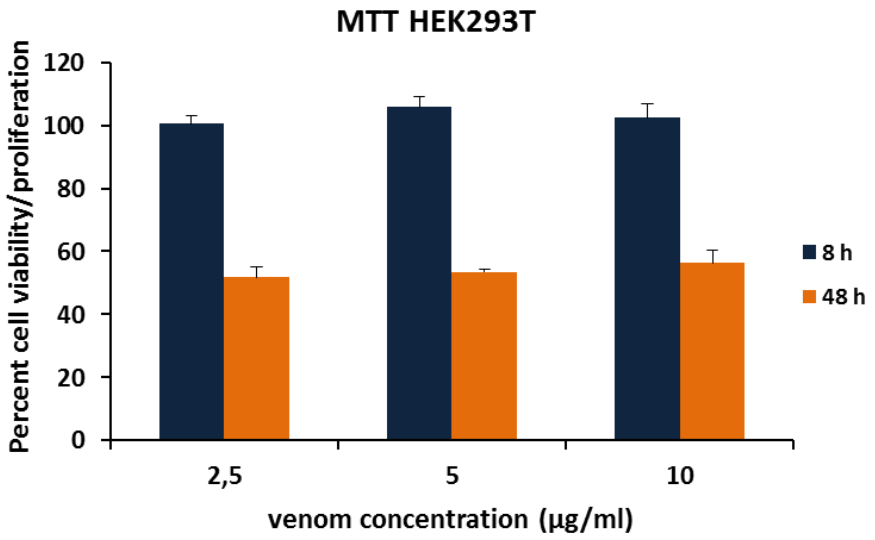

Figure 5.2: Viability assay on HEK293T cells using several venom concentrations. Viability was measured 8 and 48 hours after venom treatment. Results of one experiment are presented as cell viability/proliferation (% of control), mean ± SD. Letters indicate the significance between the different venom concentrations and time points used.

3.2. Influence of *N. vitripennis* venom on the CMV promoter and Rluc degradation

Cells transfected with a pRL-CMV or pUT651 βGal construct that were treated with several venom concentrations for 8 hours, showed no (de)activation of the CMV promoter compared to cells treated with ISB (Fig. 5.3A,C). No *Renilla* luciferase degradation was observed in venom treated cells containing the pRL-CMV or pRL-SV40 constructs, in comparison to ISB treated cells. Furthermore, the used venom concentrations did not decrease the relative light units (RLU) signals (Fig. 5.3A-D), indicating that the venom does not yet affect cell proliferation and their viability after 8 hours (see paragraph 3.1).

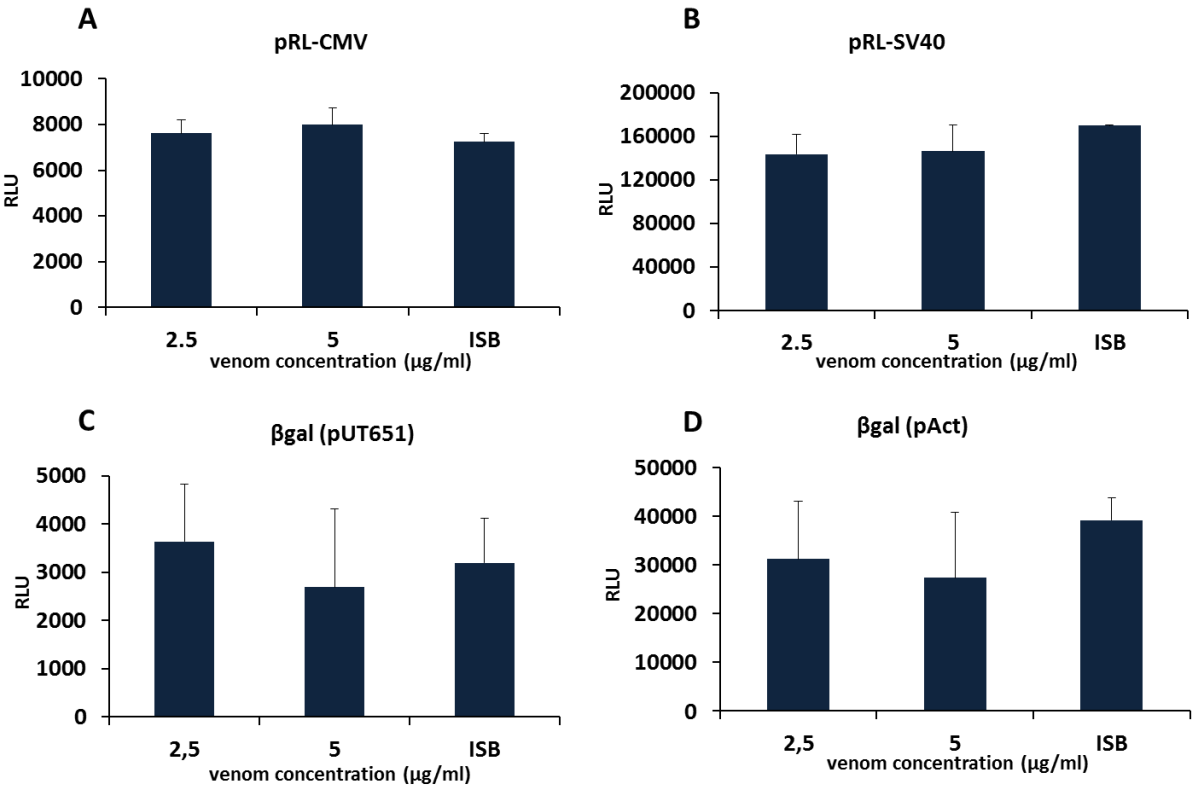

Figure 5.3: HEK293T cells transfected with several constructs were treated with 2.5 and 5 µg/ml venom during 8 hours (RLU, relative light units). *Renilla* luciferase reporter assays with a HEK293T cells transfected with a construct containing a CMV (**A**) or a SV40 (**B**) promoter. Transfected cells with β-galactosidase constructs containing either a CMV (pUT651 β-Gal, **C**) or a actin (pAct β-Gal , **D**) promoter. Results are presented as the mean ± SD of at least two independent experiments.

3.3. Venom induced pathways

Comparisons of the Cignal™ 45-Pathway Reporter Arrays indicated that several pathways were affected upon venom induction. The AARE reporter activity was found to be 4 times increased in comparison to control samples. The C/EBP, ERE, LXR and MEF2 reporters displayed a ±2 fold increased activity, while RXR reporter activity was 2.5 times increased. In addition, both the MAPK/Jnk pathway (AP1 reporter) and Xenobiotic Responsive Element (XRE reporter) reporter activity was found to be 1.5 times increased. One reporter, the octamer-binding transcription factor 4 (Oct4) had a decreased activity of 2.7 times, compared to control cells. (Fig. 5.4).

Although not significant, several other pathways showed a tendency upon venom induction. Interestingly, p53 signaling which is involved in DNA repair, cell cycle arrest, and apoptosis was found to be enhanced with a fold change of 3.86. The tendency of other reporters such as EGR1, CRE, STAT3 and PPAR also displayed an enhanced activity. The HIF, MTF1 and SRE reporters showed a decreased activity compared to ISB treated HEK293T.

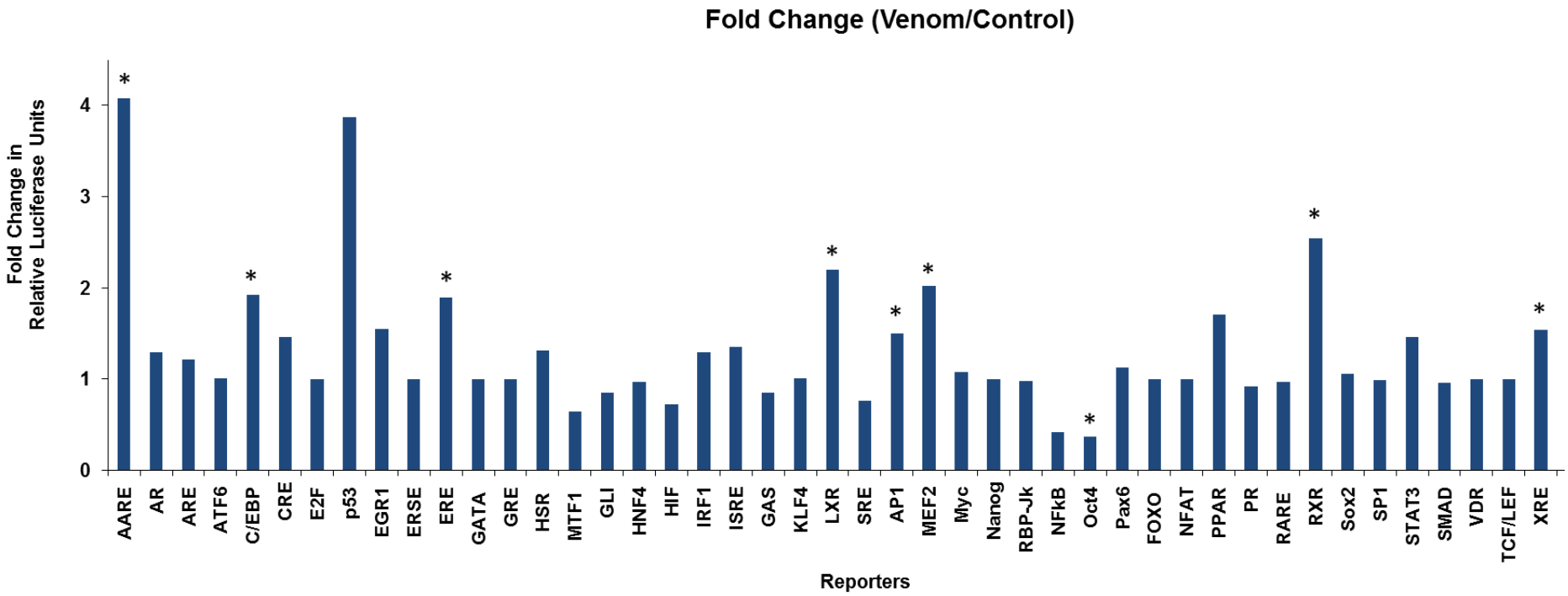

Figure 5.4: Selective (in)activation of several reporters after *N. vitripennis* venom treatment in HEK293T cells. Normalized values of both test and control samples were used to calculate the mean fold change of reporter (in)activation. A fold change of 1 indicates no differences in pathway activation between test and control samples. A fold change < 1 indicates a decreased activation of the reporter in venom treated cells, enhanced activation of the reporter upon venom treatment is indicated by a fold change > 1. Asterisks indicate the significant decrease or enhanced activation of the reporters.

