Progress is made by trial and failure; the failures are generally a hundred times more numerous than the successes; yet they are usually left unchronicled.

Sir William Ramsay (1852-1916)

Science is the acceptance of what works and the rejection of what does not. That needs more courage than we might think.

Jacob Bronowski (1908-1974)

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Glycosylation in *Tribolium castaneum:* composition, physiological significance and exploitation for pest control

ir. Tomasz Walski

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the tile:

Glycosylering in *Tribolium castaneum*: samenstelling, fysiologische betekenis en exploitatie voor ongediertebestrijding

Front cover: (upper left panel) Adult of the red flour beetle, (upper right panel) pupa-adult intermediate resulting from disruption of N-glycan processing, (lower left panel) Fluorescently-labeled lectin RSA in the midgut of the red flour beetle larva, (lower right panel) Schematic representation of a glycoprotein.

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List of abbreviations

α-Man-I	Class I α-mannosidase
α-Man-II	Class II α-mannosidase
β1,3GalTs	β-1,3-galactosyltransferase
β4GalNAcT	β-1,4-N-acetylgalactosaminyltransferase
2-AB	2-aminobenzamide
AA	Anthranilic acid
ActD	Actinomycin D
Alg1-14	Apoptosis linked gene 1-14, group of genes involved in N-glycan precursor synthesis
Bt/Cry toxins	Toxins from *Bacillus thuringensis*
C1GalTA	Core 1 Galactosyltransferase A
CF-203	A lepidopteran midgut cell line from the eastern spruce budworm, *Christoneura fumiferana*
CBD	Chitin biding domains
CMP	Cytidine monophosphate
CNS	Central nervous system
CNX cycle	Calnexin/Calreticulin cycle
Csas	CMP-sialic acid synthase
DAD1	Defender against death, essential subunit of the oligosaccharyl transferase
dHex	Deoxyhexose (e.g. fucose)
dsRNA	Double stranded RNA
EC_{50}	The concentration of a compound that gives half-maximal response
EGF	Epidermal growth factor
Eogt	EGF-domain O-GlcNAc transferase
ERAD	Endoplasmic-reticulum-associated protein degradation
ERQC	Endoplasmic-reticulum-derived quality control compartment
FDL	Fused lobes, N-acetylglucosaminidase
FITC	Fluorescein isothiocyanate
Fng	Fringe, fucose-specific β-1,3-N-acetylglucosaminyltransferase
Fuc	Fucose
FucT	Fucosyltransferase
Gal	Galactose
GalNAc	N-acetylgalactosamine
GCS1 or GCS2	Glucosidase 1 or 2
GDP	Guanidine diphosphate
Glc	Glucose
GlcA	Glucuronic acid
GlcAT	Glucuronic acid transferase
GlcNAc	N-acetylglucosamine
GNA	*Galanthus nivalis* agglutinin, a lectin from snowdrop

Hex	Hexose (e.g. mannose, galactose or glucose)
HexNAc	N-acetylhexosamine (e.g. N-acetylglucosamine or N-acetylgalactosamine)
HHA	*Hippeastrum hybrid* agglutinin, a lectin from Amaryllis
IIM	Insect Intesntinal mucins
JH	Juvenile hormone
MAA	*Maackia amurensis* agglutinin, a lectin from the Maackia tree
Man	Mannose
ManNAc	N-acetylmannosamine
Mgat1	Mannosyl (α-1,3-)-glycoprotein β-1,2-N-acetylglucosaminyltransferase
mRNA	Messenger RNA
MS	Mass spectrometry
Neu5Ac	N-acetylneuraminic acid, one of sialic acids
NeuGc	N-glycolylneuraminic acid, one of sialic acids
Nictaba	*Nicotiana tabacum* agglutinin, a lectin from tobacco
HPLC	Normal phase high pressure liquid chromatography
Ofut1	O-fucosyltransferase 1
Oga	O-GlcNAcase, O-glycan β-N-acetylglucosaminidase
OST	Oligosaccharyl transferase
PA	2-aminopyridine
PBS	Phosphate buffered saline
PGANT	Polypeptide GalNAc transferase
PM	Peritrophic matrix
POMT	protein O-mannosyl-transferase
RIP	Ribosome inactivating protein
RNAi	RNA interference
RSA	*Rhizoctonia solani* agglutinin, a lectin from a plant pathogenic fungus
S2	Schneider 2 cells line derived from the fruit fly, *Drosophila melanogaster*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sia or SA	Sialic acid
SNA-I	*Sambucus nigra* agglutinin I, a lectin from the elderberry
SNA-II	*Sambucus nigra* agglutinin II, a second lectin from the elderberry
SSA	*Sclerotinia sclerotiorum* agglutinin, a lectin from a plant pathogenic fungus
ST6Gal or SiaT	Sialyltransferase
STT3 (A or B)	Isoforms of catalytic subunit of oligosaccharyl transferase
Sxc or Ogt	Super sex combs, O-GlcNAc transferase
T antigen	O-linked Galβ1,3GalNAc
TFA	Trifluoroacetic acid
Tn antigen	O-linked GalNAc
TRITC	Tetramethylrhodamine
UDP	Uridine diphosphate
UGT	UDP-glucose-glycoprotein glucosyltransferase

WGA	Wheat germ agglutinin
Xyl	Xylose

Scope

The main aim of this PhD research was to study the importance of insect protein glycosylation and to test whether disruption of this process can be an effective novel strategy for insect control.

Chapter 1 summarizes the current knowledge on protein glycosylation and explains how glycosylation can be linked to insect control. Glycosylation is a process during which proteins are attached with sugars or sugar chains (glycans). According to estimates even up to half of all the proteins in a given organism can be glycosylated. Notably, erroneous glycosylation results in disruption of many life functions and is often lethal. Following this logic interference with insect glycosylation is likely to be an effective way to control pest insects. Unfortunately, little is known concerning the glycosylation in insects other than the fruit fly. Clearly, for effective use of this strategy for crop protection, more insight into the physiological importance of N-glycosylation in actual pest insect species is necessary. Thus this thesis focuses on N-glycosylation in the red flour beetle, *Tribolium castaneum*, which is a notorious pest but also offers the advantages of a model organism including sequenced genome and availability of molecular tools for studying gene functions.

In my PhD I tried to answer three main questions. (i) Which glycans are present on insect proteins? (ii) What are the functions of different types of glycans? (iii) Can the glycosylation be effectively disturbed for crop protection purposes?

Chapter 2 explores one of the possible ways to disrupt bioprocesses mediated by glycans, that is through the use of lectins or proteins that can bind the glycans. A range of in vitro experiments was used to select potentially most effective lectins for further examination in living insects. First, we tested toxicity of two lectins SNA-I and MAA with specificity towards complex glycans, against two insect cell lines. Additionally, confocal microscopy and fluorescently labeled lectins were used to perform a screening of lectin binding sites present on the surface of primary insect midgut cells. These experiments indicated that the insecticidal potency of lectins might depend on their glycan binding specificity and the repertoire of glycans present on the insect cell surface.

Chapter 3 comprises the verification of toxicity of the lectins selected in **Chapter 2** against the red flour beetle, *Tribolium castaneum*. For that the insecticidal effects of these lectins were first confirmed using a *Tribolium* cell line, and subsequently, the lectins were tested against *Tribolium* larvae through feeding assays. Additionally, we looked into the lectin fate in larval guts by monitoring lectin resistance to proteolysis and their ability to pass through the peritrophic matrix – a protective mesh-like structure lining the beetle gut. Finally, we performed injection assays on larvae to verify if lectins can induce systemic toxicity. Altogether these experiments showed a range of factors influencing oral toxicity of the lectins and indicated several ways for improvement of the insecticidal efficiency of these proteins.

Chapter 4 is focused on a detailed analysis of N-glycans found on beetle proteins, which is a first prerequisite for functional studies of N-glycosylation. For this purpose we employed several different strategies of glycan derivatization and analysis including high pressure liquid chromatography and mass spectrometry to characterize both monosaccharide compositions and intact N-glycans in larvae and adult beetles. The most successful approach included labeling of glycans with 2-aminobenzidine and analysis with MALDI-TOF-MS. This strategy resulted in the identification of over 20 N-glycan structures in larvae and adults and indicated quantitative differences in N-glycan composition between these life stages.

Chapter 5 describes functional studies of N-glycosylation in the red flour beetle and tests of the molecular approach for disruption of the process. First, *Tribolium* genes involved in the N-glycosylation pathway were identified using phylogenetic analyses. Second, qPCR was used to study changes in expression of genes during the post-embryonic development and find out the basis of changes in the N-glycosylation patterns between the life stages identified in **Chapter 4**. Third, through RNAi-mediated knockdown of the genes involved in the N-glycan attachment and processing we studied the involvement of the different steps of the N-glycosylation pathway in post-embryonic development. These efforts revealed that N-glycosylation is crucial for larval growth and metamorphosis in the red flour beetle.

Chapter 6 summarizes the obtained results, discusses their potential applicability and indicates further research directions.

Chapter 1

Introduction

1.1. Protein glycosylation

Glycosylation or the attachment of an oligosaccharide is a common posttranslational modification of proteins. The addition of sugar molecules to a polypeptide chain may affect protein properties including structure, solubility, charge or sensitivity to proteolysis (Staudacher, 2015). Furthermore, protein-linked sugars can directly be involved in numerous phenomena including enzyme activity regulation, secretion, cell signaling or attachment, and thus are crucial to most life functions of all organisms (Varki and Lowe, 2009). Currently several thousand of proteins have been shown to be glycosylated. However, there are still many more to be discovered since it has been estimated that half of all the proteins could be modified with glycans (Apweiler et al., 1999). In insects there are eight main monosaccharides fucose (Fuc), galactose (Gal), glucose (Glc), N-acetylgalactosamine, (GalNAc), N-acetylglucosamine (GlcNAc), glucuronic acid (GlcA), mannose (Man), xylose (Xyl) and additionally sialic acids (SA) that can be assembled to hundreds of different glycan structures. This gives rise to possibly hundreds of protein isoforms and adds another level of functional diversity beyond this conveyed by the amino acid sequence (Moremen et al., 2012).

This chapter will focus on two main types of protein glycosylation: O- and N-linked in which sugars can be attached to a protein via oxygen or nitrogen atoms, respectively.

1.1.1. N-glycosylation

The N-linked glycans are attached almost exclusively to asparagine residues in a defined sequons: N-X!-T/S (N is asparagine, X! is any amino acid except proline, T/S is threonine or serine). The N-glycosylation was also found on non-canonical sequons including N-X!-C, N-X!-V and N-G (C is cysteine, V is valine, G is glycine) but those cases were rare (Zielinska et al., 2010).

In the N-glycosylation reaction a preassembled oligosaccharide $Glc_3Man_9GlcNAc_2$ is transferred in whole from the lipid carrier to a consensus sequon in the acceptor protein. Once attached, this 14-units large oligosaccharide is trimmed down and modified by range of endoplasmic reticulum (ER) and Golgi resident enzymes to produce a variety of N-glycans (Stanley et al., 2009). These may have different terminal structures but always contain the same core unit composed of two GlcNAc residues (Fig. 1.1). The following sections will address various aspects of the biology of N-glycosylation starting from the synthesis, through the processing to the functional importance of N-glycans.

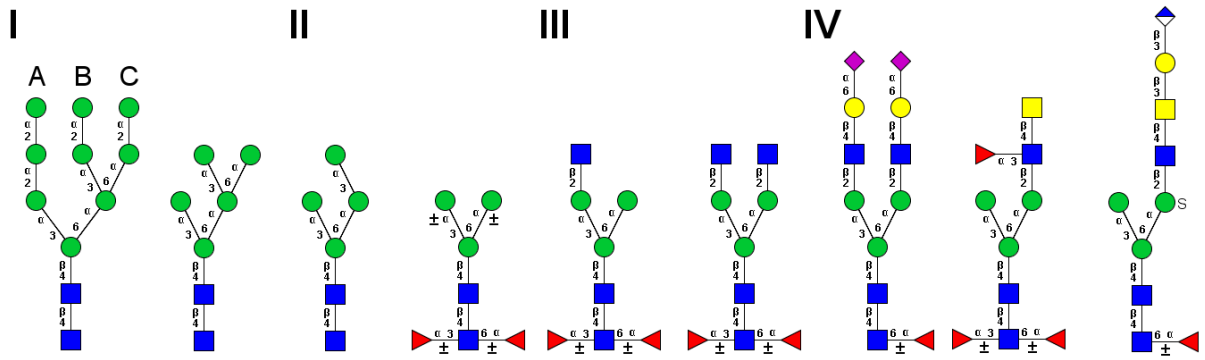


Fig. 1.1. N-glycan types present in insects. □ **- GalNAc, N-acetylgalactosamine,** ○ **- Gal, galactose,** ■ **- GlcNAc, N-acetylglucosamine,** ▲ **- Fuc, fucose,** ◇ **- GlcA, glucuronic acid,** ● **- Man, mannose,** ◆ **- Neu/Sia, Neuraminic acid/sialic acid, S – sulfate group, α, β and numbers indicate linkages. (I) High mannose glycans with nine to five mannose residues, A-C indicate the three antennas of a high mannose N-glycan, (II) Paucimannose glycans containing less than five mannoses and possible core fucoses but no terminal GlcNAc, (III) Common insect complex glycans with terminal GlcNAc residues, (IV) Rare insect complex glycans with various terminal modifications.**

1.1.1.1. Synthesis of lipid linked N-glycan precursor

Before attachment to the protein the N-glycan precursor is built in a stepwise fashion on the lipid carrier dolichol pyrophosphate (PP-Dol) embedded in the ER membrane. In the first step the enzyme Alg7 utilizes UDP-GlcNAc to synthesize the GlcNAc-PP-Dol, which would serve as the base for further extension reactions (Burda and Aebi, 1999). The activity of the Alg13/Alg14 complex adds a second GlcNAc residue to form the chitobiose core, a characteristic feature of all eukaryotic N-linked glycans. Next, through a series of reactions catalyzed by Alg1, Alg2 and Alg11, the chitobiose core is extended with five mannose residues to form the branched $Man_5GlcNAc_2$-PP-Dol intermediate (Gao et al., 2005; Larkin and Imperiali, 2011). In insects the formation of this structure is essential for the whole pathway as indicated by lethal phenotypes caused by disruption of the genes coding for the enzymes involved in its production (Donitz et al., 2014; Mummery-Widmer et al., 2009; Neumüller et al., 2011; Yamamoto-Hino et al., 2015).