4. Discussion

In the past, the cytotoxic effects of *N. vitripennis* venom were extensively studied using several insect species. These studies indicated that the venom is toxic for a broad range of insect species. Since the assembly of the venom is specially made for subduing target species such as *Sacrophaga* and *Calliphora* hosts, it is interesting to see that also non-hosts and their derived cell lines are susceptible for *N. vitripennis* venom (Rivers *et al.,* 1993; Formesyn *et al.,* 2013). In this study, the human cell line HEK293T was screened for its susceptibility upon *N. vitripennis* venom treatment using MTT viability assays. Adverse effects were not observed 8 hours after treatment, using all venom concentrations. Furthermore, previous experiments using a 24 hours treatment with the same venom concentrations displayed the same outcome, which indicates that the human cell line is far less susceptible than the insect cells tested so far. Indeed, the first cytotoxic effects in *Spodoptera frugiperda* (Sf21) cells, a non-host Lepidopteran cell line, treated with *N. vitripennis* venom were already observed 6 hours after treatment with an MTT assay and became more distinct at 24 h, using the same venom concentrations (Formesyn *et al.,* 2013). The low susceptibility of HEK293T cells is not surprising, since the evolutionary distance between insects and mammals is quite large and *N. vitripennis* venom is best suited to target its insect hosts and is not used on mammals. However, it is remarkable that the venom was able to affect the HEK293T cell lineafter a 48 hours treatment, implying that this cell line is to some degree susceptible for *N. vitripennis* venom. Importantly, since the effects became visible at a late time point (48 h), it is likely that the venom concentration used in this assay is reducing cell proliferation instead of cell viability. Screening of the 45-Pathway Reporter Arrays revealed that several pathways are affected by the venom after a treatment of 8 hours. Since the cells must not undergo cell death during the transfection procedure and venom treatment, in order to avoid deviating luciferase readouts, cells were treated with 2.5 µg/ml venom for 8 hours. This is in accordance with the observations made during the MTT assays, whereby the same concentration does not induce cell death after 8 hours.

Analysis of these arrays revealed that several reporters involved in stress responses were affected by the venom after 8 hours, although no reduction in viability was observed using MTT assays. In addition, microarrays on parasitized hosts also revealed that transcripts involved in stress, cell death, detoxification and the MAPK/JNK pathways were affected.

In the subsequent sections, an overview about the possible functions of the affected reporters is presented.

4.1. The AARE reporter

One of the most striking changes observed during a venom treatment of 8 h was the enhanced activation (4 fold) of the amino acid deprivation (AARE) reporter which is known to be an early response upon stress (Kilberg *et al.,* 2005). Malnutrition, various pathological situations and xenobiotic toxins are able to alter amino acid availability which can result in a deficit of certain amino acids and increased uncharged transfer RNAs (tRNA). Following amino acid deprivation, the general control non-derepressible-2 (GCN2) kinase is activated upon detection of these accumulated free tRNA and phosphorylates the translation initiation factor (eukaryotic initiation factor 2a) thereby attenuating protein synthesis. Since the ER regulates the production and oxidative folding of proteins, this response may prevent further accumulation of misfolded proteins and alterations in redox state (Chaveroux *et al.,* 2009). Furthermore, phosphorylated eIF2a enhances the translation of several mRNAs, including ATF4 which is rapidly induced under cell-stress conditions, such as glutathione depletion and oxidative stress. ATF4 is an important regulator of several ER stress target genes, amino acid transporters and antioxidants thereby preventing further accumulation of reactive oxygen species (ROS). However, if the stress exceeds the cellular regulatory capacity, then cell death is promoted (Kilberg *et al.,* 2005; Lange *et al.,* 2008). Although, the endoplasmic reticulum (ER) stress element (ERSE) and activating transcription factor 6 (ATF6) reporters did not differ in their activity between control and venom treated cells after 8 hours, they may be activated at different times. Furthermore, ER stress involves several responses and other signaling cascades may be activated that converge at some point, thereby resulting in the same outcome. Accumulation of unfolded proteins in the ER also induces the unfolded protein response (UPR), which consist of three main ER localized stress sensors, inositol-requiring enzyme 1 (IRE1), ATF6 and protein kinase-like ER kinase (PERK). The latter is also able to induce ATF4 and ATF3. This response is essential to maintain the homeostasis of the ER and regulates protein translation but promotes apoptosis if the stress level is too large (Breckenridge *et al.,* 2003; Lai *et al.,* 2007). ATF4 in turn phosphorylates ATF2 which is also activated by stress kinases such as JNK and p38, thereby regulating the transcription of immediate early genes involved in stress and DNA damage responses (Gupta

et al., 1995; van Dam *et al.,* 1995). ATF3 which is activated downstream of ATF4 and ATF2, is an ATF/cyclic-AMP-response element binding protein (CREB) which is induced in response to extra and intracellular stress signals, including DNA damage. Its p53 stabilizing activity promotes apoptotic cell death which makes ATF3 a putative suppressor of tumorigenesis (Yan and Boyd, 2006). The activated ATF4 as well as ATF3 and ATF2 bind to the AARE core sequence and upregulate the expression of target genes such as glutamine synthetase, calreticulin and asparagine synthase (AS). The latter is induced upon asparagine, methionine, histidine and cysteine starvation as well as deprivation of leucine, isoleucine and glutamine.

4.2. The C/EBP reporter

The CCAAT-enhancer-binding protein (C/EBP) was also up regulated in venom treated cells. Under uninduced conditions both C/EBP mRNA and protein levels are undetectable but expression is induced upon methionine starvation and ER stress involving the UPR pathway (Fafournoux *et al.,* 2000). Transcription factors of the C/EBP family such as the C/EBP homologous protein (CHOP) are activated by the ATF4 and ATF2 transcription factors and are induced upon DNA damage. For this reason these transcription factors are known to initiate the cell death machinery (Meir *et al.,* 2010; Endo *et al.,* 2006; Poli, 1998). Induction of CHOP results in downregulation of B-cell lymphoma 2 protein and upregulation of the pro-apoptotic BAX/BAD thereby increasing the production of reactive oxygen species, causing subsequent damage to the mitochondrial membrane and releasing cytochrome c into the cytosol (Lai *et al.,* 2007). Furthermore, members of the C/EBP family can form heterodimers with ATF members and are also able to recognize the AARE sequence in the asparagine synthetase gene (Kilberg *et al.,* 2005). However, the activity of C/EBP is also affected by inflammatory stimuli and cytokines such as IL-6, IL-1 and TNF-α. In addition, mouse models indicate that various C/EBP isoforms regulate the differentiation and function of the myelomonocytic lineage such as macrophages and granulocytes (Poli, 1998; Clarke and Gordon, 1998). Apart from their function in cell differentiation, C/EBP isoforms are also able to suppress cell proliferation and tumorigenesis in myeloid cells (Wang *et al.,* 2001; Johnson, 2005).

4.3. The AP-1 reporter

Another axis of the UPR pathway is mediated by IRE1 which in turn can activate the Jun N-terminal kinase (JNK) pathway by the MAPK cascade. Stress, inflammatory cytokines and genotoxic agents are all powerful inducers of JNK, which is a stress-activated protein kinase (SAPK). Subsequently the nuclear transcription factor activating protein 1 (AP-1) is activated and was found 1.5 times upregulated in this study. However, AP-1 is not a single protein but consists of a mixture of dimers composed of Jun family members, basic leucine-zipper (bZIP) proteins, Fos or ATF family members (Karin *et al.,* 1998; Ameyar *et al.,* 2003). Importantly, the outcome of AP-1 activation is dependent on the complex members which regulate cell proliferation and differentiation. In addition, AP-1 complexes are involved in the transcriptional activation of several pro-inflammatory genes and are key mediators of oxidative stress. They also function as nuclear decision-makers determining survival or cell death in response to extracellular stimuli (Verfaillie *et al.,* 2013). An interesting target of AP-1 is the tumor suppressor p53 which was found upregulated, although not significantly in this study. Other Jun targets are the proapoptotic genes FasL and TNF-α which also contain AP-1 binding sites (Hess *et al.,* 2004; Shaulian and Karin, 2001).

4.4. The MEF2 reporter

The myocyte enhancer factor-2 (MEF2) reporter which measures the transcriptional activity of the MADS box transcription enhancer factors (MEF2A, MEF2B, MEF2C and MEF2D) was found upregulated in venom treated cells. These transcription factors are just like AP-1 important for a variety of biological processes, including cell growth, differentiation, apoptosis and survival. Several cell surface receptors such as G-protein coupled receptors and the epidermal growth factor receptor determine MEF2 activity which has been shown to be involved in the induction of the c Jun promoter. However, the activity and its functionality of MEF2 also depends on the interactions with other proteins and the cell type. In general MEF2 acts as an effector of several calcium signaling pathways which are involved in these cellular processes. Transcriptional activity of MEF2 depends on the interaction with histone deacetylases which acts as a repressor, calcium/calmodulin dependent kinases and protein kinases D relieve this repression through phosphorylation of histone deacetylase (Grégoire *et al.,* 2007). In post-mitotic neurons, MEF2 was found to act as a survival factor, however it

also has a proapoptotic function during the negative selection of thymocytes (Mckinsey *et al.,* 2002). In this situation, the calcium dependent activation of MEF2 results in an upregulation of Nur77/TR3 which binds to the mitochondria thereby releasing cytochrome c (Beckenridge *et al.,* 2003).

4.5. The Oct4 reporter

The octamer-binding transcription factor 4 (Oct4), involved in the regulation of pluripotency in embryonic stem cells, is also essential for cell survival during cell stress (Kang *et al.,* 2009). Embryonic stem cells displayed increased apoptosis rates when Oct4 is knocked down (Guo *et al.,* 2008). Currently, the mechanisms behind Oct4 activation and expression are unknown. Oct4 is expressed in undifferentiated stem cells and disappears during the developmental process, it also occurs in very small embryonic-like stem cells which are present in several tissues such as epidermis, heart, pancreas, testis, bronchial epithelium and ovaries (Samardzija *et al.,* 2012). Furthermore, Oct4 expression was found to be related with the survival of patients with esophageal squamous cell carcinoma, several other cancers and stimulates cancer cell proliferation (Li *et al.,* 2012; Atlasi *et al.,* 2007; Kim and Nam, 2011; Kumar *et al.,* 2012). However, since the transcriptional activity of Oct4 is downregulated and all previously described reporters are in some way involved with cell proliferation, differentiation and cell survival/death, it is likely that a long-term venom treatment induces a growth arrest in combination with apoptotic cell death. This is in accordance to the MTT data of the HEK293T cells. Although these cells didn't display any visible toxic effects using MTT assays after a venom treatment of 8 hours, a treatment of 48 hours clearly indicates that viability is reduced since lower absorbencies were detected.

4.6. The XRE reporter

Eight hours after venom treatment the aryl hydrocarbon receptor (AhR) signaling pathway was found upregulated, indicating the activity of detoxification metabolism. Furthermore this pathway is also involved in hypoxia, hormone receptor function, development and chemically-induced carcinogenesis (Stejskalova *et al.,* 2011). This receptor is a key element in the toxic responses observed after exposure to its ligands including dioxins, polycyclic aromatic hydrocarbons and polychlorinated biphenyls. These ligands need to enter the cell in order to activate AhR and most xenobiotics do possess lipophilic characteristics which

enables them to cross biological membranes. Although AhR interacts with various proteins, in this study only the activity of the AhR/ARNT (aryl hydrocarbon receptor nuclear translocator) heterodimer was measured. Under normal conditions AhR is inactive, however upon ligand binding, AhR translocates to the nucleus and forms a complex with ARNT which recognizes the xenobiotic response element (XRE). Several xenobiotic metabolizing genes contain a XRE core sequence, including CYP1A1 (Cytochrome P450 Family-1 Subfamily-A Polypeptide-1), CYP1A2 (Cytochrome P450 Family-1 Subfamily-A Polypeptide-2), CYP1B1 (Cytochrome P450 Family-1 Subfamily-B Polypeptide-1) and NAD(P)H-Quinone Oxidoreductase (Beischlag *et al.,* 2008; Swanson, 2002). In addition, the expression of a homologue for cytochrome p450-28A1 in *S. crassipalpis* was also affected, although it was downregulated. Furthermore, AhR and estrogen signaling are known to interact with each other and binding of chemicals to ER and AhR are indicative for endocrine disruptive effects (Swedenborg and Pongratz, 2010; Medjakovic *et al.,* 2013).

4.7. The ERE reporter

Interestingly, estrogen receptor signaling was approximately 2 times upregulated upon venom induction. The receptor is activated upon binding with its ligand, estrogen, subsequently interacting with the estrogen response elements (ERE) or can interact with other transcription factor complexes such as AP-1 responsive elements where they interact with promoters that do not contain the EREs. However, the receptor also shows affinity for environmental contaminants such as polycyclic aromatic hydrocarbons, phthalates and pesticides, which may explain its enhanced activity during venom treatment. These can disrupt endocrine signaling in estrogen-responsive tissues and subsequently induce alterations in reproductive capacity and cancer. Estrogen signaling is performed by two distinct receptors (ERα and ERβ) and several splice variants. Interestingly, ER can interact with transcriptional coactivators or corepressors and their activity can switch, depending on the tissue type. The pathway plays a key role in development, including morphogenesis and proliferation, but also in cell adhesion, migration and invasion during tumorigenesis and metastasis which makes ER an interesting drug target (Heldring *et al.,* 2007; Björnström and Sjöberg, 2005).

4.8. The LXR reporter

Liver X receptor (LXRα) transcriptional activity was enhanced during venom treatment. LXR signaling is activated upon detection of oxysterols such as cholesterol and is important for regulating cholesterol homeostasis by enhancing the rate of reverse cholesterol transport, but also fatty acid, and glucose homeostasis. LXR agonists are interesting targets for treatment of atherosclerosis, diabetes, Alzheimer's disease and cancer (Chuu *et al.,* 2007). Furthermore, LXRs are involved in a wide range of biological processes like development, cell growth and immunity (Jakobsson *et al.,* 2012). They were found to regulate cell survival in mice macrophages by inhibition of anti-apoptotic factors and stimulate apoptotic cell clearance by these cells (Gonzalez *et al.,* 2009). However, activated LXRα in cancer cells are able to induce a state of apoptosis while leaving healthy cells unaffected and suppress cell proliferation (Mehrotra *et al.,* 2011).

4.9. RXR reporter

Venom treated cells displayed enhanced activity of Retinoid X Receptor (RXR)-mediated signal transduction pathway compared to ISB treated cells. This receptor is a member of the nuclear hormone receptor superfamily and is activated upon binding with vitamin A, derivatives and rexinoids, which are synthetic ligands. Besides their role in vision, multiple biological processes are mediated such as development, cellular and organ differentiation and several metabolic processes including lipid homeostasis. They are even found to regulate apoptosis. This is achieved by regulation of the Nur77-dependent apoptotic pathway Nur77 (Cao *et al.,* 2004). Furthermore, RXRs are involved in some pathological conditions such as diabetes, neoplastic formation and are seen as a promising target for cancer therapy. Interestingly, RXR forms dimers with many other nuclear receptors including LXR, retinoic acid receptor (RAR) and peroxisome proliferator-activated receptor (PPAR), which were not significantly affected in venom treated cells, except for LXR. Therefore RXRs are considered as master regulators of multiple nuclear receptor-base signal transduction pathways (Szanto *et al.,* 2004). Interestingly, methoprene which is a juvenile hormone analogue was able to activate the mammalian retinoid X receptor, but not the retinoic acid receptor pathway (RAR). Methoprene is used as a pesticide that prevents insect metamorphosis. However, the insect RXR homolog Ultraspiracle which is a component of the

Drosophila ecdysone receptor, is not activated under conditions in which RXR is responsive (Harmon *et al.,* 1995; Hall and Thummel, 1998). Activation of RXR may indicate the possibility that a retinoid analogue is present in the venom of *N. vitripennis*. It may be interesting to further investigate if the venom indeed contains one or more compounds with similar activities as insect hormones.

5. Conclusions and future perspectives

This study shows that venom of the parasitoid wasp *N. vitripennis* is affecting HEK293T cells to some degree, although the effects were only visible after 48 hours, when using MTT assays. Interestingly, from the 45 pathways tested, 9 were significantly affected. Furthermore, the majority of the pathways were found to be upregulated during venom treatment and related to stress signaling. Since each of these pathways are involved in multiple biological processes, more detailed research needs to be performed on both transcript and protein levels, in order to unravel how venom affects these processes. In addition, the possible protein interactions with other transcription factors might be investigated, because the composition of the transcription factor complexes influences their promoter specificity and hence the target pathways. Furthermore, it must be noted that this is a pilot study, in order to obtain a general overview of the affected pathways in the human cell line. Experiments using other time points or venom concentrations may also provide useful information because some pathways are active at earlier time points, while others are not yet activated at transcriptional level and may be undetected at 8 hours, using this assay. Interestingly, some pathways, such as those that involve MEF2 and Ap-1, are mediated by receptor activation, while others are regulated through binding with small chemical compounds that can pass biological membranes. This can imply that venom compounds other than proteins are present, have the ability to migrate through membranes and bind directly to transcription factors such as the nuclear receptors RXR and AhR.

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Chapter 6

Reproduction of honeybee workers is regulated by epidermal growth factor receptor signaling

Contributions

D. de Graaf and D. Cardoen assisted with the study design. J. Ravoet, E. Danneels, D. De Koker, M. Van Vaerenbergh, W. Ramon and J. Eerens assisted with the injections. E. Danneels and D. De koker assisted with the RNA and cDNA preparation, and qRT-PCR. E. Danneels and D. Cardoen assisted with the dissections. E. Formesyn was responsible for the data collection, data files and supplementary tables. T. Wenseleers performed the statistical data analyses. D. Cardoen and E. Formesyn wrote the manuscript.