The next phase involves further elongation of the glycan with mannose residues by the Alg3, Alg12, Alg9 enzymes to produce $Man_9GlcNAc_2$. The whole process is finished by the capping of the $Man_9GlcNAc_2$ with a chain of 3 glucose units by Alg6, Alg8 and Alg10. These enzymes form the final $Glc_3Man_9GlcNAc_2$-PP-Dol precursor that is ready to be transferred to a nascent polypeptide. Intriguingly, these steps are not absolutely necessary for successful N-glycosylation – some lower eukaryotes lack one

or more of these genes and still successfully N-glycosylate their proteins (Samuelson et al., 2005). For example, *Trypanosoma brucei* can use $Man_5GlcNAc_2$-PP-Dol or $Man_9GlcNAc_2$-PP-Dol as substrates for N-glycosylation (Izquierdo et al., 2012). In humans, however, mutations in any of these genes result in congenital disorders of glycosylation representing serious and often fatal diseases with numerous symptoms in nervous, muscle and digestive systems (Jaeken and van den Heuvel, 2014). In insects disruptions of some of these genes have been shown to affect development. In *Drosophila* mutations of the Alg6 gene ortholog *gny* caused larval cuticle defects and mortality during the second instar (Shaik et al., 2011). Mutation of the *xit* ortholog of the Alg8 gene disrupted apical constriction of mesoderm precursor cells and ventral furrow formation in early embryogenesis, which was attributed to incorrect intracellular localization of E-cadherin caused by defective N-glycosylation (Zhang et al., 2014c). RNAi of the Alg3 ortholog in *Tribolium* larvae prevented pupal molt and adult eclosion, while RNAi of *gny* and *xit* orthologs resulted in ovary and wing cover defects, respectively (Dönitz et al., 2014).

1.1.1.2. N-Glycan attachment and early processing

Once the lipid-linked N-glycan precursor $Glc_3Man_9GlcNAc_2$-PP-Dol is completed it can be attached by an ER membrane oligoasaccharyltransferase complex (OST) to a newly translated polypeptide chain. The insect OST has not been investigated in detail but similarities between the yeast and the mammalian OST complex provide good indication on its well conserved features among eukaryotes (Kelleher and Gilmore, 2006). The mammalian OST complex contains the catalytic subunit STT3 and non-catalytic subunits including ribophorin I, ribophorin II, OST48, DAD1, N33 or IAP, OST4, DC2, KCP2 which are involved in maintaining complex stability, substrate delivery and regulation of specificity (Roboti and High, 2012). In plants, insects and vertebrates the OST complex contains one of two possible isoforms of the catalytic subunit: STT3A or STT3B. STT3A has higher activity and N-glycosylates polypeptides co-translationally, while the less active STT3B can attach the N-glycan either during translation to the sequons skipped by STT3A or after the polypeptide translation is completed to the sequons positioned near C-termini (Ruiz-Canada et al., 2009).

Once the N-glycan is attached the glycoprotein processing can proceed. The nascent N-glycoprotein is first acted on by GCS1, a glucosidase that cleaves the outermost of the three glucose residues from the N-glycan. This reduces the binding of the glycan with OST complex and produces the substrate for another glucosidase, GCS2, which sequentially removes two remaining glucoses. Removal

of the second glucose promotes binding with calnexin or calreticulin that prevent aggregation and premature degradation of incompletely folded proteins (Helenius and Aebi, 2004). Additionally, this allows the target glycoprotein to associate with thiol-disulfide oxidoreductase (PDI/ERp57) that enhances the formation of disulfide bridges and assists in the adoption of the native conformation. Cleaving of the third glucose residue by GCS2 releases the N-glycoprotein which if properly folded can be subject to further processing steps and leave the ER. If the protein is not completely folded it can be remodified with glucose by the UDP-Glc:glycoprotein glucosyltransferase (UGT) and may bind again with calnexin/calreticulin for another round of folding. This recurrent series of glucose removal, folding and glucose attachment is called calnexin/calreticulin (CNX) cycle and is crucial for glycoprotein quality control. If target protein remains still misfolded after CNX cycle it can be retrotranslocated to the cytoplasm and eliminated through the ERAD or ER-associated degradation pathway (Lamriben et al., 2015). In *C. elegans* RNAi of the alg-1, homolog of GCS1, caused the accumulation of unfolded proteins, changes of N-glycan processing and lead to a reduction of the lifespan (Katoh et al., 2013). Similarly RNAi of GCS1, GCS2, calnexin and calreticulin in *D. melanogaster* resulted in lethal phenotypes (Mummery-Widmer et al., 2009; Neumüller et al., 2011). Reduction of activity of the CNX cycle through gene mutations or chemical inhibitors results in accumulation of misfolded proteins beyond the capacity of the ERAD pathway. This, in turn, will lead to the formation of aggregates, cause ER stress and trigger cell death (Yoshida, 2007).

After the CNX cycle glycoprotein can be processed by the ER α-mannosidase I (α-Man-Ib). This enzyme originally thought to be resident to the endoplasmic reticulum was recently shown to be localized in the ER derived quality control compartments (ERQC) and quality control vesicles (QCV) (Benyair et al., 2015b). α-Man-Ib primarily cleaves the single mannose residue from the middle antenna of $Man_9GlcNAc_2$ to form isomer B of $Man_8GlcNAc_2$. However, the prolonged exposure of an unfolded protein to α-Man-Ib allow this enzyme to cleave also additional α1,2-linked mannoses down to $Man_{5-6}GlcNAc_2$ which will prevent reattachment of glucose by UGT and reentry into the CNX cycle (Benyair et al., 2015a). Instead $Man_5GlcNAc_2$ is recognized in the ERQC and destines a protein that bears this glycan structure for degradation through ERAD (Quan et al., 2008). Coversely, the properly folded glycoproteins are exported to the Golgi where the glycans can be processed towards paucimannose or complex glycans (Schachter, 2009).

1.1.1.3. Golgi mannosidase trimming

Most of the final N-glycan structures are produced in the Golgi apparatus. These glycans can be classified as high mannose, paucimannose, complex glycans and hybrid structures (Fig. 1.1). In insects the first two types are most abundant, while in vertebrates relatively little paucimannose glycans occur but much higher amounts of complex N-glycans are present compared to insects (Schachter, 2009).

In the Golgi apparatus the N-glycan is first processed by the Golgi class I α-mannosidase (α-Man-Ia) which can trim the high mannose glycans down to $Man_5GlcNAc_2$ (Kawar and Jarvis, 2001). In insects the removal of the α1,2-linked mannose from the B antenna is the bottle neck of the process (Kawar et al., 2000; Nemcovicova et al., 2013), therefore it is likely that conversion to $Man_5GlcNAc_2$ is aided by the α-Man-Ib activity which preferentially cleaves this mannose residue. For a large number of glycoproteins glycan processing ends at this stage and they are ready to be delivered to organelles, to the plasma membrane or to be secreted from the cell. In fact the $Man_{5-9}GlcNAc_2$ glycans represent approximately 80% of all detected glycans in silkworm silk glands (Mabashi-Asazuma et al., 2015) or 30-50% in fruit fly adults and embryos (Aoki et al., 2007; Fabini et al., 2001; Leonard et al., 2006; Sarkar et al., 2006).

Genetic ablation of α-Man-Ia in *D. melanogaster* is not lethal, but impairs the immune response and causes minor defects in the wings and the eyes (Kerscher et al., 1995; Mortimer et al., 2012). Surprisingly, Liu et al. (2009) observed that reduction of the α-Man-Ia activity can even have positive effect on the life-span of the fruit flies. The lack of serious defects was attributed to the presence of an alternative N-glycan processing route suggested by the observation that mannose trimming was not blocked completely in α-Man-Ia mutant flies (Roberts et al., 1998).

Indeed insects encode another enzyme that can be involved in the processing of high mannose glycans. SfMan-III identified in the cell line derived from the fall armyworm (*Spodoptera frugiperda*) effectively cleaves $Man_6GlcNAc_2$ to $Man_{3-5}GlcNAc_2$ glycans but can also process $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ (Kawar et al., 2001). Similar enzymatic properties were demonstrated for recombinantly produced α-Man-IIb from *D. melanogaster* (Nemcovicova et al., 2013). Since mutations in α-Man-IIb cause also only minor defects in eye development (Rosenbaum et al., 2014) and immune response (Mortimer et al., 2012) it is likely that this enzyme does not play a crucial role in high mannose trimming but can partially rescue it in the absence of α-Man-Ia.

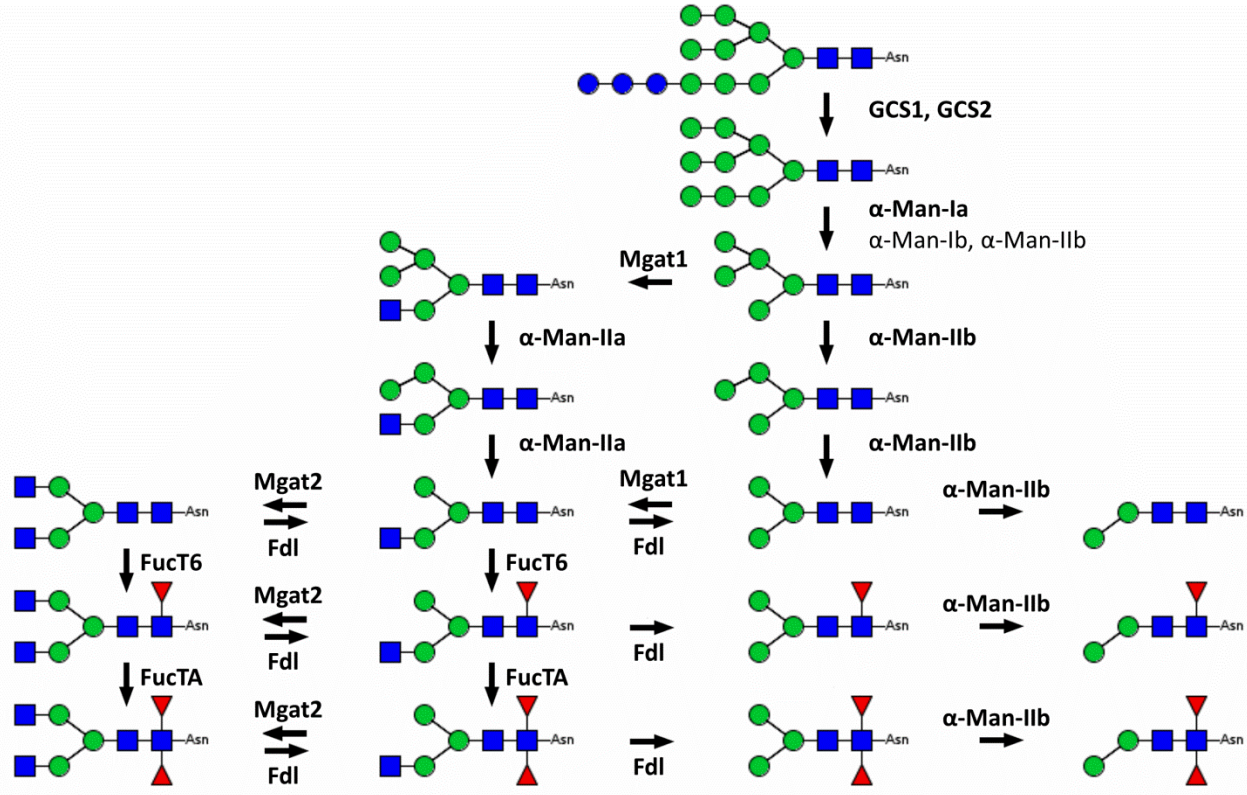


Fig. 1.2. N-glycan processing in insects. Simplified scheme of the pathway leading to production of major insect N-glycans, based on (Hang et al., 2015). ■ - GlcNAc, N-acetylglucosamine, ▲ - Fuc, fucose, ● - Man, mannose, ● - Glc, glucose, Asn - asparagine in a polypeptide chain. Note that the contribution of α-Man-Ib and α-Man-IIb to the trimming of $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ is not completely understood in insects. Based on the similarity with the vertebrate enzyme α-Man-Ib may be primarily involved in folding, quality control and targeting of misfolded proteins for ERAD (Benyair et al., 2015a). α-Man-IIb mainly processes paucimannose N-glycans (Rosenbaum et al., 2014). However, studies involving recombinant enzymes form *D. melanogaster* or *S. frugiperda* indicate that it can also process high mannose N-glycans (Kawar et al., 2001; Nemcovicova et al., 2013).

As mentioned earlier mannose trimming in the ERQC is important for glycoprotein folding and quality control but it is still an open question what is the impact of the final number and position of mannoses, resulting from Golgi processing on the functionality of mature folded proteins. The phenotypes observed in *D. melanogaster* give some indication but it is likely that these defects are not caused by erroneous number of mannoses but rather by disruption of the downstream processing towards paucimannose and complex glycans or blockage of the stepwise deglycosylation (Rosenbaum et al., 2014).

1.1.1.4. Paucimannose and fucosylated N-glycans produced in the Golgi

Further processing in the Golgi apparatus results in the production of paucimannose and complex glycans (Fig. 1.2). In insects paucimannose glycans, containing 2-4 mannoses and up to 2 core fucoses, are highly abundant and together with high mannose glycans usually account for over 90% of all the identified N-glycans (Aoki et al., 2007; Fabini et al., 2001; Leonard et al., 2006; Sarkar et al., 2006).

The $Man_5GlcNAc_2$ produced by class I mannosidase can be extended with a GlcNAc residue by the mannosyl (α-1,3-)-glycoprotein β-1,2-N-acetylglucosaminyltransferase (GNT-I or Mgat1) to form a $GlcNAcMan_5GlcNAc_2$ (Altmann et al., 1993; Sarkar et al., 2006). This terminal GlcNAc residue is required for subsequent action of Golgi class II mannosidase (α-Man-IIa) which removes two mannose residues to form $GlcNAcMan_3GlcNAc_2$ (Altmann and Marz, 1995; Jianxin et al., 1997; Rabouille et al., 1999) the key intermediate for production of paucimannose, fucosylated and complex N-glycans (Paschinger et al., 2005; Staudacher and Marz, 1998).

RNAi and mutations of genes coding for Mgat1 and α-Man-IIa in *D. melanogaster* can be partially lethal before the end of the larval stage (Yamamoto-Hino et al., 2015; Sarkar et al., 2006). Surviving *Mgat1* null mutants exhibited an altered N-glycan profile with greatly reduced paucimannose and complex glycan levels and in consequence showed brain morphology defects, a reduced life span and abnormal movement (Sarkar et al., 2006). Those phenotypes could be rescued by neuronal expression of *Mgat1* which indicated that Mgat1 dependent N-glycans are particularly important for central nervous system (CNS) function (Sarkar et al., 2010). This stage of glycan processing was also shown to be involved in eye development (Rosenbaum et al., 2014) and larval immune response (Mortimer et al., 2012).

Most of the N-glycans carrying terminal GlcNAc and trimmed by class II mannosidase are then processed by the fucosyltransferase FucT6 that attaches fucose residues to the chitobiose core of the N-glycan at α1,6-linkage (Aoki et al., 2007; Fabini et al., 2001; Kurz et al., 2015; Paschinger et al., 2005). A subset of α1,6-fucosylated N-glycans can be further processed by FucTA which attaches another fucose residue in the α1,3-linkage to produce the core difucosylated glycan structure that is unique to insects, nematodes or plants and cannot be synthesized in vertebrates (Fabini et al., 2001; Paschinger et al., 2009; Rendić et al., 2006; Rendić et al., 2010). FucTA can also add a fucose residue to $GlcNAcMan_3GlcNAc_2$ but α1,3-fucosylated glycans cannot be further processed by FucT6 (Paschinger et al., 2005; Staudacher and Marz, 1998).