This chapter is redrafted from:

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1. Introduction

The honeybees' reproductive division of labor between fertile queens and largely sterile workers has fascinated scientists for centuries. In fact, Darwin considered the near sterility of the workers a major evolutionary paradox and a 'special difficulty' to his theory of natural selection (Ratnieks *et al.,* 2011). Since 2006 the honeybee genome became publically available (The Honeybee Genome Sequencing Consortium), which made the honeybee into a key model system to study the underlying molecular mechanisms of insect polyphenisms, such as those involved in queen-worker caste determination (Chan *et al.,* 2006; Foret *et al.,* 2012; Kamakura, 2011; Kucharski *et al.,* 2008; Schwandler *et al.,* 2010).

Honeybee queen development is initiated early on in larval development. A specific diet, consisting of high amounts of royal jelly, containing high titers of major royal jelly protein 1 or 'royalactin', causes differentiation into queen-destined larvae (Kamakura, 2011). Downstream, caste determination relies on the insulin/insulin-like (de Azevedo *et al.,* 2008; Wheeler *et al.,* 2006; Wolschin *et al.,* 2011) target of rapimycin (TOR) (Patel *et al.,* 2007) and epidermal growth factor receptor (EGFR) signaling pathways (Kamakura, 2001; 2002; 2011). In addition, the altered gene expression responsible for queen development has been shown to be caused partly by differential DNA methylation (Foret *et al.,* 2012; Kucharski *et al.,* 2008). These gene expression changes may result in an increased juvenile hormone (JH) titer in queens (Hartfelder *et al.,* 2002; Rembold, 1987), and in a maturation of their ovaries. Adult queens can contain up to 200 ovarioles in contrast to the 3 to 26 ovarioles present in typical workers (Snodgrass, 1984). Furthermore, alternative splicing of the *Gemini* transcription factor was also found to be involved in ovary activation (Jarosh *et al.,* 2011).

The queen signals her presence to the colony using pheromones, which generally results in an inactivation of the ovaries of nearly all, 99.99%, of the workers (Kocher and Grozinger, 2011). Upon the irreversible loss of the queen, up to 30% of the worker bees activate their ovaries and start laying unfertilized, male-destined eggs (Ratnieks, 1993). Large-scale screenings of the genome (Linksvayer *et al.,* 2009; Oxley *et al.,* 2008), transcriptome (Cardoen *et al.,* 2011b; Grozinger *et al.,* 2007; Thompson *et al.,* 2006; 2008) and the proteome (Cardoen *et al.,* 2011a; 2012) delivered many candidate genes and proteins that might underlie this shift in the reproductive capacity of worker bees. A large-scale

microarray study also revealed that there was significant overlap in the genes that were involved in regulating worker reproduction and queen-worker caste determination, including the epidermal growth factor receptor (Grozinger *et al.,* 2007). Given this overlap and the fact that a recent study showed EGFR signaling to play a key role in queen-worker caste determination (Kamakura, 2011), we decided to test the involvement of EGFR signaling in honeybee worker reproduction. Additional evidence was provided by a microarray study comparing gene expression patterns in reproductive versus non-reproductive honeybees in queenless colonies and showed that orthologues of three *Drosophila melanogaster* EGFR inhibitors (*Argos*, *Sprouty* and two paralogues of *Drosophila Cbl isoform A*) were upregulated in non-reproductive worker bees. Furthermore, a significant enrichment (ca. 8 fold) of genes categorized in the gene ontology function 'negative regulation of epidermal growth factor receptor activity' was observed in sterile workers, which implies that a decreased EGFR signaling likely induces worker sterility (Cardoen *et al.,* 2011a). Since down-regulation of EGFR, using RNA interference (RNAi) in queen-destined larvae resulted in a defective queen phenotype with undeveloped ovaries (Kamakura, 2011), we decided to test whether EGFR knock-down likewise inhibits ovary development in adult workers in a queenless environment.

2. Materials and Methods

2.1. Honeybees

For our experiments, we used *Apis mellifera carnica* honeybees that were reared at the experimental apiary of the Laboratory of Zoophysiology (Ghent, Belgium). Brood frames with emerging brood of six different honeybee colonies were collected on March 18th 2012 and incubated overnight at 34 °C and high relative humidity. The next morning, 300 newly emerged workers (max 24 h old) were collected from the six colonies and randomly mixed. For each of the experimental or control groups, 150 healthy honeybee workers were used and kept in three cages (approx. 10.5 x 9 x 7 cm^3) containing 50 honeybees each. Every cage was equipped with a piece of beeswax, water, pollen paste and sugar dough containing 77% powdered sugar and 23% honey. All cages were incubated for 21 days at 34 °C and high relative humidity. Previous studies on honeybee ovary development were also successfully performed using cage experiments in order to mimic a queenless colony (Miller *et al.,* 2001),

thereby obtaining workers with activated ovaries (Grozinger *et al.,* 2007; Hoover *et al.,* 2003; 2005). This approach also ensures that all treated bees can be analyzed without possible influences of untreated bees present in a natural queenless hive (Katzav-Gozansky *et al.,* 2006).

2.2. RNAi experiment

Newly emerged worker bees (< 24 h old) were injected with siRNA targeting either the target EGFR gene or the non-target control gene GFP (a non-honeybees gene). Three different siRNA sequences to knock down EGFR and three sequences for GFP were mixed (Table S6.1; supplemental materials can be obtained by contacting Prof. de Graaf). All fragments were purchased at Sigma-Aldrich (France). All siRNA fragments were dissolved in insect saline buffer (150 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid), vortexed, then shortly sonicated and spun down. An overall amount of 60 pmol of siRNA mix (dissolved in a volume of 2 µl) was injected in the back of the thorax with a 33 gauge needle. For each target one needle was used. The injection spot was sealed using melted synthetic wax (Syncera) at approximately 60 °C. Differences in survival among treatments were also monitored.

2.3. qRT-PCR validation of knockdown

Seven days post injection, 5 honeybees of each cage (i.e. 15 per experimental group) were randomly sampled, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. Total RNA was extracted from individual whole honeybees with the RNeasy lipid tissue mini kit (Qiagen) following the manufacturer's guidelines. Reverse transcription was performed *in duplo* starting with 5 µg RNA of each sample using Oligo(dT) primers (0.5 µg/µL) and was carried out according to the RevertAid H Minus First strand cDNA Syntheis kit protocol (Fermentas). Concentration and sample quality after each protocol was determined using Nanodrop 2000 (Thermo Fisher Scientific). Quantitative reverse transcriptase PCR (qRT-PCR) was performed using Platinum SYBR Green qRT-PCR Supermix-UDG (Invitrogen) and CFX96 Real-Time PCR Detection System (Biorad). Primers were developed with Primer Express 2.0 (Applied biosystems) and validated by standard and melt curve protocols. The three reference genes (Table S6.2) used in this study were selected according to Cardoen (2011), who studied the same phenotype.

Normalized target gene expression levels were calculated for every bee, using the comparative Ct method and the geometric mean expression level of the three best (most stably expressed) reference genes: GB10903 (ribosomal protein L32), GB16844 (elongation factor 1-alpha) and GB12747 (eukaryotic translation initiation factor 3 subunit C). Log2 transformed relative expressed levels were statistically compared using a general linear mixed model, in which cage was included as a random factor. This was done using the statistical software R 2.15, using function function lmer in package lme4, and assessing signficance using the pvals.fnc function in package languageR.

2.4. Assessment and comparison of worker ovary development

Three weeks post injection, dissection of the remaining workers was performed. Bee abdomens were dissected and the ovary activation was scored. Ovary activation was scored on a scale from 0 to 3, based on the scale described in ref. (Lin *et al.,* 1999), with score 0 being used for ovaries that were undeveloped and in which no oocytes could be distinguished, a score of 1, 2 or 3 being awarded when ovarioles contained visible round oocytes, sausage-shaped oocytes, or at least one fully developed egg, respectively (Fig. 6.1B). Worker ovary development was compared among the different treatment groups using an ordered mixed logit model in which cage was coded as a random factor. This was done using function clmm in package ordinal in R 2.15.

3. Results

Quantitative real-time PCR revealed that EGFR knock-down was successful in bees sampled 7 days post injection (2.6-fold down-regulation, p = 2.2E-6, compared to green fluorescent protein (GFP) siRNA-injected control bees) (Figure 6.1A; Table S6.3). In addition, mortality was not significantly different between the treatment groups. Bees injected with siRNA targeting EGFR showed significantly reduced levels of workers ovary activation compared to control bees injected with siRNA targeting the non-honeybee gene GFP (Figure 6.1B; Table S6.4; ordered mixed logit model, respectively p=0.005). This provides supporting experimental proof that EGFR signaling is involved in regulating reproduction in honeybee workers.

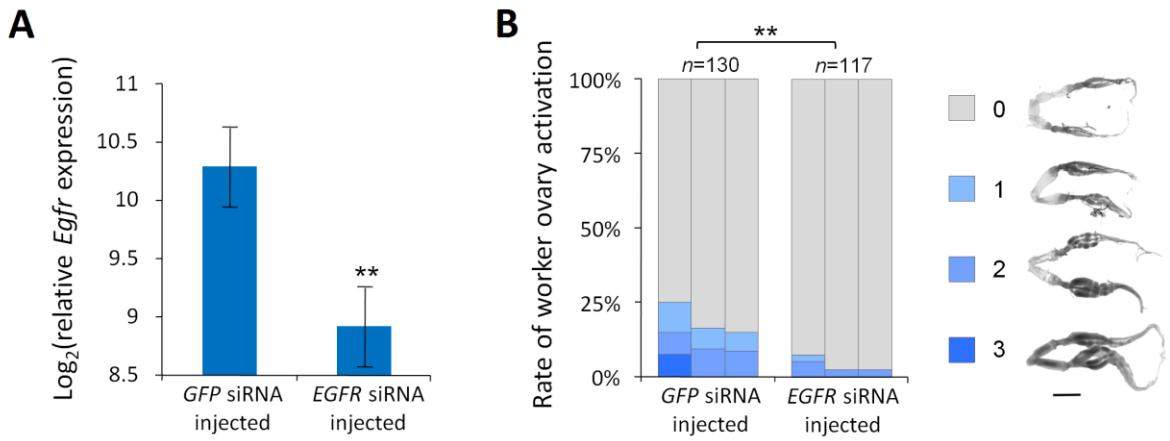

Figure 6.1: A) qRT-PCR validation of the RNAi knock-down of the *Egfr* gene. Log2 tranformed relative EGFR expression levels measured using qRT-PCR 7 days post-injection. Error bars indicate the 95 % confidence intervals. GFP siRNA bees were injected with GFP non-target control siRNA and EGFR siRNA bees were injected with siRNA targeting the EGFR gene. EGFR was 2.6-fold down-regulated (p = 2.2E-6) compared to GFP siRNA-injected control bees. Significance levels were calculated using a general linear mixed model in which cage was coded as a random factor. **B**) Epidermal growth factor receptor (EGFR) knock-down induces worker sterility. EGFR knock-down induces near-sterility in honeybee workers. Bees injected with siRNA targeting EGFR showed significantly reduced levels of workers ovary activation compared to control bees injected with siRNA targeting the non-honeybee gene GFP (p=0.005). Significance levels were calculated using an ordered mixed logit model in which the ovary development score of each bee (cf. legend, scale bar = 1 mm) was compared among treatments and with cage being included as a random factor.

4. Discussion

EGF signaling has been very well investigated in *Drosophila* due to its involvement in a wide array of physiological processes (Shilo and Raz, 1991). In honeybees, EGFR has previously been shown to be a key element in queen development (Kamakura, 2011). Our data, are the first to provide supporting evidence, based on RNA interference knockdown, that EGFR signaling is also involved in regulating reproduction in honeybee workers. In particular, down-regulation of EGFR resulted in a situation in which workers could no longer activate their ovaries in a queenless environment. This is interesting, because even though in former microarray (Cardoen *et al.,* 2011b; Grozinger *et al.,* 2007) and proteomic analyses (Cardoen *et al.,* 2011a, 2012) neither EGFR or its likely ligand, gurken, were differentially expressed between reproductive and nonreproductive workers, and the EGFR inhibitors *Argos*, *Sprouty* and *Cbl* were all upregulated in sterile workers. Furthermore, the genes upregulated among sterile workers in queenless colonies were ca. 4 fold enriched with the gene ontology function 'negative regulation of epidermal growth factor receptor signaling pathway' (p=0.01), ca. 8 fold enriched for the GO term 'negative regulation of epidermal growth factor receptor activity' (p= 0.02) and ca. 2 fold enriched for the GO term 'transmembrane receptor

protein tyrosine kinase signaling pathway' (p=0.02) (Cardoen et al, 2011b). This implies that down-regulation of the EGFR signaling pathway likely plays a key role in the suppression of worker ovary activation, whereas conversely, its upregulation plays an important role in initiating worker egg-laying in queenless colonies. In addition, downstream factors such as Raf kinase and suppressor of cytokine signaling 5 (SOCS-5, which causes negative regulation of EGFR signaling), were also upregulated among sterile workers (Cardoen *et al.,* 2011b). All these data strongly support that EGFR signaling plays a key role not only in queen determination in the larval stage (Kamakura *et al.,* 2011), but also in regulating reproduction in adult honeybee workers. This is consistent with the fact that EGFR signaling is known to be involved in both embryonic gonad development (Weyers *et al.,* 2011) and oocyte maturation in adult individuals in other invertebrate model organisms (Shilo and Raz, 1991; Poulton *et al.,* 2006; Van Buskirk *et al.,* 1999) and that EGFR signaling is also involved in the negative regulation of apoptosis (Parker, 2006). Indeed, both worker ovary development in the larval stage and the suppression of worker ovary development in queenright colonies has been shown to involve specific patterns of cell apoptosis (Cardoen *et al.,* 2012; Tanaka *et al.,* 2004).

5. Conclusions

This study provided the first direct evidence that there is indeed an overlap in the molecular pathways involved in both caste differentiation and worker reproduction. Here, EGFR downregulation was found to inhibit ovary activation, while control bees were able to activate their ovaries. This study shows that EGFR plays a key role in honeybee worker ovary activation.

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PART III

DISCUSSION

Chapter 7

Discussion and future perspectives

1. *Nasonia vitripennis*

1.1. The effects of *N. vitripennis* venom revealed in natural hosts

Parasitoid wasps such as *N. vitripennis* target fly species that are known to be harmful for poultry and livestock and are therefore considered as natural pest agents (Rivers, 2004). In the past decades, *N. vitripennis* was extensively studied and its venom is known to regulate several physiological processes in their natural hosts. Moreover, it became obvious that this parasitoid wasp contains a highly complex venom composition of 80 venom proteins, needed for successful parasitation (de Graaf *et al.,* 2010). Parasitation of both endo and ectoparasitoids generally results in several host responses, including alterations in development, metabolism and immunity (Asgari and Rivers, 2011; Danneels *et al.,* 2010). Despite the large amount of information concerning endoparasitoid-host relationships, including several transcriptomic studies on parasitized hosts, detailed information was lacking about the affected pathways in natural hosts parasitized by ectoparasitoids. Part of this work focuses on the impact of *N. vitripennis* venom in natural hosts. However, since it is difficult to track parasitized hosts infected by this wasp, without opening the pupae, a screening tool was developed that targets *Nv_nos* mRNA in parasitoid eggs using RT-PCR. This approach can be extended to other parasitoids-host relationships, while choosing the right molecular markers, in order to verify successful parasitation of hosts. Furthermore, this avoids unnecessary manipulations of the host, which are not desired in the scope of transcriptome studies and ensures that the samples are indeed infected with parasitoids. Some studies performed with parasitoids also uses pooled samples of hosts ranging from 6 to 48 hours post parasitation or exposes hosts to the parasitoids for approximately two days before starting the post parasitation time (Etebari, *et al.,* 2011; Zhu *et al.,* 2013; Schlenke *et al.,* 2007). As a consequence, little information is available about the gene expression of entire hosts at early post parasitation times and hence the effects of venom only. This study aimed to determine *S. crassipalpis* host responses at well-defined time points, 3h and 25h after parasitation with *N. vitripennis*. At these time points only the effects of the venom are observed since the parasitoid eggs are not hatched yet. This study revealed that only one transcript was affected 3h post parasitation. In addition, differential expression of heat shock proteins was only recorded in whole body total RNA, 13 hours after parasitation of *S. bullata* pupae by *N. vitripennis* (Rinehart *et al.,* 2002). These findings are in contrast to other

endoparasitoid-host transcriptome studies, such as the *Drosophila melanogaster-Asobara tabida* interaction, which showed that 1 hour post parasitation immune processes were already affected (Wertheim *et al.,* 2005). Moreover, a recently published transcriptome study using hemocytes of *Musca domestica* parasitized by *N. vitripennis* revealed that gene expression is already affected 1h after parasitation (Qian *et al.,* 2013). Furthermore, other studies using host hemocytes of *S. bullata* or specific tissues such as the fat body and brains revealed that envenomation by *N. vitripennis* induces alterations in cell behavior and toxic effects, which were already visible after 1h (Rivers *et al.,* 2002, 2011). In vivo and in vitro examination of several hosts and insect cell lines also revealed that they were affected shortly after exposure or treatment with venom of their corresponding parasitoids (Er *et al.,* 2010; Ergin *et al.,* 2006; Deyrup *et al.,* 2006). Moreover, immunohistochemical stainings of *S. crassipalpis* pupae 4h post parasitation revealed that hosts are indeed enduring detrimental effects by *N. vitripennis* venom, which are manifested through cell death. However, at that time, cell death was only observed at localized areas in the abdomen and possibly indicates the injection spot of the ovipositor. A possible scenario is that whole body RNA can contain transcripts that are upregulated in some cell types or tissues, while the same transcripts are downregulated in others which cancel each other out. An indication for this scenario may be the finding that several transcripts involved in programmed cell death were differentially expressed, although some of them are known to promote cell death while others suppress this process. Interestingly, transcripts related to stress, apoptosis, metabolism, transport, cytoskeleton, cell cycle and transcription/translation regulation were found in hemocytes of *S. bullata* 1 hour after parasitation by *N. vitripennis* (Qian *et al.,* 2013). In this PhD thesis, transcripts that belong to the same categories were found in the transcriptomic study of *S. crassipalpis*, 25h post parasitation. Here, much more transcripts were found to be affected by the venom compared to 3h post parasitation. Since *N. vitripennis* lacks the capacity to synthesize lipids themselves, their progeny are completely dependent on host lipid metabolism (Visser *et al.,* 2012). Indeed, the expression of several genes involved in nutrient mobilization supports previous research on parasitized *S. bullata*, which implies that metabolism is altered and synchronized with the developing larvae (Rivers *et al.,* 1994). Interestingly, transcripts involved in programmed cell death and autophagy also had an altered expression profile upon parasitation. The latter plays a role in the degradation of cytoplasmic components, required for cell survival during starvation, but

is also associated with cell death when starvation is prolonged. Programmed cell death is involved in several physiological processes including immunity, development and metabolism. Furthermore, localization of cell death in parasitized *S. crassipalpis* pupae revealed that 26 and 50 hours post parasitation, a substantial amount of trophocytes, posterior in the abdomen are undergoing cell death, which may be involved in the metabolic reorganization to increase fat body lipid levels. However, it is unclear if these trophocytes are undergoing cell death as a consequence of autophagy. Immunity is also commonly affected by parasitoid venoms and results in evoking programmed cell death in host hemocytes (Teramoto *et al.,* 2004). However, since programmed cell death affects many processes, it still remains unknown in which of these processes cell death is involved.