α1,3-fucosylated glycans are rather low abundant at the level of whole organisms but are particularly enriched in neuronal tissues (Kurz et al., 2016; Rendić et al., 2010). This appears to be due to

temporal and spatial regulation of FucTA expression in *D. melanogaster* – the highest transcript abundance of this gene was detected in adult heads (Rendić et al., 2006). The reduction of FucTA expression caused defects in brain morphology (Yamamoto-Hino et al., 2010) which is also in agreement with CNS-related phenotypes in *Mgat1* mutants. Interestingly, overexpression of FucTA and thus overproduction of fucosylated N-glycans caused wing blistering and aberrant neuronal growth. These defects were the result of a distorted balance with O-fucosylation (see section 1.1.2.4.) because the two processes compete for the same substrate: GDP-fucose (Rendić et al., 2010). Insect genomes encode also additional putative α1,3-fucosyltransferases: FucTB, C and D – but these proteins are apparently not involved in the core fucosylation (Rendić et al., 2006).

At present the role of α1,6-fucosylation is less clear. *FucT6* mutant *D. melanogaster* larvae are viable but exhibit a somewhat impaired immune response to wasp infection (Mortimer et al., 2012). In other studies on fruit flies whole-body suppression of *FucT6* was lethal before adult eclosion, while specific silencing in the wing disks and histoblasts resulted in wing vein defects and abdomen depigmentation, respectively (Yamamoto-Hino et al., 2015).

N-glycan processing in insects involves action of an unusual β-N-Acetylglucosaminidase (FDL) which is another element of the pathway responsible for marked differences between insect and vertebrate N-glycan composition (Leonard et al., 2006). FDL removes the terminal GlcNAc from $GlcNAcMan_{3-4}GlcNAc_2$, or its fucosylated forms (Altmann et al., 1995; Dragosits et al., 2015). This directs the N-glycan processing towards paucimannose products and reduces the final abundance of the complex N-glycans (Geisler and Jarvis, 2012; Leonard et al., 2006). Additionally, there might be a reciprocal regulation between FDL and fucosyltransferases, because FucT6 and FucTA cannot act on substrates lacking the terminal GlcNAc (Fabini et al., 2001), while core fucosylation of the substrate might reduce the activity of the FDL (Dragosits et al., 2015). In *Drosophila*, complete lack of FDL is lethal (Spradling et al., 1999), while reduced *fdl* expression causes brain and eye phenotypes consistent with other genes involved in paucimannose glycan production (Leonard et al., 2006; Rosenbaum et al., 2014).

Following FDL action the N-glycans can be further trimmed by the Golgi mannosidase IIb (α-Man-IIb or Man-III; Rosenbaum et al., 2014). This enzyme has a broad substrate specificity and is able to process $Man_5GlcNAc_2$ to $Man_3GlcNAc_2$ and smaller glycans (Kawar et al., 2001; Nemcovicova et al., 2013). Interestingly, Yamamoto-Hino and colleagues (2010) observed that the reduction of *α-Man-IIb* expression through RNAi resulted in elevated α1,6-fucosylation of chaoptin. Thus in wild type flies α-

Man-IIb seems to contribute to production of non-fucosylated paucimannose oligosaccharides through an alternative route bypassing Mgat1, α-Man-IIa, fucosyltransferases and FDL.

1.1.1.5. Complex N-glycans

Alternatively to production of paucimannose glycans, some of the oligosaccharides retain their terminal GlcNAc and can be further elaborated with additional GlcNAc, GalNAc, Gal or sialic acid residues (Fig. 1.3.) to form complex N-glycans (Geisler and Jarvis, 2012). Contrary to vertebrates, these glycans account for the minor fraction of the insect N-glycomes (1-20%) but still appear to play important roles in insect physiology (Aoki et al., 2007; Baas et al., 2011; Fabini et al., 2001; Kajiura et al., 2015; Leonard et al., 2006; Sarkar et al., 2006).

N-Glycans produced by Mgat1 can be processed by N-acetylglucosaminyltransferase II (GNT-II or Mgat2) which attaches another GlcNAc to the α1,6-arm of the glycan and forms a biantennary structure (Altmann et al., 1993; Geisler and Jarvis, 2012). In lepidopteran cells Mgat2 activity is about two orders of magnitude lower than this of Mgat1 – which might be one of the reasons for low abundance of complex glycans (Altmann et al., 1993). Mgat2 and additional putative N-acetylglucosaminyltransferases (e.g. CG9384, CG17173, CG31849 in *Drosophila*) homologous to mammalian enzymes responsible for the production of tri- or tetra- antennary complex glycans may be required for fruit fly development but their functions have not been studied in detail so far (Yamamoto-Hino et al., 2015).

Terminal GlcNAc residues can be further extended by GalNAc/Gal transferases (β4GalNAcT's). In insects these enzymes usually exhibit a preference towards GalNAc as a substrate (Haines and Irvine, 2005; Vadaie and Jarvis, 2004; van Die et al., 1996). Still, N-glycans containing both LacNAc (Galβ1,4GlcNAc) and LacdiNAc (GalNAcβ1,4GlcNAc) motifs were found in mosquito larvae (Kurz et al., 2015) and *Drosophila* embryos (Aoki and Tiemeyer, 2010). At whole insect level these glycans are usually produced in trace amounts or even below the detection limits (Leonard et al., 2006; Mabashi-Asazuma et al., 2015). However, they account for up to 10% of all glycans found on bee venom glycoproteins (Kubelka et al., 1995; Kubelka et al., 1993).

Mutations in the *D. melanogaster* β4GalNAcT genes affect the function of neuromuscular junctions, impair movement and cause egg defects (Chen et al., 2007; Haines and Irvine, 2005; Haines and Stewart, 2007). Biochemical evidence suggests that these phenotypes can be caused by lack of

GalNAc on complex N-glycans but also by the inability to elongate glycosphingolipids with LacdiNAc (Stolz et al., 2008).

The final stage of complex glycan processing involves capping of Gal or GalNAc residues with the negatively charged sialic acid which is carried out by α2,6-sialyltransferase, SiaT or ST6Gal (Kajiura et al., 2015; Koles et al., 2004). So far, among insects the existence of the sialylated N-glycans has been only proven in *D. melanogaster* adult heads and embryos (Aoki et al., 2007; Aoki and Tiemeyer, 2010; Koles et al., 2007). In those cases the sialylated N-glycans accounted for up to 0.1% of the total N-glycan pool.

Genes coding for sialyltransferase and CMP-sialic acid synthase (Csas) that provides the sugar donor for ST6Gal are expressed throughout the development almost exclusively in the neurons (Islam et al., 2013; Repnikova et al., 2010). In line with this, mutations in either of the genes resulted in locomotor abnormalities, increased sensitivity to heat and reduced life span, caused by defects in neuromuscular junctions and neural transmission (Islam et al., 2013; Repnikova et al., 2010).

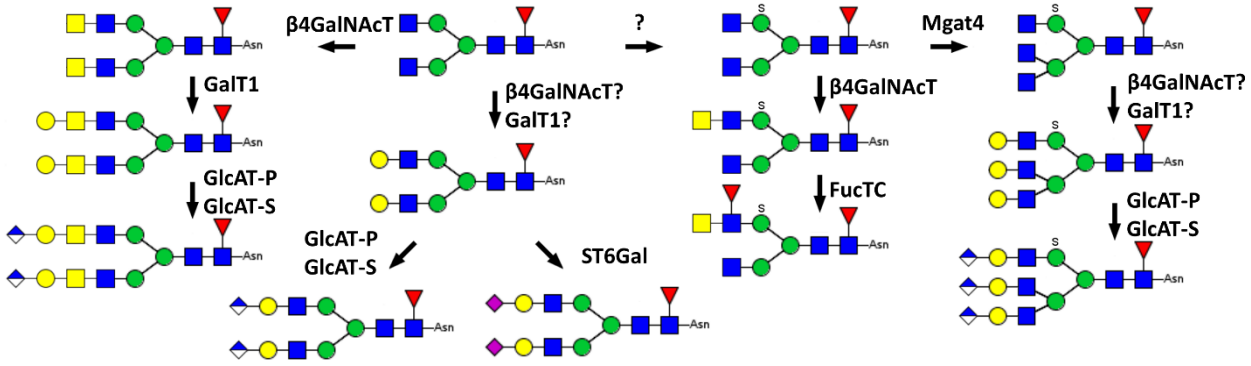


Fig. 1.3. Complex N-glycan diversity in insects. Selected complex N-glycans and enzymes involved in their synthesis in identified in various insects. □ - GalNAc, N-acetylgalactosamine, ○ - Gal, galactose, ■ - GlcNAc, N-acetylglucosamine, ▲ - Fuc, fucose, ◇ - GlcA, glucuronic acid, ● - Man, mannose, ◆ - Sia, sialic acid, S – sulfate group, Asn- asparagine in a polypeptide chain. Note that it is not clear yet which enzymes catalyzes Gal attachment to GlcNAc. In vitro studies demonstrated that β4GalNAcT's have a clear preference for UDP-GalNAc over UDP-Gal as a donor (Haines and Irvine, 2005; Vadaie and Jarvis, 2004), while GalT1 has so far been shown to attach Gal to terminal GalNAc (Ichimiya et al., 2015; Yamamoto-Hino et al., 2015). The enzyme(s) involved in N-glycan sulfation in insects has not been identified so far. N-glycans bearing no or two core fucoses can also be processed in a similar fashion.

Other modifications of the complex N-glycan include antenna fucosylation, T-antigen synthesis (Galβ1,3GalNAc), glucuronic acid attachment or sulfation (Aoki and Tiemeyer, 2010; Kurz et al., 2015; Kurz et al., 2016). *Drosophila* GlcAT-P and GlcAT-S were shown to add glucuronic acid to N-glycoproteins

in vitro (Kim et al., 2003). Additionally, the first of the two enzymes was found to be involved in nervous system development, similarly to other enzymes in the complex glycan pathway (Pandey et al., 2011). Enzymes involved in galactose or fucose transfer to terminal residues were also recently identified. However, the biological relevance of these N-glycan modifications has yet to be uncovered (Ichimiya et al., 2015; Kurz et al., 2016; Yamamoto-Hino et al., 2015).

1.1.1.6. Functional implications of N-glycosylation diversity

Apweiler and colleagues (1999) estimated that approximately half of all the proteins can be glycosylated. So far, N-glycoproteomic studies in insects identified over one thousand N-glycosylated proteins (Baycin-Hizal et al., 2011; Koles et al., 2007; Zielinska et al., 2012). All the known N-glycoproteins are either secreted or located in plasma membrane or membranes of the Golgi, lysosomes and the ER. No nuclear, cytoplasmic or mitochondrial N-linked glycosylation have been firmly proven in insects. Most common functional classes of identified N-glycosylated proteins involve cell adhesion proteins, transporters, receptors, proteases, enzymes involved in sugar and lipid metabolisms, ion channels and extracellular matrix components (Fig. 1.4.) (Baycin-Hizal et al., 2011; Koles et al., 2007; Zielinska et al., 2012). Approximately 9% of *Drosophila* N-glycoproteins are involved in development (Baycin-Hizal et al., 2011). Comparative analysis of N-glycoproteins from aphids, bees, beetles, silkworms and flies revealed that N-glycoproteins in all these insects representing different orders are also involved in cell communication, vesicle-mediated transport and response to stress (Vandenborre et al., 2011a). Interestingly this analysis showed substantial order-specific differences in glycoproteome profiles clearly indicating that studies on insects other than *D. melanogaster* are needed to fully understand the importance of glycosylation.

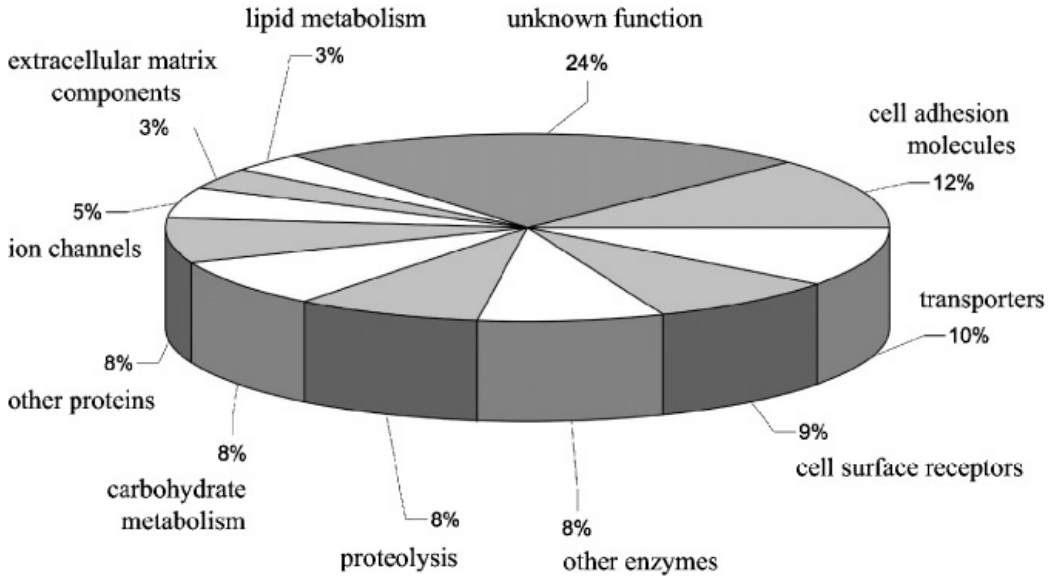


Fig. 1.4. Functional classes of N-glycosylated proteins identified in the fruit fly brain. Figure taken from Koles et al. (2007).

Standard methods based on chromatography and mass spectrometry applied to insect N-glycans usually identify between 15 and 20 different structures (Ailor et al., 2000; Fabini et al., 2001; Kajiura et al., 2015; Kubelka et al., 1993; Leonard et al., 2006; Mabashi-Asazuma et al., 2015; Rendić et al., 2007; Sarkar et al., 2006). However, more sophisticated approaches revealed that the actual array of the N-glycans produced in insect cells can reach over 40 or even nearly 90 distinctive forms (Aoki et al., 2007; Aoki and Tiemeyer, 2010; Baas et al., 2011; Kurz et al., 2015). Taking this into account each glycoprotein can be present in tens of glycoforms expanding the diversity encoded by the gene/protein sequence by an order of magnitude. However, it is rather unlikely that each of the possible N-glycans would have unique effects on the protein that carries it. Instead phenotypes caused by disruption of specific parts of the N-glycan processing pathway indicate that N-glycans rather have type-specific functions. High-mannose N-glycans appear to be primarily involved in protein folding and sorting (Benyair et al., 2015a), paucimannose and fucosylated glycans play a role in immune response (Mortimer et al., 2012) as well as in the development of the CNS and the eye (Leonard et al., 2006; Rosenbaum et al., 2014; Yamamoto-Hino et al., 2010), while complex glycans modulate neural transmission, locomotor behavior and life span (Haines and Stewart, 2007; Islam et al., 2013; Repnikova et al., 2010; Sarkar et al., 2010). Additionally, a large majority of the genes involved in N-glycan processing might be required for development (Yamamoto-Hino et al., 2015).

Relatively few studies demonstrated the impact of particular glycans on insect protein activity. In one of such papers diminished N-glycosylation was shown to abolish the activity of *Drosophila* chaoptin. This was due to retention of the protein in the ER, decreased abundance and impaired transport to the cell surface where it normally functions (Hirai-Fujita et al., 2008). Also in case of smoothened, a GCPR signal transducer of the Hedgehog pathway, loss of N-glycosylation prevented its translocation to the membrane and disrupted signaling (Marada et al., 2015). A different situation was observed for α-mannosidase III in lepidopteran cells, which requires α-glucosidase trimming of the N-glycan for efficient secretion but not for activity (Francis et al., 2002).