In order to detect and localize early and late responses in parasitized pupae, it may be interesting to analyze gene expression or proteomic alterations in separate tissues such as fat body, midgut, gonads, glands and brains. In addition, antibodies can be raised against the identified targets which are subsequently used for immunoblotting or staining, approaches that were successfully performed to detect certain proteins in *S. perigrina* hemocytes (Kobayashi *et al.,* 1991; Hori *et al.,* 1997). Currently, only a restricted amount of assays can be performed on *Sarcophaga* and no transgenic flies were produced so far, since this species is not that well characterized as *Drosophila*. Albeit *N. vitripennis* does not parasitize *Drosophila melanogaster*, this fruit fly species is sensitive for its venom, although to a lesser extent than natural hosts (River *et al.,* 1993). Since many tools are available for *Drosophila* research and a wide range of transgenic strains are present, it may be an option to investigate the effects of *Nasonia* venom on *Drosophila* pupae at several levels. For instance, assessments of immune responses in *Drosophila melanogaster* and two *Leptopilina* species were already performed to validate microarray data. Here, transgenic *Drosophila melanogaster* strains were used that contain green fluorescent protein GFP under the control of several antimicrobial peptide (AMP) promoters in fat body tissues (Schlenke *et al.,* 2007). Other processes including apoptosis and autophagy can be evaluated as well using *D. melanogaster* (Meier *et al.,* 2000b; Mcphee *et al.,* 2010; Klionsky *et al.,* 2012).

Although, this study provides us better insights in how the host is affected upon parasitation by the ectoparasitoid wasp *N. vitripennis*, more research needs to be performed on the affected pathways and in particular host tissues, in order to construct a meaningful

framework. The fact that only a limited amount of genes in whole hosts were differentially expressed may imply that venom targets only specific pathways and tissues.

1.2. Impact of *N. vitripennis* venom on non-host insect cell lines

In general, *N. vitripennis* venom is toxic for a broad range of insect species and cell lines, including lepidopteran species, which are labeled as an adequate model for wasp venom research (Rivers *et al.,* 1993; 1999). In addition, many genes involved in cell death in *Drosophila* have also been identified in Lepidoptera. The Sf21 cell line tested in this study showed that venom of *N. vitripennis* is highly toxic towards these cells, even when cultured as adherent and suspension cells. This is interesting since this cell line originates from the ovary tissues of the Fall Army worm, *Spodoptera frugiperda*, of which the caterpillar stage is regarded as a pest species in the New World for crops (Nagoshi and Meagher, 2008). Another frequently used cell line in *Nasonia* venom research are BTI-TN-5B1-4 cells from the cabbage looper (*Trichopulsia ni*), which are also derived from ovary tissues (Rivers *et al.,* 1999; 2005; 2010). Although *N. vitripennis* does not parasitize lepidopteran species, they are susceptible for its venom and future research may be headed towards insect pest control. Interestingly, the venom is also able to induce apoptosis in a subset of the Sf21 cells when cultured in suspension. The occurrence of apoptosis, as a consequence of envenomation can impair proper development, immune responses and metabolic activities, including midgut functions in insects. Insect control approaches are generally targeting one of these processes (Hakim *et al.,* 2010; Bulmer *et al.,* 2009; Pener *et al.,* 2012). In addition, microarrays on parasitized *S. crassipalpis* showed that several targets involved in programmed cell death had an altered expression level. On the other hand, apoptosis is a temporarily process that rapidly progresses in secondary necrosis when clearance is not possible, which is the case in cell culture systems. It is very likely that apoptosis is only one of the several cell death types induced by the venom, which supports earlier reports on BTI-TN-5B1-4 cells (Rivers *et al.,* 2010). However it is currently unknown how and which apoptotic signaling pathways are targeted by the venom and its effect on other processes in Lepidopteran species.

1.3. Effects of *N. vitripennis* venom on mammalian cells

Currently, only the impact of *N. vitripennis* venom on insects is studied and has led to a better understanding on how hosts and insect cells react upon envenomation (Rivers *et al.,* 1994; 2010). In general, venoms of parasitoid wasps are designed to target host species and alter several physiological processes. However, this study is the first one that also explored the effects of parasitoid wasp venom in the human cell line HEK293T and demonstrates their susceptibility for the venom of this parasitoid. This cell line demonstrated a reduced cell viability or proliferation, which was detected with MTT assays only after a venom treatment of 48 h, in contrast to the Sf21 cell line that showed already a reduced viability 6 h post envenomation. Interestingly, 9 of the 45 pathways tested were affected 8 h after venom treatment and a majority of these are related to stress signaling. Furthermore, some of the affected pathways seem to be involved in tumor progression and several cancers, including Oct4, estrogen receptor and LXR signaling which are popular research targets (Kumar *et al.,* 2012; Siegfried *et al.,* 2009; Schiff *et al.,* 2003; Chuu *et al.,* 2007). In addition, microarrays on parasitized hosts also revealed that transcripts involved in stress, detoxification and the MAPK/JNK pathways were affected. Pathway (in)activation, for instance those that involves the MEF2 and Ap-1 transcription factors, is mediated by receptor activation, others are regulated through binding with small chemical compounds that can pass biological membranes. Insecticides which are normally considered to be species specific, often have potential targets in mammals as observed with methoprene and octapamine (Harmon *et al.,* 1995; Marrs, 2012). Furthermore, several studies have shown that environmental contaminants and insecticides act on both estrogen and aryl hydrocarbon receptors, which may imply the occurrence of endocrine disruptive effects (Medjakovic *et al.,* 2013). The latter is a well-known target for insecticides and AhR agonists have the ability to disrupt endocrine signaling. In addition, most ligands for AhR are hydrophobic aromatic compounds with a planar structure, although these structures can deviate (Giesy *et al.,* 2002; Denison *et al.,* 1998). However, all these compounds that bind to both ER and AhR are chemicals and so far no proteins that possess AhR or ER binding affinity are currently known. This may indicate that there are even more venom compounds present, in addition to the already identified protein compounds. Future research is necessary to confirm the (in)activation of these signaling pathways, to elucidate the

subsequent responses and to determine the venom components that trigger these responses. In accordance to bee venom, it would be interesting to test several cancer cell lines with *N. vitripennis* venom as well.

1.4. Uncovering the actions of venom proteases and other venom compounds

Characterization of the venom revealed that *N. vitripennis* contains a toolbox of biological weapons which are used to control its hosts and induce several responses (de Graaf *et al.,* 2010; Danneels *et al.,* 2010). In the present study, both serine and metalloproteases were found to be toxic for the Sf21 cell line. Moreover, venomous serine proteases were found to be involved in apoptotic cell death. Both protease families are known for their functions in immune responses (Toubarro *et al.,* 2009; You *et al.,* 2003). Although these two protease families comprise only one fifth of the total venom protein ID's, a large part in the cytotoxic response observed in Sf21 resulted from these proteases. Screening of these proteases may elucidate their individual toxicity and other functions. However, other venom compounds are also worthwhile to investigate, because inhibition of both serine and metalloproteases was not enough to rescue all cells from death. Since venom of *N. vitripennis* is toxic for a broad range of insects, it may contain promising compounds for insect biological control research, which are environmentally safe. Interestingly, a teratocyte secretory protein (TSP14) from the endoparasitoid wasps *Microplitis croceipes* was successfully expressed in transgenic tobacco which resulted in a larger resistance against lepidopteran pests. *Heliothis virescens* and *Manduca sexta* larvae that were fed on these leaves displayed a higher mortality and an altered development (Maiti *et al.,* 2003). A chitinase produced by teratocytes of *Toxoneuron nigriceps* was also used to create transgenic tobacco plants. Feeding tobacco leaves to larvae of *Heliothis virescens* affected survival (Rossi *et al.,* 2012). This enzyme is able to disrupt the perithrophic membrane in the gut epithelium and interferes with the digestive process of the pest species. Venom of *N. vitripennis* also contains a chitinase which may be investigated in the future for its potential use in the development of pest strategies. In addition, venom may contain other compounds of non-protein nature, such as hormone mimics that may play major roles in subduing hosts. Venom that was filtered, resulting in only components smaller than 10kDa, was not able to induce visible cytotoxic effects after 24h in Sf21 cells using MTT assays. In addition, denaturation and inactivation of venom proteins resulted in an absence of cytotoxic effects.

However, these observations does not imply that only venom proteins are important for venom functionality, since other processes are affected too, which cannot be measured with MTT assays. Instead it is possible that the remaining components are not cytotoxic at all but affect other pathways. Although there are hypotheses that the observed developmental arrest in parasitized hosts could be the result of metabolic changes or cell death in the brains, ecdysteroid treatment of the host could not abolish this arrest (Rivers *et al.,* 1994, 2011). Apart from this finding, presently no information is available about the influence of endocrine signaling pathways in *Nasonia* infected hosts, albeit several parasitoid wasps are known to modify the endocrine system of their host, as observed in the *P. puparum-Pieris rapae* (Zhu *et al.,* 2009), *Glyptapanteles liparidis-Lymantria dispar* (Schafellner *et al.,* 2007) and the *Eulophus pennicornis-Lacanobia oleracea* (Edwards *et al.,* 2006) interactions.

2. The role of EGFR in *Apis mellifera* worker ovary activation

A honeybee colony consists of fascinating polyphenism which consist of two alternative forms of adult females, namely one fertile queen and many thousands of sterile workers. The molecular pathways that determine caste differentiation are presently well studied and has led to a better understanding of the underlying mechanisms (Maleszka, 2008). A recent study conducted by Kamakura (2011) showed that EGFR signaling plays a crucial role in queen-worker determination. Furthermore, EGFR signaling was found to be involved in embryonic gonad development, oogenesis and negative regulation of apoptosis in *Drosophila* (Weyers *et al.,* 2011; Van Buskirk *et al.,* 1999; Parker *et al.,* 2006). However, far less is known about the mechanisms involved in the shift from sterile to fertile workers, which occurs on a small scale in queen right colonies but increases in queenless situations. Interestingly, transcriptome and proteome studies revealed that there is a possible overlap of the molecular pathways involved in both caste determination and worker reproduction, including EGFR signaling (Cardoen et al. 2011b; Grozinger *et al.,* 2007). In this study, for the first time EGFR signaling was found to be involved in regulating worker reproduction. By RNAi knockdown of EGFR in caged workers, ovary activation was inhibited compared to the control group. This is also the first direct evidence that there is indeed an overlap in the molecular pathways involved in both caste differentiation and worker reproduction. However, since previous large-scale studies pointed out that the EGFR inhibitors *Argos*, *Sprouty* and *Cbl* were all upregulated in non-reproductive worker bees (Cardoen *et*

al., 2011b), the next step could be to down regulate these targets by RNAi. This should result in the increased ovary activation in workers. Although EGFR RNAi knockdown was successfully confirmed by qRT-PCR on complete honeybee workers, additional information about the location of EGFR expression and knockdown is lacking. Therefore it would be interesting to investigate the transcriptome or proteome of certain tissues like the ovaries upon RNAi treatment. Another way to investigate overlapping molecular mechanisms is by targeting the nutrition sensing insulin/IGF and TOR signaling pathways in caged workers.

However, in honeybees epigenetic regulation was also found to be involved in caste differentiation (Kucharski *et al.,* 2008). Interestingly, methylation levels are associated with the nutritional input the larvae receive during their development, which are higher in workers (Kucharski *et al.,* 2008). Investigation of these methylation levels during EGFR knockdown or RNAi treatment targeting other components involved in these nutrition sensing pathways may give us the possibility to attain a broader view on worker ovary activation.

3. Final conclusions

In this work, *N. vitripennis* venom was extensively studied and revealed some of its possible functions in both natural hosts and non-host cell lines, which was found to target specific pathways. Some of these are involved in immunity and stress resulting in cytotoxic effects such as apoptosis, which is in accordance with the available literature. Two protease families, metallo-and serine proteases, present in the venom were found to exert these cytotoxic effects of which the latter is involved in apoptosis in Sf21 cells. Furthermore, merging of the obtained results suggest that also other venom components of non-protein nature are present in the venom. The second part of this thesis, supports the notion that there is an overlap in the molecular pathways involved in both caste differentiation and worker reproduction. Here, EGFR was found to play a key role in honeybee worker ovary activation.

Summary / Samenvatting

SUMMARY

Hymenoptera such as honeybees and parasitoid wasps are considered as beneficial insects due to their role in plant pollination and as biological control agents for both agricultural and medically important pests. Their importance has led to the sequencing of both the honeybee, *Apis mellifera* and the parasitoid wasp, *Nasonia vitripennis* genomes. Both insect species are considered as novel model species, which may complement and expand the existing knowledge, acquired with *Drosophila*. From that point on, advanced research was performed in order to understand the biological processes of several related topics including venomics, reproduction, developmental biology and others.

The main part of this work encompass studies that were performed using the ectoparasitoid wasp *Nasonia vitripennis*. These wasps parasitize fly pupa of the genera *Sarcophaga* and *Calliphora*, while using their venom to modify several physiological processes in their hosts. These changes include the alteration of immune responses, arrestment of development and a modified metabolism which is synchronized with the development of the parasitoid larvae. In this thesis we successfully used RT-PCR targeting *Nv_nos* to verify parasitation success of the exposed pupae. Subsequently, the early changes in the pupal transcriptome of *Sarcophaga crassipalpis*, 3h and 25h after parasitation by the ectoparasitoid wasp, *Nasonia vitripennis* were examined. Using these time points, only the responses to venom injections were measured since the wasp eggs are not hatched yet. Three hours after parasitation only one gene was differentially expressed, while 25h post parasitation, 128 genes were differentially expressed. These data were validated by qRT-PCR of some selected genes. 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. This demonstrates that the venom alone is able to alter the metabolic landscape and developmental fate of the fly host prior to subsequent penetration of the pupal cuticle by the wasp larva.

However, since we are also interested in the location where the venom of *N. vitripennis* induces cell death their natural hosts, immunohistochemical stainings of *S. crassipalpis* pupae were made at 4h, 26h and 50h post parasitation. Furthermore, the parasitoid eggs were removed in half of the 50h group while leaving the other pupae untouched in order to

assess the effects of the feeding larvae. Observations of these pupae pointed out that when the envenomation time proceeds, more and more cell death is occurring in both hemocytes and trophocytes, especially at the exposed abdominal regions where wasps could inject their venom and eggs. This could indicate that immune responses and metabolism are indeed targeted upon parasitation, although it must be mentioned that more data are needed in order to confirm this. Interestingly, pupae with parasitoid larvae displayed more pronounced cell death around the legs of their hosts, in contrast to pupae without larvae. This probably indicates that larvae prefer host tissues with a fragile cuticle and epidermis that is easily damaged in order to rapidly access host hemolymphe.

Since proteases are predominant venom components of the ectoparasitoid *N. vitripennis*, two protease families were targeted using protease inhibitors, in order to reveal their cytotoxic effects on the Lepidopteran *Spodoptera frugiperda* (Sf21) cells. As complete venom is able to induce apoptosis in Sf21 cells, both serine and metalloproteases were found to be cytotoxic venom components, although their activity differs between adherent and suspension cells. Under physiological conditions, these venomous proteases may possibly function in immune related processes and tissue destruction enabling venom distribution.

Furthermore, we explored the possible functions of parasitoid wasp venom in the human cell line HEK293T. Surprisingly this cell line appears to be susceptible for *N. vitripennis* venom, which results in a reduced viability 48h post envenomation. However, compared to the Sf21 cell line, this cell line is far less susceptible for parasitoid wasp venom. In addition, this cell line was used to screen for the activity of 45 pathways upon venom treatment. Eight pathways were upregulated and one was found to be downregulated after a venom treatment of 8h. Most of these pathways are related to stress responses. Interestingly, members of the nuclear receptor family, such as the liver X receptor, retinoid X receptor and the estrogen receptor were all upregulated in venom treated cells, as well as the aryl hydrocarbon receptor. These receptors are activated upon ligand binding, although their ligands are chemical compounds. This may indicate that not only proteins are present in the venom, but also smaller compounds of non-protein nature.

Another topic that was investigated in this work encompasses *Apis mellifera* worker fertility. A honeybee colony consists of one fertile queen and many thousands of sterile workers.

However a small part of the workers can become fertile, depending on the conditions, as observed in queenless situations. According to previous studies that suggested the overlap in molecular pathways involved in both caste differentiation and worker reproduction, we found direct evidence for this overlap using EGFR knockdown. Furthermore, EGFR was found to be a key player in honeybee worker ovary activation, since EGFR knockdown in caged workers resulted in sterility. This is in contrast to the control group of which a substantial percentage of honeybee workers contained activated ovaries.

SAMENVATTING

Talrijke hymenoptera zoals honingbijen en parasitoide wespen worden beschouwd als nuttige insecten omwille van hun belangrijke rol in de bestuiving van planten en hun potentieel als biologisch bestrijdingsmiddel tegen verscheidene plaagsoorten. Omwille van hun impact op het ecosysteem werden zowel de genomen van de honingbij, *Apis mellifera* als dit van de parasitoide wesp, *Nasonia vitripennis* gesequeneerd. Tegenwoordig worden beide soorten beschouwd als nieuwe model organismen die de kennis, verworven tijdens het *Drosophila* onderzoek verder kunnen aanvullen. De beschikbaarheid van deze genomen heeft bijgevolg ook geleid tot nieuwe inzichten in verschillende onderzoeksthema's zoals ondermeer het gifonderzoek, reproductie en ontwikkelingsbiologie.

Een groot deel van dit doctoraatsonderzoek handelt over de ectoparasitoide wesp *N. vitripennis*. Deze soort parasiteert vliegenpoppen van zowel *Sarcophaga* als *Calliophora* genera, waarbij het gif van deze wesp tal van fysiologische processen in de gastheer beïnvloedt. Opvallende effecten van dit gif zijn onder andere de gewijzigde immuun responsen, ontwikkelingsstop en een gewijzigde metabolische activiteit die gesynchroniseerd is met de ontwikkeling van de parasitoide larven. Om na te gaan of de gastheerpoppen effectief geparasiteerd zijn werd via RT-PCR gescreend naar de aanwezigheid van *Nv_nos* dat tot expressie komt in de eitjes van deze wesp. Vervolgens werden de vroege transcriptoom veranderingen onderzocht in *Sarcophaga crassipalpis* poppen, 3 uur en 25 uur na parasitatie door de ectoparasitoide wesp *N. vitripennis*. Op deze tijdstippen werden enkel de effecten van het gif waargenomen, want op dit moment zijn de parasitoide larven nog niet zijn uit het ei gekomen. Drie uur na parasitatie had slechts één gen een gewijzigd expressieprofiel, terwijl 25 uur post parasitatie reeds 128 genen een gewijzigd expressie profiel vertoonden, wat kon gevalideerd worden met qRT-PCR. Deze 128 responsieve genen werden vervolgens in 8 clusters geplaatst die een rol spelen in het wijzigen van metabolische activiteit, ontwikkeling, inductie van immuun responsen, detoxificatie responsen en geprogrammeerde celdood. Deze studie toont aan dat het gif alleen in staat is om de fysiologische processen in de gastheer te wijzigen voordat de gastheer wordt aangetast door de parasitaire larven.