Intriguingly, particular N-glycans can take part in the fine modulation of protein activity. Such a phenomenon was observed for mosquito dopachrome conversion enzyme, which carries single, mainly fucosylated, paucimannose N-glycan. Removal of the whole N-glycan reduced enzymatic activity and thermal stability of the enzyme, while fucose removal or trimming of the mannoses had a positive effect on these two factors (Li et al., 2007). Another study on *Drosophila* voltage-gated K^+ channel SBb demonstrated that sialylation may increase gating efficiency through impact on electrostatic interactions (Johnson and Bennett, 2008).

1.1.1.7. Regulation of N-glycosylation diversity

Another natural question appears considering the immense diversity of N-glycans: can a glycoprotein bear any of the possible N-glycans? Recent advances indicate several factors that modulate the final outcome of the N-glycan processing.

As mentioned in section 1.1.1.2. a first of the factors that affect the N-glycosylation process is the folding dynamics and thus the length of the interaction with the ER-Man-I which would determine the number of mannose residues (Benyair et al., 2015a). Clearly, the production of more processed N-glycans depends on availability of the enzymes involved in particular steps. It has been shown for *FucTA* or genes involved in sialylation that their expression is restricted to specific tissues and developmental stages (Islam et al., 2013; Rendic et al., 2006; Repnikova et al., 2010).

Several papers indicated that protein structure can be a key determinant for the N-glycan processing. Kanie and co-workers (2009) studied *Drosophila* chaoptin which contain 13 N-glycosylation sites. Although several different N-glycans were found on each of the N-glycosylation sites, a subset of glycosylation sites was modified exclusively with high mannose glycans while the others had

paucimannose and complex glycans. They showed that the extent of the processing was defined by the protein 3D structure in the vicinity of the N-glycosylation site and thus by the physical accessibility of the N-glycan for processing enzymes.

A recent study involving the cabbage looper (*Trichoplusia ni*) cell line revealed additional aspects of N-glycan maturation control (Hang et al., 2015). In these cells the glycosylation profile of Pdi1p depended on whether this protein was retained in the ER and early Golgi or was secreted. In the first case the protein contained exclusively high mannose glycans, while in the latter the bulk of the glycans were more trimmed and modified with core fucose and terminal GlcNAc. Yet one of the site still contained primarily high mannose glycans. Further experiments indicated that due to the interaction with protein surface this glycan was inaccessible for processing enzymes (Hang et al., 2015).

Interestingly, the N-glycan profile of human transferrin, containing only two glycosylation sites, produced in a homogenous insect cell line included 16 different oligosaccharide structures (Ailor et al., 2000). This might suggest additional elements affecting the N-glycan profile beyond protein structure and expression level of the glycan processing enzymes. The insect specific mechanism leading to N-glycan diversity may be linked to organization of the Golgi. Unlike the single large Golgi ribbon as in mammalian cells, insects cells may contain thousands of dispersed transitional endoplasmic reticulum (tER)-Golgi units (Herpers and Rabouille, 2004). Because mRNAs are individually targeted to these Individual tER-Golgi units they may differ in enzyme composition ands consequently they can independently perform distinct glycosylation reactions (Kondylis and Rabouille, 2009).

Another source of the glycosylation diversity may originate from the organization of the Golgi trafficking. A protein delivered to Golgi in mammalian cells is partitioned into processing domains enriched in enzymes and export domains. The latter may result in rapid secretion from Golgi and limited exposure to enzymatic processing (Patterson et al., 2008). A similar phenomenon might be present also in insects.

Clearly glycans and glycoproteins are involved in most biological phenomena going from development to function of the nervous system and life span determination. Notably, the disruption of glycosylation leads to serious consequences including mortality. Following this logic interference with insect glycosylation appears to be a promising strategy for pest insect control. Unfortunately, most of the knowledge concerning insect N-glycosylation comes from the fruit fly model which lacks relevance in the agricultural context. Limited data from other insects point out significant differences between insect orders and indicate that data coming from *D. melanogaster* cannot be translated easily into other

insects. So to employ this strategy for pest control purposes more insight into glycobiology of relevant pest insects is required.

1.1.2. O-glycosylation

O-glycosylation is initiated by the attachment of a single glycan molecule to serine or threonine residues in a polypeptide chain. In insects the first sugar can further be extended in a stepwise fashion to form oligosaccharide chains consisting usually up to four sugar residues (Fig. 1.5.) but more elaborated glycans are also possible (Aoki et al., 2008; Gaunitz et al., 2013). O-glycans are classified into several main types based on the monosaccharide that is attached first. Notably different O-glycan compositions may mediate distinct functions.

1.1.2.1. O-GalNAc or mucin type glycans

Up to 90% of all the O-glycans in *D. melanogaster* belong to the mucin type (Aoki et al., 2008) that contains N-acetylgalactosamine (GalNAc) directly linked to serine or threonine. Mucin type glycans are abundantly present on insect secreted proteins or on extracellular domains of membrane proteins. This modification is involved in a plethora of processes including recognition, adhesion, signaling or immune system function (Kurz et al., 2015; Tran and Ten Hagen, 2013).

The first step in the synthesis of these glycans, the attachment of GalNAc to a polypeptide is catalyzed by UDP-GalNAc:polypeptide α-N-acetylgalactosaminyltransferases (PGANTs) a multi-gene family of enzymes. Insect genomes encode more than 10 pgant genes which have distinct spatial and temporal expression during development (Tian and Ten Hagen, 2006). Consequently, some of the pgants have specialized roles in *Drosophila*. For instance, *pgant35* is implicated in the formation of the respiratory tract (Tian and Ten Hagen, 2007b), *pgant5* is required for proper gut acidification (Tran and Ten Hagen, 2013), while *pgant4* is involved in the regulation of protein secretion (Zhang et al., 2014b)

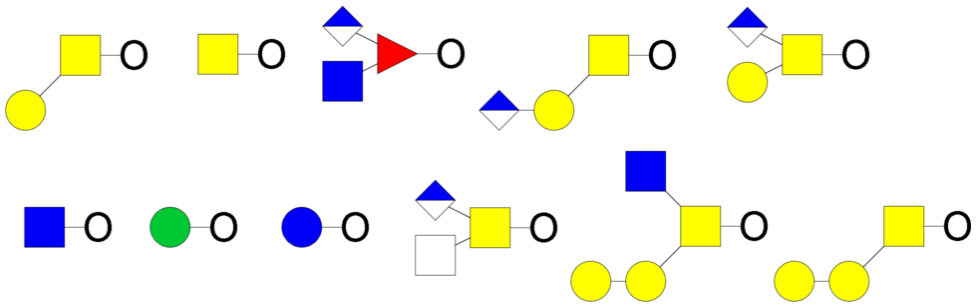


Fig. 1.5. Major O-glycans identified in fruit fly embryos, fruit fly larvae and mosquito larvae (Aoki et al., 2008; Kurz et al., 2015). □ - GalNAc, N-acetylgalactosamine, ○ - Gal, galactose, ■ - GlcNAc, N-acetylglucosamine, ▲ - Fuc, fucose, ◇ - GlcA, glucuronic acid. ● - Man, mannose, ● - Glc, glucose, □ - HexNAc, N-acetylhexosamine., O – Oxygen atom of serine or threonine in a polypeptide chain.

Based on the observation that several tissues require more than one PGANT gene Tran and Ten Hagen (2013) suggested that PGANTS may work in a collaborative or hierarchical fashion. Interestingly, orthologs of *pgant3* and *pgant6* are required for post-embryonic development and metamorphosis in the red flour beetle, *Tribolium castaneum* as indicated by a larval RNAi approach (Dönitz et al., 2014)

Peptide linked GalNAc can be further extended with galactose residues by glycoprotein-N-acetylgalactosamine 3-β-galactosyltransferases (β1,3GalTs) to form the core 1 structure or T-antigen. Multiple putative β1,3GalTs have been identified in *Drosophila* which similarly to pgant enzymes are expressed in a tissue and stage specific manner. Their mRNA was detected in the embryonic gut, salivary gland and nervous system while in adults prominent expression was observed mainly in the reproductive organs (Lin et al., 2008). Biochemical assays revealed that C1GalTA has the highest galactosyltransferase activity of the tested family members in *Drosophila* (Müller et al., 2005). Consequently, mutation of the gene coding for this enzyme reduced T-antigen levels in embryos and lead to ventral nerve cord as well as brain defects and eventually to pupal mortality (Lin et al., 2008). The product of C1GalTA was also found on the surface of the circulating hemocytes suggesting the involvement of core 1 glycan in immunity (Yoshida et al., 2008). T-antigens are among the most abundant glycans in fruit fly and mosquito larvae (Kurz et al., 2015) thus are likely to have a broad tissue distribution and potentially more diverse functions. Insect mucin type glycans can be further extended with N-acetylglucosamine but the enzymes involved in this step have not been characterized yet.

So far only a handful of proteins carrying mucin-type glycans has been identified. Among them were extracellular matrix proteins involved in the organization of tissue structure in salivary glands or the

gut: laminin γ and tenectin (Schwientek et al., 2007; Syed et al., 2012). Additionally, O-GalNAc glycans have also been found on imaginal disc growth factors or tiggrin that play role in tissue proliferation and cell adhesion during wing development (Schwientek et al., 2007; Zhang et al., 2008). Another group of O-GalNAcylated proteins include drosocin, attacin-C, peroxidosin and serine proteases which are implicated in immune defense responses (Rabel et al., 2004; Uttenweiler-Joseph et al., 1998).

1.1.2.2. O-GlcNAc glycans

The O-GlcNAc modification is found in proteins localized in all cellular compartments and organelles including the nucleus and nuclear membrane, the cytoplasm, the Golgi apparatus and mitochondria (Love and Hanover, 2005). Intracellular O-GlcNAcylation is carried out by a single enzyme O-GlcNAc transferase (sxc/Ogt) and is in dynamic equilibrium with O-GlcNAcase (Oga), the enzyme capable of removing O-GlcNAc from proteins. Although O-GlcNAc accounts for less than 1% of all O-linked glycans in *Drosophila* (Aoki et al., 2008) this modification is central to a variety of physiological processes. The O-GlcNAc attachment to nuclear Ph protein, a component of the polycomb-group repressive complex 1, was found to be involved in regulation of cell cycle and expression of developmental genes (Gambetta et al., 2009). In some proteins, O-GlcNAcylation can occur at the same sites as phosphorylation. This specific competition between two modifications is crucial for adjusting circadian rhythm by regulation of cyclic translocation of Period protein from the cytoplasm to nucleus (Kaasik et al., 2013; Kim et al., 2012). Because O-GlcNAcylation is dependent on cellular glucose levels this modification plays a role in nutrient sensing and response to starvation (Park et al., 2015). Through this link O-GlcNAc is also implicated in insulin signaling and specification of body size (Sekine et al., 2010). During embryonic development O-GlcNAc is required for heat sensing, adaptation to temperature change (Radermacher et al., 2014) and for fibroblast growth factor signaling (Mariappa et al., 2011). Detection of O-GlcNAc residues on the 26S proteasome subunit of *Drosophila* suggest that this modification may be involved also in regulation of protein degradation (Sümegi et al., 2003)

Long-lasting consensus on exclusively intracellular roles of O-GlcNAcylation has been recently revised by the discovery of O-GlcNAc on extracellular EGF domains of several proteins including Notch and Dumpy (Sakaidani et al., 2011). Phenotypes caused by mutation in Eogt, a single enzyme catalyzing extracellular O-GlcNAc attachment, resemble those caused by defective O-GalNacylation and include defects in cell-matrix interactions, cuticle structure, trachea formation and adhesion of the wing epithelium (Matsuura et al., 2008; Sakaidani et al., 2011).

This vast diversity of basic physiological functions of intracellular and extracellular O-GlcNAcylation relies on unique non-redundant enzymes Ogt and Eogt. Consequently knockdowns or mutations of the genes encoding these enzymes in *Drosophila* resulted in severe developmental defects and mortality (Gambetta et al., 2009; Matsuura et al., 2008; Sakaidani et al., 2011). Knockdown of Ogt ortholog in *Tribolium* larvae caused a disruption of metamorphosis, body deformation and complete mortality (Donitz et al., 2014). This feature positions these genes as particularly promising targets for pest control.

1.1.2.3. O-mannosylation

O-linked mannose constitutes less than 1% of the O-glycans identified in *Drosophila* embryos. Insects encode two enzymes responsible for O-mannose attachment: POMT1 and POMT2. Unlike the PGANT enzymes that could be both specialized and functionally redundant, POMT1 and POMT2 require each other for proper function. Ichimiya et al. (2004) demonstrated that knockdown of either of the POMTS resulted in a complete abolishment of O-mannosyltransferse activity. Mutations in genes encoding these enzymes produced characteristic rotated abdomen phenotype in adults, defective synaptic transmission and muscle dystrophy caused by disrupted myosin production and fiber attachment abnormalities (Martín-Blanco and García-Bellido, 1996; Wairkar et al., 2008). Additionally, some studies indicated that flies lacking POMTS expression are non-viable at elevated temperatures and sterile (Cooley et al., 1988; Ueyama et al., 2010). So far Dystroglycan is the only protein confirmed to be a substrate of the O-mannosyltransferases (Nakamura et al., 2010). As revealed by mutagenesis and RNAi studies this protein is responsible for proper muscle formation through actin cytoskeleton organization and linking with extracellular matrix, but is also required for oocyte polarization (Haines et al., 2007; Yatsenko et al., 2007). These phenotypes overlap with those of defective POMT expression clearly indicating that O-mannosylation is crucial for Dystroglycan activity and physiological function. Parental RNAi of Dystroglycan in *Tribolium* caused embryonic and early larval developmental defects including abnormal segmentation and irregular musculature. RNAi in larvae resulted in mortality or disruption of metamorphosis in only a minor subset of beetles (Donitz et al., 2014). The *Tribolium* genome also encodes two POMT genes but functional studies have not been performed yet.

1.1.2.4. O-linked fucose and other rare O-glycans on EGF domains

EGF domains are 30-40 amino acid sequences similar to epidermal growth factor protein and are found on multiple secreted and cell surface proteins that are involved in cell-matrix interaction,

adhesion, signal transduction and protein trafficking. Characteristic features of EGF domains include multiple cysteine residues forming disulfide bridges and multiple serine and threonine residues that can be subject to unique O-glycosylation (Haltom and Jafar-Nejad, 2015). The O-linked glycans found so far exclusively on EGF domains include O-fucose, O-glucose and extracellular O-GlcNAc (although O-GlcNAc is commonly found on intracellular proteins, the extracellular O-GlcNAc have been found only on proteins with EGF domains, see section 1.1.2.2) (Acar et al., 2008; Okajima and Irvine, 2002; Sakaidani et al., 2011).

O-fucose is attached by Ofut1 protein and it is required for proper Notch signaling (Ishio et al., 2015). Ofut1 mutant flies show misshapen wings, rough eyes and sensory organ defects (Okajima and Irvine, 2002), while the lack of both maternal and zygotic Ofut1 expression causes severe overproduction of neurons and is lethal (Sasamura et al., 2003). O-linked fucose can be extended with β1,3-GlcNAc by the GlcNAc transferase called Fringe (Fng). This modification is crucial for Notch signaling in wing development but not for regulation of embryonic neurogenesis (Okajima et al., 2008). Other functions of Fng inferred from mutant phenotypes involve the establishment of the proventriculus (foregut organ), egg chamber formation and eye development (Dominguez et al., 2004; Fuss et al., 2004; Grammont and Irvine, 2001). Phenotypes produced by *Tcfng* RNAi in *T. castaneum* suggest that this modification is also required for proper adult eclosion and survival (Donitz et al., 2014).

Another type of glycan found uniquely in EGF domains is O-glucose. Insects have a single glucosyltransferase named Rumi that transfers glucose exclusively to serine residues (Acar et al., 2008). This modification together with O-fucose is essential for Notch transport from the ER to the membrane (Ishio et al., 2015). Additionally, through O-glucosylation of the protein Eyes shut it is also involved in development of *Drosophila* photoreceptors (Haltom et al., 2014). Rumi mutants display bristle and leg defects and fail to eclose at elevated temperatures indicating a role of O-linked glucose in the protection against heat induced loss of signaling (Leonardi et al., 2011). O-glucose can be further extended with xylose by the xylosyltransferase called Shams, which restricts Notch signaling in pupal wing (Lee et al., 2013).

1.1.2.5. Glucuronic acid

Both the mucin type glycans and O-fucose can be further extended with glucuronic acid (Aoki et al., 2008; Breloy et al., 2008; Kurz et al., 2015). This particular sugar is negatively charged and is believed to act in insects as a functional equivalent of sialic acids, a group of acidic glycans commonly found in

vertebrates but not detected in insect O-glycans (Breloy et al., 2008). There are at least three enzymes possessing glucuronyltransferase activity in insects, GlcAT-I, GlcAT-P and GlcAT-S. Mutations or knockdowns of these genes in *Drosophila* result in leg, wing and nervous system defects and can be lethal (Pandey et al., 2011; Yamamoto-Hino et al., 2015). In *Tribolium* larval RNAi of GlcAT-I caused malformed antenna and wings as well as adult mortality (Donitz et al., 2014). However, these enzymes have a broad acceptor specificity and can attach glucuronic acid also to glycolipids and proteoglycans and N-glycans (Kim et al., 2003), thus it is difficult to infer the functions specific for glucuronic acid containing O-glycans.

1.2. Insects: agricultural pests

Pest insects pose a serious challenge for a global agriculture. They are responsible for 10-20% decrease in the worldwide agricultural output but locally they may cause a complete yield loss (Dhaliwal et al., 2010; Kranthi et al., 2002; Oerke, 2006; Ouyang et al., 2016). This generates direct losses and costs required for insect control measures accounting to billions of dollars. Notable pest insects in Lepidoptera order (moths and butterflies) include cotton boll worms, *Heliothis zea* and *Helicoverpa armigera*, major pests of cotton and corn, or armyworms *Spodoptera exigua* and *Spodoptera frugiperda* notorious for damaging wide range of crops. Other infamous examples of pest insects are the cotton aphid, *Aphys gossipi*, the brown plant hopper, *Nilaparvata lugens*, which damges the rice fields or *Myzus nicotiane* – tobacco aphid. Obviously this does not exhaust the list of pest insects that can pose a threat to successful farming. In fact there are thousands of species belonging to most of the insect orders that are capable of causing serious agricultural damage (Pimentel, 2009).

This thesis, however, will focus on the red flour beetle *Tribolium castaneum* (Herbst) a representative of the Coleoptera order. Beetles are the most successful order on the earth comprising more than 350 000 species, which is one fourth of all species described so far (Hunt et al., 2007; McHugh and Liebherr, 2009). Beetles developed characteristic sclerotized front wings (elytra) that provide substantial mechanical protection, while membranous hindwings hidden below still allow the flight. This feature gave rise to the greek name of beetle. “Koleos”, means shield, while “pteron”, means wing. Most of the beetles have a depressed body shape, legs positioned at the ventral side of the body and heavily sclerotized sternites (ventral portion of the body) that together with elytra and a sclerotized head and thorax provide tight protection against mechanical damage, predators and parasitoids (Fig. 1.6). All beetles are holometabolous (McHugh and Liebherr, 2009) that is their life cycle includes morphologically

distinct larval, pupal and adult stages (See section 1.2.2. for more details). Within Coleoptera order approximately 50% of species adapted to herbivory – the ability to feed on plant material (Farrell, 1998) and many of them are serious agricultural pests. These include one of the most widespread pests the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) but also other major pests of important crops including Western corn rootworm, *Diabrotica virgifera virgifera* (LeConte), the coffee berry borer, *Hypothenemus hampei* (Ferrari), the cotton boll weevil, *Anthonomous grandis* (Boheman) or the rapeseed beetle, *Meligethes aneus* (Fabricius). Some beetles, for instance the lesser grain borer, *Rhizoperta dominica* (Fabricius) or the red flour beetle, *Tribolium castaneum* (Herbst) can also infest grain and flower storage facilities.

1.2.1. The red flour beetle, *Tribolium castaneum*

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Coleoptera

Family: Tenebrionidae

Genus: *Tribolium*

Species: *Tribolium castaneum*

Tribolium castaneum is notorious for its remarkable ability to develop resistance to all classes of insecticides. This together with high fecundity (females can lay hundreds of eggs) and longevity (up to 3 years) makes the red flour beetle an extremely difficult pest to control (Blaser and Schmid-Hempel, 2005; Roth and Kurtz, 2008). *Tribolium castaneum* is easy to culture in a laboratory setting and is resistant to inbreeding. This species offers also additional benefits because of its fully sequenced and annotated genome, the relative ease of genetic manipulation as well as the possibility of the robust, systemic gene silencing through RNA interference, which made the red flour beetle a convenient model for multiple evolutionary, developmental and gene function studies (Bucher et al., 2002; Lorenzen et al., 2007; Tomoyasu and Denell, 2004; *Tribolium* Genome Sequencing Consortium, 2008). On top of that *T. castaneum* has also been used to verify novel pest control approaches and to study the mechanisms of action of insecticidal compounds (Caballero-Gallardo et al., 2012; Contreras et al., 2015; Contreras et al., 2013a; Contreras et al., 2013b; Kim et al., 2015; Oppert et al., 2005; Perkin et al., 2016; Zapata and Smagghe, 2010).

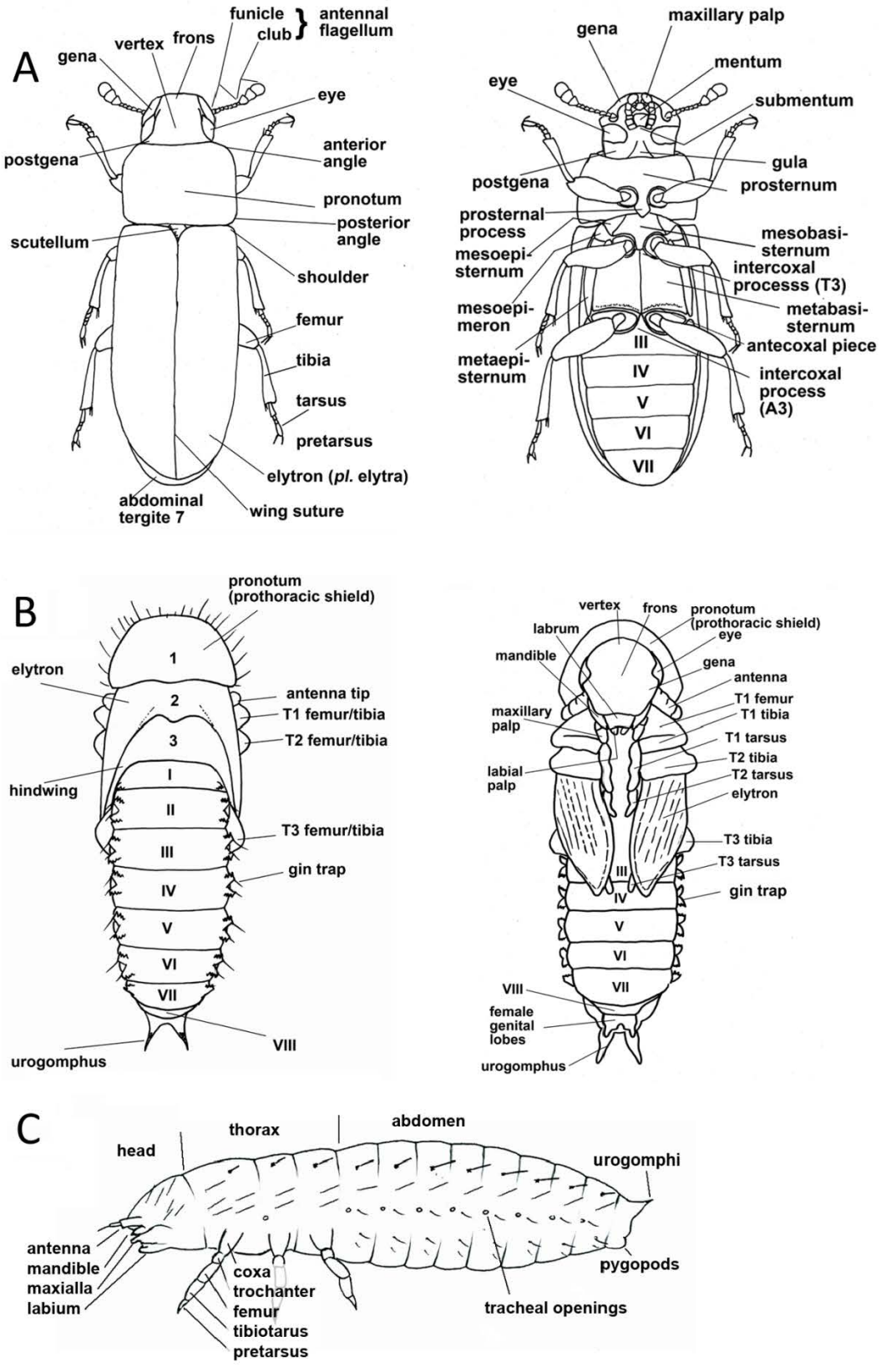


Fig. 1.6. Morphology of *Tribolium castaneum.* (A) adult, (B) pupa, (C) larva. From Dönitz et al. (2013).

1.2.2. Life cycle of *T. castaneum* and the metamorphosis

Like all the other beetles *T. castaneum* is a holometabolous insect. That means that its life cycle includes complete metamorphosis from morphologically distinct larval to pupal and adult stage. First instar larvae hatch from the eggs after 4-6 days of incubation and begin feeding. After another few days of growing larvae shed the old cuticle to molt into the next instar which allows further development. Under the optimal temperature (approx. 30°C), humidity (60-80% r.h.) and food conditions *T. castaneum* will go through 5-7 larval instars growing from 0.1 mg to over 3 mg within approx. 3 weeks. Last instar larvae molt into immobile pupae with visible characteristic features such as wing covers, compound eyes and genital lobes. In 5-6 days the adults will eclose which after the next several days will be ready to mate and lay eggs to complete the life cycle (Fig. 1.7).

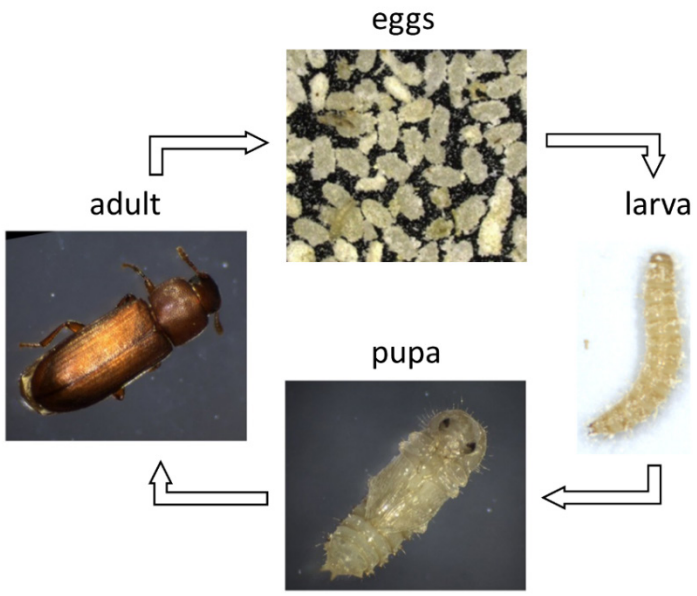


Fig. 1.7. Life cycle of *Tribolium castaneum.*

Insect metamorphosis is regulated by balance between juvenile hormone (JH) and molting hormones (ecdysteroids). In beetles increased levels of ecdysteroids in the presence of high juvenile hormone titers will induce molting into another larval stage (Konopova and Jindra, 2008). The antimetamorphic action of JH is mediated through the Methoprene-tolerant (Met) receptor, which induces the expression of Krüppel homolog 1 transcription factor (Kr-h1). This in turn prevents induction

of Broad-Complex (BR-C) expression required for pupation (Konopova and Jindra, 2007). Low JH titers in the last larval stage result in elevated expression of BR-C and entry into the pupa morphogenesis program. In pupae JH level remains low but also Kr-h1 and BR-C expression drop which is necessary for adult development (Konopova and Jindra, 2008; Minakuchi et al., 2009).

1.2.3. RNAi – a powerful tool to study gene functions in *T. castaneum*

One of the advantages of the red flour beetle as a model is that introduction of specific dsRNA induces robust and systemic gene silencing. This phenomenon is called RNA interference (RNAi) and is frequently used as a method to study gene functions through observation of the consequences of gene expression knockdown (Noh et al., 2012).

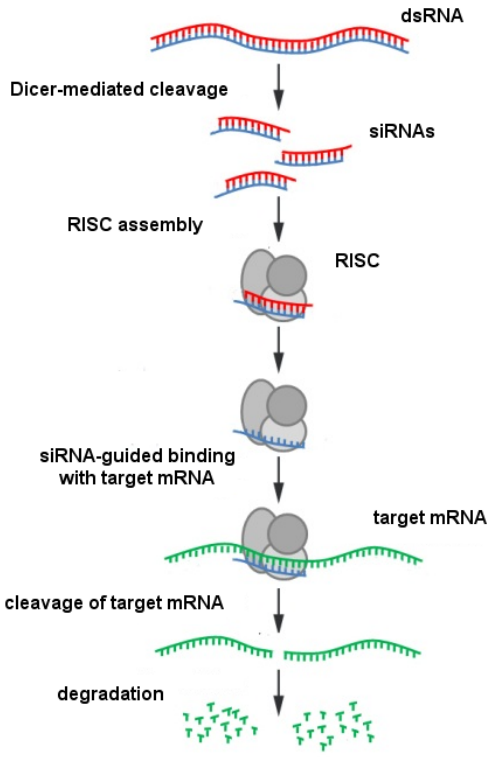


Fig. 1.8. Schematic model of RNA interference. Adapted from Petrova et al. (2013).

The RNAi is initiated by uptake of extracellular long dsRNA into cells through Sid-like transmembrane channels or through endocystosis (Huvenne and Smagghe, 2010; Tomoyasu et al., 2008). Internalized dsRNA is cleaved in the cytoplasm by Dicer proteins (RNAse III) into 19-23 base-pair short interfering RNAs (siRNAs). Additionally, Dicer-like proteins cooperate with proteins containing dsRNA

binding motif (Loquacious, R2D2, C3PO, Drosha) to load the siRNAs into the RNA-induced silencing complex (RISC).