Voortbouwend op de resultaten uit de vorige studie, werden immunohistochemische kleuringen uitgevoerd op complete *S. crassipalpis* poppen, met als doel celdood te lokalizeren in de verschillende weefsels. De kleuringen werden uitgevoerd op 4 u, 26 u en 50 u post parasitatie, waarvan bij de helft van de poppen de eitjes werden verwijderd in de 50 u groep. Op deze manier werden poppen onderzocht die enkel met gif werden geïnjecteerd, terwijl men bij de andere helft het effect van zowel het gif als de voedende larven kon bestuderen. Naargelang de inwerkingstijd van het gif toenam, werd meer celdood waargenomen in hemocyten en trofocyten van geparasiteerde poppen. De locatie waar de meeste celdood optreedt situeert zich in de abdominale regio's die blootgesteld waren voor gif injectie en het afleggen van de parasitoide eitjes. Deze gegevens kunnen er enerzijds op wijzen dat immuun en metabolische processen inderdaad één van de doelwitten zijn van dit gif. Anderzijds moet worden opgemerkt dat meer data nodig is om deze veronderstellingen te bevestigen. Poppen in de 50 u groep, waarvan de wespen larven niet waren verwijderd vertoonden meer celdood rond de poten, in tegenstelling tot controle poppen. Dit kan erop duiden dat de larven een voorkeur hebben voor weefsels met een broze cuticula en epidermis, die gemakkelijk te beschadigen is om snel toegang te krijgen tot hemolymfe van de gastheer.

Proteases zijn één van de meest voorkomende gif componenten in *N. vitripennis*. In deze studie werden twee protease families onderzocht op mogelijk cytotoxische effecten in *Spodoptera frugiperda* (Sf21) cellen (Lepidoptera). Met behulp van protease inhibitoren werd de werking van deze proteasen in het gif uitgeschakeld. Volledig gif is cytotoxisch voor Sf21 cellen en veroorzaakt apoptosis. Serine en metalloproteasen in het gif spelen hierbij een belangrijke rol, want beide families zijn toxisch voor deze cellijn. Bovendien blijken serine proteasen ook betrokken te zijn in apoptosis. Verassend genoeg verschilt hun activiteit tussen adherente en suspensie cellen. In de natuurlijke gastheer kunnen deze proteasen mogelijks een rol spelen in immuun processen en weefselbeschadiging die vervolgens de verspreiding van het gif bevorderen.

In dit project werden voor de eerste maal ook de effecten van gif afkomstig van parasitoide wespen bestudeerd in de humane cellijn HEK293T. Ook deze cellen blijken gevoelig te zijn voor *N. vitripennis* gif, die de cel vitaliteit en proliferatie beïnvloedt na een gif behandeling van 48 u. Deze cellijn is echter veel minder gevoelig voor dit gif dan de Sf21 cellijn. Daarnaast

werd ook de activiteit van 45 humane pathways nagegaan in HEK293T cellen, na een gif behandeling van 8 u. Van de 45 pathways waren er 8 met verhoogde activiteit en 1 met een verlaagde activiteit in vergelijking met de controle groep. De meerderheid van deze pathways spelen een rol in stress responsen. Verder was er ook een verhoogde activiteit van enkele nucleaire receptoren zoals de lever X receptor, retinoïde X receptor en de oestrogeen receptor. Dit was ook het geval bij de aryl hydrocarbon receptor. Deze receptoren worden geactiveerd door ligandbinding, waarbij dit overwegend chemische componenten zijn. Dit kan erop wijzen dat er niet enkel proteïnen aanwezig zijn in het gif, maar ook kleinere chemische componenten.

Een andere onderzoekslijn in deze doctoraatsthesis handelt in het kader van honingbij fertiliteit, onder meer het vermogen van *Apis mellifera* werksters om hun ovaria te activeren. Hoewel een bijenkolonie bestaat uit 1 vruchtbare koningin en vele onvruchtbare werksters, blijkt een klein deel van deze werksters geactiveerde ovaria te bezitten. Het aandeel werksters dat hiertoe in staat is, hangt af van de omstandigheden, zoals bij het ontbreken van een koningin. Voorafgaande studies hebben reeds aangehaald dat mogelijks dezelfde moleculaire pathways aan de basis liggen van kaste differentiatie en de reproductie van werksters. In deze studie werd dit bevestigd via neerregulatie van EGFR. Bovendien blijkt EGFR een hoofdrol te spelen in de ovaria activatie van werksters. Neerregulatie van EGFR resulteerde immers in steriele werksters, terwijl een groter aandeel geactiveerde ovaria had in de controle groep.

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Curriculum vitae

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- Dissertation topic: Exploring diverse molecular processes in two genome sequenced hymenopteran model systems: *Nasonia vitripennis* – (non)host interactions and honeybee reproduction
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Publications

- Cardoen D., Ernst U.R., Boerjan B., Bogaerts A., Formesyn E.M., de Graaf D.C., Wenseleers T., Verleyen P., Schoofs L. (2012). Worker honeybee sterility: a proteomic analysis of suppressed ovary activation. Journal of Proteome Research

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- Danneels E.L.*, Formesyn E.M.*, Hahn D.A., Denlinger D.L., Cardoen D., Verleyen P., Wenseleers T., Schoofs L., de Graaf D.C. (2013). Early responses in the pupal transcriptome of the flesh fly *Sarcophagha crassipalpis*, to parasitation by the ectoparasitic wasp, *Nasonia vitripennis*. Insect Biochemistry and Molecular Biology (*Shared first authors)

- Formesyn E.M.*, Cardoen D.*, Ernst U.R., Danneels E.L., Van Vaerenbergh M., De Koker D., Verleyen P., Wenseleers T., Schoofs L., de Graaf D.C. (2013). Reproduction of honeybee workers is regulated by epidermal growth factor receptor signaling. Manuscript submitted in: General and Comparative Endocrinology. (*Shared first authors)

International stays

- Agricultural institute of Slovenia. Analysis of parasitized and control pupae, from 17 June to 21 June 2013 in cooperation with Prof. Dr. Aleš Gregorc.

Conventions

- Formesyn E.M., Danneels E.L., Jacobs F.J. & de Graaf D.C. (2010). – poster presentation. Twenty-three unique venom proteins of *Nasonia vitripennis* recombinant expressed for functional analysis. 17th Benelux Congress of Zoology , Gent, België, 22-23 oktober 2010, p. 127.

- Formesyn E.M., Danneels E.L., Jacobs F.J. & de Graaf D.C. (2010). – poster presentation. Exploring the functionality of *Nasonia vitripennis*' unknown venom proteins. Bee-together: conference on pollinators with emphasis to stimulate interactions in the field, Gent, België, 21 december 2010, p. 25.

- Formesyn E.M., de Graaf D.C. (2011). – Lecture. The venom of *Nasonia vitripennis*: only effective against insect cells? The Nasonia Meeting 2011, Nashville, U.S.A., Vanderbilt University, 14-17 juni 2011, p. 8.

- Formesyn E.M., Danneels E.L., de Graaf D.C. (2012). – poster presentation. Involvement of several venomous proteases in the viability of the Sf21 cell line. 60th Annual Meeting of the Entomological Society of America (Entomology 2012), 11-14 november, p. 141.

Workshops

- Danneels E.L., Formesyn E.M., Jacobs F.J. & de Graaf D.C. (2010). – poster presentation. 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, Gent, België, 30 augustus – 1 September 2010, p. 19.

- Formesyn E.M., (2012). Summer course Beneficial, Pest and vector insects 2012. Leuven, België, 11-15 June.

Guidance of students

Practical courses

- Integrated practical exercises 1st Bachelor in Medicine and Dentistry: Aspects of peripheral blood research, Prof. Dr. Frans Jacobs
- Practical exercises Biomedical physiology (2nd Bachelor in Biochemistry and Biotechnology), Prof. Dr. Dirk de Graaf
- Practical exercises Insect Physiology (1st and 2nd Master in Biology), Prof. Dr. Dirk de Graaf

Bachelor projects

- 2010-2011: Assessing the viability of the Sf21 cell line using thiazolyl blue tetrazolium bromide (MTT) after *Nasonia vitripennis* venom exposure. Lien Steenwinckel, Lisa Van den Broeck, Jolien Van Hecke

Master projects

- 2010-2011: RACE technology and recombinant expression of venom compounds from the ectoparasitoid *Nasonia vitripennis*. Ine Opsteyn and Hannes Demaré
- 2011-2012: Confirmation of microarray data : the fleshfly *Sarcophaga crassipalpis* whether or not envenomated by *Nasonia vitripennis*. Chengetai Mpamhanga

Master thesis

- 2009-2010: The venom of the parasitoid wasp *Nasonia vitripennis*: recombinant expression of new venom compounds and the effect on cellular immune responses in the host. Wim Jonckheere
- 2009-2010: The venom of the parasitoid wasp Nasonia vitripennis: recombinant expression of new venom compounds and the effect on humoral immune responses in the host. Dieter De Koker