One strand of the siRNA incorporated into the RISC is dissociated while the other serves as a "guide" to target the mRNA molecules containing the complementary sequence. Upon recognition such mRNA is cleaved by Argonaute endonucleases which are part of the RISC. Cleavage of the mRNA precludes its translation into protein and results in posttranscriptional silencing of the gene expression (Huvenne and Smagghe, 2010; Noh et al., 2012; Tomoyasu et al., 2008).

In *T. castaneum* injection of a dsRNA solution into embryos, larvae or adults results in gene knockdown in all the tissues, which is in contrast to only local silencing triggered by dsRNA injection in *D. melanogaster*. Larval RNAi in *T. castaneum* persists for several weeks and continues through pupal and adult stages, thus this approach is especially suited to study functions of genes in the process of metamorphosis (Tomoyasu and Denell, 2004).

1.2.4. Insect gut and midgut cells

The gut is one of the biggest organs in insects and also one of the main interfaces between the beetle and the environment. As such it appears particularly interesting from an insect control point of view (Perkin et al., 2016). The beetle gut is composed of three main parts: foregut, midgut and hindgut (Fig. 1.9). The main roles of the foregut are fragmentation and delivery of ingested food into the midgut where the main digestion and absorption of the nutrients take place. Hindgut is involved in salt and amino-acid adsorbtion to maintain the osmotic pressure in the hemolymph and in the excretion of the remnants of the food bolus with feces (Ameen and Rahman, 1973; Chapman and De Boer, 1995; Lehane and Billingsley, 2012).

The insect midgut is composed of enterocytes (columnar cells), endocrine cells, goblet cells and stem cells which can differentiate in to one of the former. These cells are supported by a basement membrane and layers of circular and longitudinal muscles (Hakim et al., 2010).

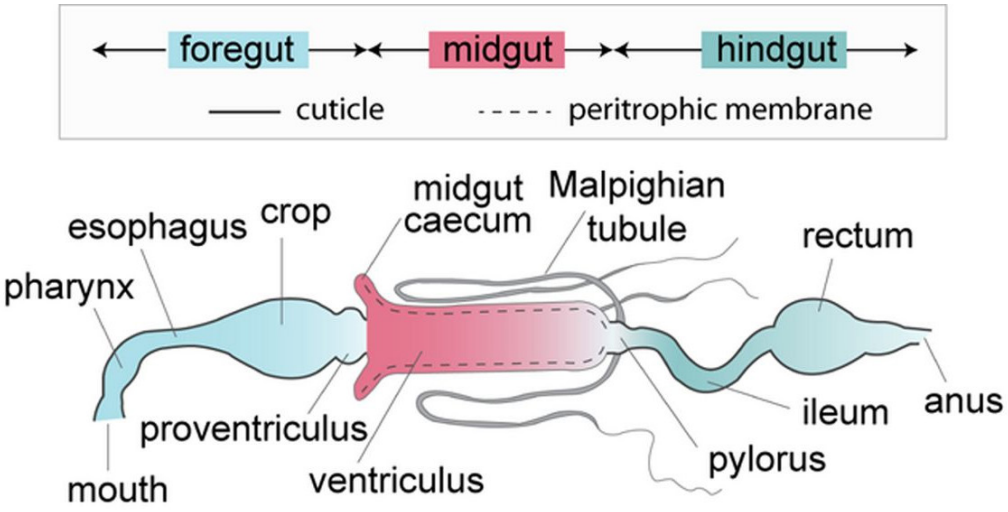


Fig. 1.9. Generalized scheme of insect digestive system. From Engel and Moran (2013).

Goblet cells are mainly involved in ion transport and maintenance of the gut pH, while enteroendocrine cells take part in chemosensory processes. In response to gut contents the latter cells produce peptide hormones that adjust the release of digestive hormones or food intake (Lehane and Billingsley, 2012; Moffett and Koch, 1992; Park and Kwon, 2011).

Columnar cells are predominant in midgut epithelium and perform a plethora of roles. They adsorb and store the nutrients from the gut lumen as well as produce and release the digestive enzymes. Both secretory and adsorptive functions are aided by the characteristic morphological features of the columnar cells: a highly folded apical membrane with microvilli - tiny membrane protrusion that expand the effective surface of contact between the cells and the interior of the gut (Lehane and Billingsley, 2012; Loeb and Hakim, 1996).

Additionally, columnar cells secrete chitin and glycoproteins which are components of a peritrophic matrix (PM). The PM is a semi-permeable structure that lines the insect midgut and divides it into two regions: endoperitrophic space or region enclosed by the peritrophic matrix and ectoperitrophic space between the peritrophic matrix and a surface of the midgut epithelium. This division allows spatial organization of digestion. Furthermore, the PM is involved in lubrication of the ingested food and acts as a shield protecting midgut cells from pathogens, toxins and abrasive food particles (Lehane and Billingsley, 2012; Wang and Granados, 2001).

Chitin, a basic component of the PM, is a linear polymer composed of β1,4-linked GlcNAc residues. Hundreds of chitin chains condense into microfibrils with diameters ranging from 2 to 6 nm, which in turn, assemble into 20 nm thick bundles (Fig. 1.10). These intersecting chitin bundles form a lattice with mesh size of approx. 150 nm which serves as a scaffold for the PM (Lehane, 1997).

Peritrophins and insect intestinal mucins are two main types of proteins which are associated with the PM. The peritrophins contain one up to 19 chitin binding domains (CBDs) and are often N- and O- glycosylated. The exact functional roles of these proteins have not been deciphered completely, but they appear to be crucial for preservation of PM structure and its barrier function. Peritrophins with multiple CBDs might assist in assembly of the chitin chains into bundles and in maintenance of the lattice structure. On the other hand, proteins with single CBD are likely to play a role in protection of ends of the chitin chains from chitinolytic enzymes (Agrawal et al., 2014; Hegedus et al., 2009).

Another group of proteins associated with the PM consists of insect intestinal mucins (IIMs). These proteins contain both CBDs and mucin domains. Mucin domains are short, often tandem-repeated sequences (up to 27 amino acids) enriched with serine or threonine residues, which are heavily O-glycosylated. In fact, glycans may comprise up to 90% of the molecular weight of some IIMs (Hegedus et al., 2009). Insect intestinal mucins assume a characteristic “bottle brush” structure with O-glycan chains projecting outward This promotes binding of water and salts and contributes to the viscosity, adhesiveness as well as to lubricating properties of the PM (Hegedus et al., 2009; Lehane and Billingsley, 2012; Terra, 2001). The majority of known insect gut mucins is located to the PM and only few cases of cell surface mucins have been identified (in mosquitoes and fruit flies). That is most likely because the PM plays analogous role to the mucin layer of vertebrate epithelia (Toprak et al., 2010).

Proteins associated with the PM tightly fill most of the space within chitin meshwork and render the PM permeable only to smaller and predigested molecules. The width of remaining openings, or PM pores, differs between insects; it can reach even 50 nm in some species but most often falls between 7 and 10 nm (Terra, 2001). This range of PM pore sizes allows compartmentalization of digestion (Lehane, 1997). Insect amylase and trypsin have average particle diameters of 6.2 and 6.5 nm, respectively. Therefore they can pass through 7 nm wide PM pores into endoperitrophic space and begin the digestion process. In contrast larger enzymes, such as trehalase, maltase and aminopeptidases are trapped within the PM, confined to the space between PM and the gut wall or bound to the midgut cell membranes. Consequently, these enzymes can act only on predigested nutrients that were able to pass through the PM (Ferreira et al., 1994; Terra, 2001). Importantly, the pore sizes appear not to be fixed but

are subject to a constant regulation. One of mechanisms for such regulation involves proteins with chitin deacetylase domains. Activity of these enzymes may locally modify chitin chains which will preclude binding by CBD domains and increase the PM permeability (Hegedus et al., 2009).

The anterior part of the *T. castaneum* larval midgut has acidic pH and is dominated by cysteine peptidases, while in the posterior part the pH increases towards mildly alkaline and serine peptidases are more abundant. A complex mixture of diverse peptidases allows efficient digestion and avoidance of negative effects of protease inhibitors that plants use in their defense against insects (Perkin et al., 2016; Vinokurov et al., 2009).

Similarly to the vertebrate digestive system the insect guts host rich microbiota which increase the capability to adapt to new host plants and overcome pest control measures (Chu et al., 2013).

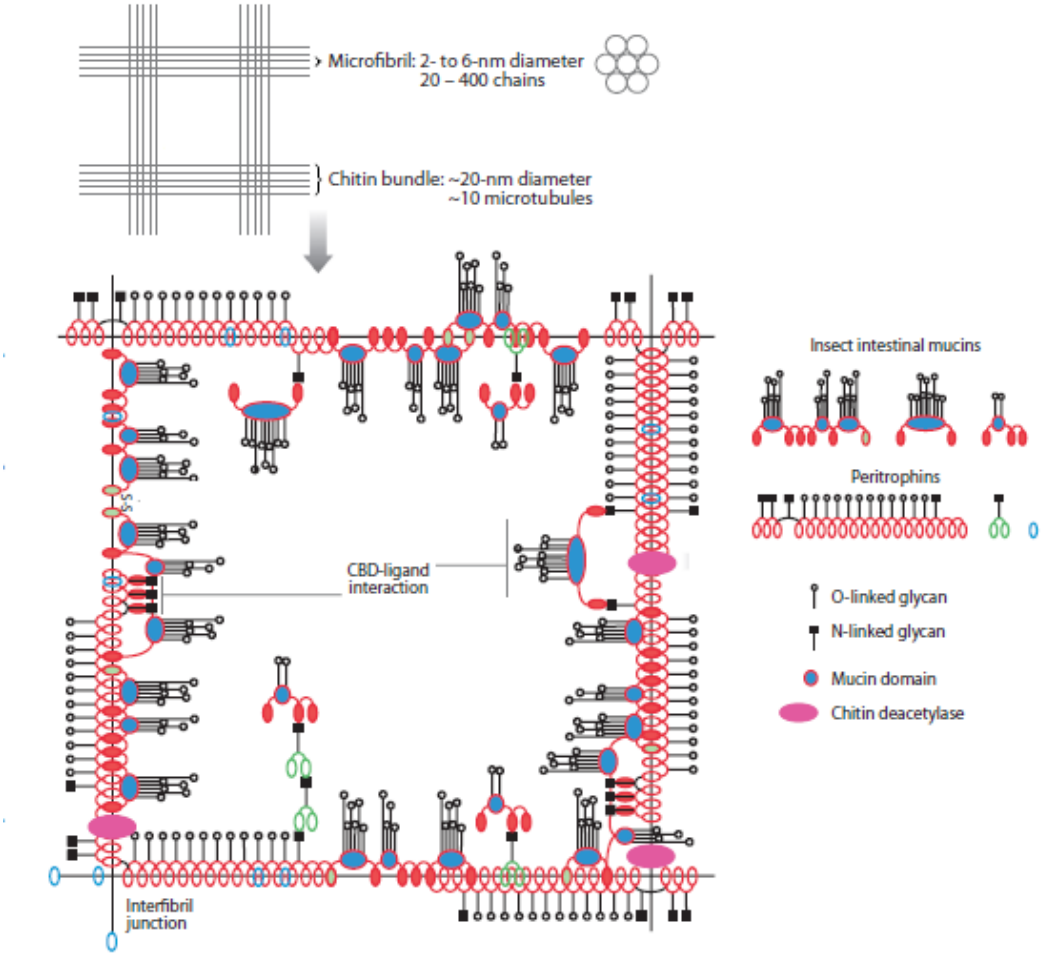


Figure 1.10. Architecture of the peritrophic membrane. From Hegedus et al. (2009)

1.2.5. Insect control measures: resistance problem and need for new solutions

The use of synthetic insecticides is the most popular approach to control pest insect populations and reduce the damage they cause (Alyokhin, 2009). The choice of the compound depends mainly on the target pest, but the most commonly used insecticides are organophosphates and carbamates (acetylcholinesterase inhibitors), organochlorines (GABA-gated chloride channel blockers), pyrethroids (sodium channel modulators) as well as neonicotinoids and spinosyns (nicotinic acetylcholine receptor modulators) (Alyokhin, 2009; Alyokhin et al., 2007; Mota-Sanchez et al., 2006; Scharf et al., 2000; Traugott et al., 2015). However the effectiveness of the currently used insecticides is threatened by the rapidly arising problem of insecticide resistance. The Colorado potato beetle and the red flour beetle are among the most resistant insect species with reported cases of resistance to 51 and 32 different active ingredients, respectively (Arthropods Resistant to Pesticides Database, 2016).

Additionally, the use of synthetic insecticides has had detrimental effects on the environment: by reducing populations of the non- target insects (natural enemies and pollinators), and by the contamination of ground or surface water. Poisoning of farm workers and consumers has also been described (Isman, 2006). Thus potential and proven negative effects of insecticides triggered the authorities to impose the restrictions or even prohibition of use of some pesticides (Commission Implementing Regulation (EU) No 485/2013). Therefore, there is a high demand for alternative more environmental-friendly methods that could replace the synthetic insecticides. The currently available alternatives include botanical products: pyrethrum, neem, essential oils, antifeedants and repellents. However, due to regulatory barriers and lower cost-effectiveness they lose the competition with microbial or synthetic insecticides (Isman, 2006). Crop rotation or the introduction of natural enemies can also reduce the number of pest insects. However, these methods are less applicable in a large scale production or become ineffective due to insect adaptation (Alyokhin, 2009; Gray et al., 2009).

Most of the problems introduced by chemical insecticides can be alleviated by the use of insecticidal crystal proteins produced by the bacteria *Bacillus thuringensis*. These so called Cry or Bt toxins form pores in insect midgut cells which leads to their lysis, septicemia and eventually to insect death (Bravo et al., 2007). Cry toxins are highly selective (Cry1 against Lepidoptera, Cry3 against Coleoptera, Cry4 against Diptera) and require to be ingested to exert their toxic action which greatly reduces the unwanted effects for non-target organisms (Whalon and Wingerd, 2003). Cry toxins can be sprayed as synthetic insecticides but the proteins can also be constitutively produced in transgenic crops

which gives additional benefits by the elimination of the application costs (Reed et al., 2001; Whalon and Wingerd, 2003).

This method has been particularly effective in the management of the Western corn rootworm. However, the widespread planting of transgenic maize expressing Cry3 toxins in US generated a strong selective pressure which resulted in numerous instances of resistance to Cry3 (Gassmann et al., 2014; Gassmann et al., 2011). To tackle this problem a new concept of resistance gene pyramiding was proposed. Transgenic pyramided crops produce multiple Bt toxins that can target the same pests and are believed to delay development of the insecticide resistance. It has been assumed that even if the insect becomes unsusceptible to one toxin it will still be killed by the others (Tabashnik et al., 2015). Experimental evidence, however, contradicts this assumption and indicates that for Cry toxins development of resistance to one such toxin results in a reduced susceptibility to the others (Brévault et al., 2013). Moreover, there are cases of reduced efficiency of crops expressing multiple Cry toxins compared to single ones. These antagonistic effects and the development of cross-resistance were explained by the similar mode of action and the high amino acid sequence similarity between Cry toxins (Carrière et al., 2015). These observations point towards the conclusion that the pyramids must consist of multiple unrelated toxins with different modes of action to effectively counteract resistance development.

A growing number of studies indicate that lectins, sugar binding proteins, might also be effective as agents conferring insect resistance in crop plants. Lectins could be effective both as stand-alone insecticidal factors and as elements of the resistance gene pyramid.

1.3. Lectins as insect control agents

Lectins (or agglutinins) are proteins containing at least one non-catalytic domain that can irreversibly bind to mono- or oligosaccharides (Vandenborre et al., 2011). Such carbohydrate binding proteins can be found throughout all the kingdoms of life and many of them, particularly plant and fungal lectins, have been shown to be toxic to insects (Dodd and Drickamer, 2001; Michiels et al., 2010; Varrot et al., 2013). Unlike antibodies, which are very selective, lectins usually can bind to an array of carbohydrate structures. For instance ASAL from garlic (*Allium sativum)* is specific for glycans containing mannose residues, while UDA from stinging nettle (*Urtica dioica*) prefers chitooligosaccharides (Vandenborre et al., 2011). Other lectins such as XCL from mushroom *Xerocomus chrysenteron* show

binding to diverse structures including Gal-GalNAc O-glycans and N-glycans carrying terminal GlcNAc residues (Sabotič et al., 2016).

In general the insecticidal activity of lectins appears to be linked with their ability to bind to glycans. Diverse oligosaccharide structures are present on the surface of insect gut cells, the peritrophic matrix as well as on proteins secreted to the gut lumen and can be recognized by the ingested lectins. Although glycan binding appears to be one of the prerequisites for lectin entomotoxicity, the diversity of the glycan structures, their localization and diversity of lectin binding characteristics result in various and often complex modes of insecticidal action (Vandenborre et al., 2011). However, most commonly lectins tend to impair the digestive enzyme production and secretion as well as nutrient digestion and absorption. Such severe disruption of the digestive processes incapacitates the insect growth and often results in death out of starvation. Several lectins have been also shown to trigger cell death upon binding (Macedo et al., 2015).

Plant lectins have been classified in twelve families based on the amino acid sequences and structural features of the carbohydrate-binding domains (Van Damme et al., 2008). These families are named after the prototype lectins and include: *Agaricus bisporu*s agglutinin homologs, amaranthins, class V chitinase homologs, cyanovirin family, *Euonymus europaeus* agglutinin family, *Galanthus nivalis* agglutinin family, lectins with hevein domains, jacalins, legume lectins, LysM (lysin motif) domains, *Nicotiana tabacum* agglutinin family, and ricin-B family. Most of these lectin families include at least several lectins with proven insecticidal activity (Table 1.1.; Vandenborre et al., 2011).

1.2.6. GNA-related lectins

One of the best studied groups of insecticidal lectins consists of GNA–related lectins. The prototype of the group, *Galanthus nivalis* agglutinin or GNA, was isolated from the bulbs of snowdrop. Similar lectins were found in most of tissues of multiple monocot species, such as garlic, onion, tulip or summer snowflake, but tend to be particularly abundant in bulbs (up to 50% of all protein). Most of known GNA-related lectins are specific towards mannose, mannose oligomers or high-mannose N-glycans. Therefore, these lectins are usually isolated by affinity chromatography using immobilized mannose. Most of GNA-related lectins are dimers or tetramers composed of non-glycosylated 11-15 kDa subunits (Van Damme et al., 1998a; Van Damme et al., 1998b).

GNA and related lectins from garlic (ASA-I, ASA-II, ASAL) showed a broad insecticidal activity against hemipteran and lepidopteran insects both in artificial diet assays as well in trials with transgenic

crops expressing these proteins (Vandenborre et al., 2011). Ectopically expressed GNA in rice could effectively reduce the fecundity and survival of green leafhopper *Nephotettix virescens*, as well as planthoppers *Nilaparvata lugens* and *Sogatella furcifera*, which resulted in a diminished plant damage (Foissac et al., 2000; Michiels et al., 2010; Vandenborre et al., 2011). Exposure of the lepidopteran stalk borers, *Eorreuma loftini* and *Diatraea saccharalis* to GNA produced in sugar cane negatively affected insect survival, adult emergence, female fecundity and pupal weight (Setamou et al., 2002). The expression of garlic lectin in transgenic tobacco conferred resistance against the moth *Spodoptera litura* and the peach aphid (*Myzus persicae*; Upadhyay and Singh, 2012a). The mechanisms of insecticidal activity of GNA and related lectins have been partially characterized. Binding to glycosylated proteins present in the membrane of insect gut epithelium microvilli appears to one of key events for toxicity. Identified receptors of GNA-related lectins include ferritin, cytochrome P450 cell and cadherin (Upadhyay and Singh, 2012b), which suggests that lectins might interfere with maintenance of iron homeostasis, production of the insect-moulting hormone (20-hydroxyecdysone) and cell adhesion. Additionally, lectins were found to bind with insect midgut enzymes such as aminopeptidase N, sucrase and alkaline phosphatases which might explain observed antinutritional effects (Macedo et al., 2015). On top of that some of these lectins can pass through the gut and be transported to other tissues through the hemolymph which can interrupt the development and induce insect death through a yet unknown mechanism (Upadhyay and Singh, 2012).

1.2.7. Legume lectins

Legume lectins are another lectin family that includes several insecticidal proteins. As indicated by their name lectins belonging to this family have been isolated exclusively from legume plants (*Fabaceae*). Most of legume lectins have been isolated from seeds, however some legume trees, such as *Maackia amurensis* or black locust (*Robinia pseudoacacia*), store high amounts of lectins in the bark. Legume lectins share a high amino acid sequence similarity but present very diverse glycan binding specificities, which cover the majority of known carbohydrates. For instance ConA isolated from *Canavalia ensiformis* (jackbean) binds preferably to α-D-glucose and α-D-mannose, peanut agglutinin, PNA, recognizes terminal galactose, while soybean agglutinin, SBA, is specific to GalNAc. This lectin family also includes proteins such as *Maackia amurensis* agglutinin, MAA, and *Lotus tetragonolobus* (asparagus pea) agglutinin, LTA, which display rare binding properties towards Neu5Acα(2,3)Gal/GalNAc and fucose, respectively (Van Damme et al., 1998a; Van Damme et al., 1998b). Consequently, diverse

ligands used to isolate those lectins by affinity chromatography include immobilized mannose, galactose, GalNAc derivatives or the sialylated protein fetuine (Van Damme et al., 1998b).

Most of the legume lectins are primarily translated as approx. 30 kDa protomers which usually assemble into tetrameric structures. However, protomers of pea lectin, PSA (*Pisum sativum* agglutinin) are internally cleaved into two polypeptides (approx. 17 kDa and 6 kDa) which form a heterotetrameric protein. Contrary to GNA-related lectins the polypeptides building agglutinins from kidney bean, soy bean or mistletoe can be glycosylated with one or two N-glycan chains (Van Damme et al., 1998a).

Insecticidal properties of ConA have been partially characterized. This lectin inhibited development of tomato moth (*Laconobia oleracea*), several species of planthoppers as well as peach and pea aphids (Gatehouse et al., 1999; Rahbe et al., 1995). In tomato moth dietary ConA bound efficiently to gut microvilli and reduced activity of the gut α-glucosidase. Apparently, this lectin was able to cross the gut barrier and accumulated in several organs such as Malpighian tubules, fat body and hemolymph (Fitches and Gatehouse, 1998; Fitches et al., 2001). Additionally, Melander et al. (2003) showed that transgenic expression of PSA, another lectin specific to glucose/mannose from common pea (*Pisum sativum*), in rapeseed flowers reduced the growth of pollen beetle (*Meligethes aneus*) larvae. Also a GlcNAc-specific lectin GS-II, isolated from the seeds of *Griffonia simplicifolia,* has been shown to exert strong insecticidal activity against cowpea weevil (*Callosobruchus maculatus*), presumably due to binding and disruption of the peritrophic matrix structure (Zhu-Salzman et al., 1998).

1.2.8. Lectins with hevein domains

Hevein related lectins form another lectin family which groups proteins with insecticidal activity. The structural determinant of this family is a presence of the chitin-binding domain similar to a hevein polypeptide found in latex of the rubber tree (*Hevea brasiliensis*). Consequently, hevein-related lectins are usually specific towards GlcNAc and its oligomers (Van Damme et al., 2008). Yet, wheat germ agglutinin, WGA, has been shown to bind also sialylated glycans. Proteins with hevein domains can be found in multiple species throughout the plant kingdom. There is also a great diversity of tissues in which these lectins can be found including, but not limited to tubers, roots, fruits and leaves.

Lectins belonging to this family are composed of one to seven tandem-arrayed hevein domains. Additionally, there are remarkable differences in the overall structure between particular hevein-like lectins. In that WGA is composed of two 18 kDa subunits, UDA from stinging nettle (*Urtica dioica*) is a 9

kDa monomer, while potato lectin is built by two 65 kDa monomers (Van Damme et al., 2008; Van Damme et al., 1998a; Van Damme et al., 1998b).

Dietary WGA could inhibit growth of cowpea weevil and Southern corn rootworm (*Diabrotica undecimpunctata*). This lectin also induced mortality in European corn borer (*Ostrinia nubilalis*) and blowfly (*Lucilia cuprina;* (Czapla and Lang, 1990; Eisemann et al., 1994; Harper et al., 1998; Murdock et al., 1990). Transgenic expression of WGA in Indian mustard resulted in an enhanced resistance against mustard aphid (*Lipaphis erysimi*). Additionally, Huesing et al. (1991) demonstrated toxicity of dietary UDA to cowpea weevil. Lectins, such as WGA can bind to chitin (GlcNAc polymer) chains which form a scaffold of the peritrophic matrix. This has been shown to result in a disorganization of the PM, loss of its protective function and disruption of the digestive process. This, in consequence, led to damage of the microvilli of the epithelium cells by food particles and gut bacteria normally restrained by the intact PM (Harper et al., 1998; Hopkins and Harper, 2001; Li et al., 2009).

1.2.9. Ricin-B-related lectins

Another family of lectins with insecticidal properties groups all proteins with ricin-B like domains. The family was named after ricin, a lectin from castor bean seed (*Ricinus* communis) which is composed of a carbohydrate-binding B-chain linked by disulfide bridge to an A-chain. Ricin A-chain has a N-glycosylase activity and is capable of cleaving adenine residues from nucleic acids. Such cleavage inactivates ribosomes and results in inhibition of protein translation, therefore polypeptides sharing homology with ricin A-chains are termed ribosome-inactivating domains or RIPs (Van Damme et al., 2008). Multiple lectins with ricin-B domains have been isolated from plant seeds (e.g. Abrin from Crab's eye, *Abrus precatorius*), bark (e.g. SNA-I, II, IV from elderberry, *Sambucus nigra*) or bulbs (e.g. lectin from *Iris hybrid;* Van Damme et al., 1998b). Homologous lectins were also identified in sclerotia (overwintering structures) of phytopathogenic fungi as in the case of RSA, *Rhizoctonia solani* agglutinin and SSA, *Sclerotinia sclerotiorum* agglutinin (Hamshou et al., 2010; Van Damme et al., 1998a).

The majority of ricin-B related lectins is specific towards GalNAc or galactose, however several lectins found in different elder species (e.g. SNA-I, or SSA-I from *Sambucus sieboldiana*) bind preferentially to sialylated glycans, i.e. Neu5Acα2,6Gal/GalNAc (Van Damme et al., 1996). Structurally, numerous ricin-B related lectins are composed of one, two or four protomers consisting of an A-chain (approx. 30 kDa) and a B-chain (approx. 35 kDa). Furthermore, there are several Gal/GalNAc lectins

belonging to this family, such as RSA, SNA-II or SNA-IV, which are built exclusively of B-chains, which assemble into homodimers or homotetramers (Van Damme et al., 2008).

Cinnamomin, a lectin isolated from seeds of the camphor tree (*Cinnamomum camphora*), was shown to have insecticidal properties against bollworm (*Helicoverpa armigera*) and common mosquito (*Culex pipiens*; Zhou et al., 2000). Recently several ricin-B like lectins, SNA-I, SNA-II and RSA, which are specific to sialic acid and GalNAc/Gal, have also been shown to exert strong toxic effects against several lepidopteran and hemipteran species, such as pea aphid, green peach aphid, cotton leafworm and beet armyworm. These lectins were shown to bind insect cell membrane receptors and induce inhibition of proliferation or apoptosis. Interestingly, SNA-I and SNA-II were effectively internalized in insect midgut cells, which was not observed for RSA (Hamshou et al., 2010; Hamshou et al., 2013; Shahidi-Noghabi et al., 2010a; Shahidi-Noghabi et al., 2010b; Shahidi-Noghabi et al., 2009). Among the identified RSA receptors were Neuroglian, Latrophilin Cirl, Integrin α-PS3 or Crumbs, which are involved in signaling, cell differentiation and maintenance of tissue structure. Therefore, RSA toxicity might be linked to interference with those processes (Hamshou et al., 2012).

There are relatively fewer reports on lectin activity towards coleopterans compared to aphids or caterpillars. However, the few available studies on the pollen beetle, the cowpea weevil or the Colorado potato beetle indicate that lectins might be potentially applicable for the control of pest beetles (Macedo et al., 2007; Melander et al., 2003; Sadeghi et al., 2006; Wang et al., 2003).

Table 1. 1. Classification and mechanisms of action of selected insecticidal lectin.s

Lectin family	Carbohydrate specificity	Notable examples and their sources	Structure	Susceptible insect	Insecticidal mechanisms
GNA-related lectins	High mannose N-glycans, mannose oligomers	**GNA** *Galanthus nivalis* (snowdrop) agglutinin **ASAI, ASAII, ASAL** *Allium sativum* (garlic) bulb and leaf lectins	$[P12]_4$ $[P(11+12)]$ $[P12]_2$	Legume pod borer Cotton leaf worm Red cotton bug Mustard aphid Brown planthopper	Disruption of gut epithelium microvilli, Interference with iron homeostasis, hormone production and nutrition
Legume lectins	α-D-mannose, α-D-glucose, GlcNAc oligomers	**ConA** *Canavalia ensiformis* (jackbean) agglutinin **PSA** *Pisum sativum* (pea) agglutinin **GS-II** *Griffonia simplicifolia* agglutinin	$[P26]_4$ $[P(17+6)]_2$ $[P30]_4$	Pea aphid Tara planthopper Cowpea weevil Pollen beetle	Binding to peritrophic matrix and midgut microvilli, Reduction of gut enzyme activity
Hevein-related lectins	GlcNAc oligomers, sialic acid	**WGA** *Triticum aestivum* Wheat germ agglutinin	$[P18]_2$	Cowpea weevil European corn borer Southern corn rootworm	Disruption of peritrophic matrix and microvilli,
Ricin-related lectins	GalNAc/Gal, Neu5Acα2,6Gal/GalNAc	**Ricin** *Ricinus communis* (castor bean) agglutinin **SNA-I** *Sambucus nigra* (elderberry) agglutinin **RSA**[1] *Rhizoctonia solani* (fungus) agglutinin	$[P(32+34)]$ $[P(32+35)]_4$ $[P15.5]_2$	Cotton leafworm Beet armyworm European corn borer Pea aphid Green peach aphid	Ribosome inactivation, Inhibition of protein translation, Binding to gut epithelium microvilli, Antiproliferative activity, Induction of apoptosis

Numbers in square brackets indicate approx. molecular weight of polypeptides that compose the lectin subunit/protomer. Digits in subscript indicate numbers of subunits that build up the native lectins.

Chapter 2

In vitro studies of lectin interactions with insect cells

2.1. Abstract

Multiple lectins (glycan binding proteins) are toxic to insects and therefore have attracted a lot of attention as potential agents for insect control. To date most of the research focused on mannose binding lectins. However, recent studies showed that lectins specific to complex glycans can also be effective. This chapter presents the preliminary study of the toxicity of two such lectins SNA-I and MAA against insect cell lines. Additionally, the distribution of the glycan epitopes present on primary insect midgut cells was analyzed.

In vitro toxicity tests indicated that both lectins MAA and SNA-I can reduce the viability of CF-203 and S2 insect cells. Both lectins showed high toxicity against the midgut CF-203 cell line, and SNA-I was the more potent of the two lectins. Confocal imaging of cells incubated with fluorescently labeled SNA-I and MAA indicated that both lectins internalized in insect cell lines and had a similar intracellular distribution. Both lectins caused membrane blebbing, cell shrinkage and formation of vesicles similar to apoptotic bodies. Additionally, SNA-I induced internucleosomal DNA fragmentation in CF-203 cells, which suggested that this lectin can induce apoptosis in midgut cells. On the contrary only non-specific DNA degradation was observed in a fraction of the S2 cells incubated with lectins which pointed out that lectin action might be cell-type specific.

Screening of primary midgut cells isolated from *Spodoptera litoralis* with fluorescently labeled lectins showed that the GalNAc/Gal-binding lectins SSA and RSA, sialic acid binding lectin SNA-I and the terminal GlcNAc-recognizing WGA bound preferentially to the apical microvillar zone of the midgut cells. In contrast the mannose-binding lectins GNA and HHA and the GlcNAc binding lectin Nictaba showed the highest binding to the basal pole of the cells. Only weak binding was observed for MAA.

These results showed that polarization of the midgut cells is also reflected by an asymmetric distribution of glycans, with clear differences between the basal and the microvillar pole. Additionally, these observations further supported sialic acid and GalNAc/Gal binding lectins (SNA-I and RSA or SSA) as promising proteins for insect control.

2.2. Introduction

In recent years lectins (or agglutinins) have attracted a lot of attention as potential alternative insect control agents. The bulk of research has focused on mannose binding lectins, including GNA from snowdrop (*Galanthus nivalis*), ASAL and ASA-II from garlic (*Allium sativum*) or ConA from jackbean (*Canavalia virosa*) (Foissac et al., 2000; Michiels et al., 2010; Upadhyay and Singh, 2012; Vandenborre et al., 2011b). However, in the last several years studies conducted in our group indicated that lectins binding to complex N-glycans and O-glycans can be effective against pest insects. One of such lectins is SNA-I, isolated from the bark of elderberry (*Sambucus nigra*), which is specific to Siaα2-6Gal/GalNAc. This lectin has previously been shown to be toxic to aphids and caterpillars (Hamshou et al., 2013; Shahidi-Noghabi et al., 2010a). The unique specificity of MAA lectin from the bark of Maackia tree (*Maackia amurensis*) towards glycans containing Galβ1-4GlcNAc, Galβ1-3GalNAc motifs and their α2,3-sialylated or sulphated forms (Geisler and Jarvis, 2011: Van Damme et al., 1997a) suggested that this lectin might also have insecticidal properties although this has not been extensively tested yet.

The insect midgut consists mainly of columnar and goblet cells, with stem cells located among their basolateral surfaces and resting on the basal lamina. The midgut columnar cells, also called enterocytes, are the most abundant cell type and typically are polarized into basolateral and apical poles. On the apical pole, these cells produce microvilli which are responsible for the secretion of enzymes and the absorption of nutrients. Notably, this apical brush border is well known to contain targets for insecticidal proteins such as the Bt toxins and some lectins (Hakim et al., 2010; Lehane and Billingsley, 2012)

The interaction of lectins with the midgut cell glycoproteins can lead to disruption of cell metabolism, enzyme secretion and nutrient absorption. This in turn results in the impairment of the digestive processes, growth retardation and even death of starvation. Some lectins have also been shown to induce a signaling cascade that leads to the programmed cell death or inhibition of cell proliferation. Since the lectin binding to the midgut cells is a prerequisite for their toxicity it is crucial to study which glycan motifs are present in these cells (Hamshou et al., 2013; Macedo et al., 2015; Shahidi-Noghabi et al., 2008). Especially, decoding the composition of glycans in the brush border of the midgut epithelium will allow for a rational selection of the proteins that are most likely to bind to the cell and exert a toxic action.

Relatively little data is available with respect to the glycan composition in insects. The few available analyses focused either on whole organisms (Aoki et al., 2007) or single proteins (Kim et al.,

2009; Knight et al., 2004) and rarely on specific tissues or structures, such as the digestive tract (Vancova et al., 2012). The glycan composition is most commonly analyzed using various mass spectrometry and chromatography techniques (Gutternigg et al., 2007; Johswich et al., 2009; Kurz et al., 2015) or with aid of the more recently introduced lectin microarray approach (Pilobello et al., 2005). These methods offer high sensitivity and reliability but are not suitable for analysis of spatial heterogeneity in glycan distribution in tissues or cells. Therefore, microscopic techniques are preffered for the concurrent analysis of different glycan types and their spatial distribution. The use of metabolic glycan labeling (Laughlin and Bertozzi, 2009; Ning et al., 2008), antibodies (Laughlin and Bertozzi, 2009) or lectins (Tian and Ten Hagen, 2007a) allows to detect specific monosaccharides or glycan motifs across N-, O-glycans, glycolipids or glucosaminoglycans simultaneously and precisely localize them taking advantage of the high resolution of electron or light microscopy.

The first goal of this chapter was the preliminary analysis of the insecticidal activity of two lectins specific for complex glycans, in particular SNA-I and MAA. The CF-203 insect midgut cell line and S2 insect embryonic cell line were used to study impact of the two lectins on cell viability and morphology as well as on DNA fragmentation. The second objective was to explore the distribution of the glycans in the insect midgut epithelium. For that primary cell cultures from the midgut of cotton leafworm *Spodoptera littoralis* (Lepidoptera) were established and probed with a selection of fluorescently labeled lectins. Subsequently, cells were imaged by confocal microscopy and lectin binding to various cell surface regions of the columnar cells and the stem cells was analyzed quantitatively.

2.3. Materials and methods

2.3.1. Lectin purification and labeling with fluorescent tags

SNA-I was purified from the lyophilized elderberry bark (*Sambucus nigra*) according to Van Damme et al. (1996). Briefly, elderberry bark was pulverized and extracted with 20 mM diaminopropane (DAP). The extract was clarified by filtration and centrifugation. Extracted protein was precipitated using saturated ammonium sulphate, pelleted by centrifugation and re-dissolved in phosphate buffered saline (PBS) pH 7.6. SNA-I was specifically purified by two rounds of affinity chromatography on fetuin Sepharose 4B column and desorbed using 20 mM DAP. In the next steps the lectin was further purified by gel filtration and dialysis, and finally lyophilized to obtain pure lectin powder. Lectin purity was confirmed by SDS-PAGE. Coomassie blue staining of the gels led to detection of bands with sizes

corresponding to lectin subunits. No other bands were detected, which suggested that lectin preparations were essentially free of potential protein contaminants.

MAA was isolated from *Maackia amurensis* bark according to Van Damme et al. (1997a) using a similar protocol as for SNA-I, except that 50 mM acetic acid was used for extraction and ion-exchange chromatography was used to cfractionate the protein extract before lectin purification on fetuin-Sepharose 4B chromatography.

GNA (*Galanthus nivalis* agglutinin) was obtained from *G. nivalis* bulbs, HHA (*Hippeastrum* hybrid agglutinin) from *Hippeastrum* hybrid bulbs, WGA (wheat germ agglutinin) from *Triticum aestivum* germs, Nictaba from jasmonate-treated *Nicotiana tabacum* leaves, RSA (*Rhizoctonia solani* agglutinin) from the sclerotes of the fungus *Rhizoctonia solani*, SSA (*Sclerotinia sclerotiorum* agglutinin) from sclerotes of the fungus *Sclerotinia sclerotiorum*. The lectins were purified previously using combination of glycan affinity chromatography, ion-exchange chromatography and gel filtration as described in Vranken et al. (1987), Van Damme et al. (1998b) and Vandenborre et al. (2010). See Table 2.1. for more details.

Table 2.1. Lectins used for screening of glycan epitopes in midgut cells.

Lectin	Lectin family	Glycan binding specificity	Source, tissue / organ	Affinity ligand used for isolation
MAA	Legume lectins	Neu5Acα(2,3)Gal/GalNAc	Bark of Maackia tree *(Maackia amurensis)*	Fetuin
SNA-I	Ricin-related lectins	Neu5Acα(2,6)Gal/GalNAc	Bark of elderberry (*Sambucus nigra*)	Fetuin
RSA	Ricin-related lectins	terminal Gal or GalNAc	Sclerotia of plant pathogenic fungus *Rhizoctonia solani*	Immobilized galactose
SSA	Ricin-related lectins	terminal Gal or GalNAc	Sclerotia of plant pathogenic fungus *Sclerotinia sclerotiorum*	Immobilized galactose
WGA	Hevein-related lectins	GlcNAc oligomers, sialic acid	Wheat germs	Immobilized GlcNAc
Nictaba	Nictaba-related lectins	GlcNAc oligomers,	Tobacco leaves after induction with jasmonate	Combination of acetylated chitin and ovomucoid
GNA	GNA-related lectins	α1,3-mannose units	Snowdrop bulbs	Immobilized mannose
HHA	GNA-related lectins	α1,3-and α1,6-mannose units	Amaryllis bulbs	Immobilized mannose

Lectins were labeled with fluorescein isothiocyanate (FITC) or Tetramethylrhodamine (TRITC) as described previously (Hamshou et al., 2012). Briefly, lectins were dissolved in 50 mM sodium borate buffer (pH 8.5) and mixed with 24-fold molar excess of FITC dissolved in dimethylformamide. After incubation at room temperature in the dark for 2 h, the free label was removed by gel filtration on a Sephadex G25 column equilibrated with PBS. The protein concentrations were determined with Bradford's method (Bradford, 1976) and measurement of absorbance of the protein solution at 280 nm. The labelling resulted on average in attachement of 2-3 FITC molecules per protein molecule, as estimated based on absorbance of the solutions at 280 nm and 488 nm. FITC-labeled bovine serum albumin (FITC-BSA) was purchased from Sigma Aldrich. Ratio of labeling for FITC-BSA was 7-12 FITC molecules per protein molecule.

2.3.2. Cell cultures

The FPMI-CF-203 cell line was originally established from the midgut of the spruce budworm *Choristoneura fumiferana* (Lepidoptera; Sohi et al., 1993). This cell line was maintained in Insect-Xpress™ medium (Lonza) supplemented with 2.5% fetal bovine serum. The Schneider-2 cell line (S2) was derived from the embryos of the fruit fly *Drosophila melanogaster* (Diptera; Schneider, 1972). S2 cell were grown in SFX-Insect medium (Thermo Scientific). Both cell lines were kept at 27 °C and subcultured weekly at 1:10 dilution.

2.3.3. Cell viability assay and morphological observations

SNA-I and MAA were dissolved in PBS to prepare a range of stock solutions. Cells at logarithmic phase of growth (3 days after subculturing) were harvested, counted under the microscope using a hemocytometer and diluted with fresh medium to 2 000 000 cells/ml in case of S2 cells and 200 000 cells/ml in case of CF-203 cells. Stock solutions were mixed at a ratio 1:10 with suspension of S2 or CF-203 cells to give final lectin concentrations of 0.1 – 1000 nM for SNA-I and 0.1-10000 nM for MAA. PBS was used as a negative control. Cell suspensions were then transferred to 96-well plates (100 µl per well in 4-5 replicates). Cells were incubated with lectins for 4 days, which is usually to observe lectin effects on cell viability and proliferation. After incubation cell viability was measured using the MTT assay (Twentyman and Luscombe, 1987). Viable cells convert this compound to insoluble formazan crystals, which is soluble in organic solvents. After incubation for 2 hrs with MTT reagent formazan crystals were collected by centrifugation and dissolved in 100 µl of isopropanol. The amount of the product, which is

proportional to number of viable cells, was measured by the absorbance at 560 nm using a plate reader (PowerWave X340, Bio-Tek Instruments Inc.). EC_{50} values or effective concentrations causing 50% reduction in cell viability were calculated using sigmoidal dose response curve fitting in GraphPad Prism 4.0. Statistical comparisons between lectins and cell lines were performed in the same software using the F-test for comparison of nonlinear regression parameters (log EC_{50}).

The morphological changes induced by lectins were observed directly in 96-well plates with transparent bottom using an inverted microscope (4-20x objectives) equipped with a digital camera.

2.3.4. Confocal microscopy of insect cell lines

For confocal microscopy cells were diluted with medium and seeded on poly-L-lysine coated cover glasses. Subsequently, the cells were washed with medium and incubated with fluorescently-labeled lectin (0.5 µM SNA-I, 1.5 µM MAA) for 1 hour in darkness. Then cells were washed 3 times using PBS, fixed with 3.7% formaldehyde in PBS (5 mins), washed 3 times (1 min each) and stained with nuclear dye Hoechst 33258 at 1 µg/ml in PBS (15 mins) and washed for final 3 times. Cover glasses were mounted on the microscope slides using Vectashield medium (Vector Labs). Images were obtained using confocal microscope Nikon A1R. Following set up was used: 60x oil immersion Plan Apo objective (1.4 NA). A 405.3 nm laser and 450/50 nm filter were used for Hoechst 33258, 488.0 nm laser and 525/50 nm bandpass filter were used for FITC-labeled lectins, while 561.7 nm laser and 595/50 nm filter were used for TRITC-MAA. Pixel sizes: 0.41 µm for CF-203 cells (0.09 for single cell imaging), 0.24 µm for S2 cells (0.06 µm for single cell imaging). The thickness of the optical section was set to 1 µm. Obtained images were edited for brightness and contrast in FiJi software (Schindelin et al., 2012).

2.3.5. Evaluation of genomic DNA fragmentation by gel electrophoresis

To analyze DNA fragmentation 2 ml of cell suspensions were treated with lectins or Actinomycin D for 24h. One day incubation is usually sufficient for DNA fragmentation to occur upon induction with known apoptosis inducers (Fink and Cookson, 2005). Subsequently cells were collected by centrifugation, washed with PBS and disrupted by freeze-thawing. Then cells were further homogenized in DNA extraction buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA-NaOH, 0.1% SDS, pH 8.0) using a pestle. The extract was digested with RNase A (20 µg/ml) for 30 min at 37°C and proteinase K (100 µg/ml) for 60 min at 50°C. Finally, DNA was purified using a standard phenol-chloroform method and the yield was

estimated with the Nanodrop spectrophotometer. Approximately 3 µg of DNA were resolved in a 2% agarose gel at 100 V and visualized by staining with ethidium bromide.

2.3.6. Analysis of DNA fragmentation in S2 cells by flow cytometry

S2 cells (2 ml) treated with lectins were collected by centrifugation and fixed using 70% methanol at -20°C for 30 mins. Fixed cells were collected by centrifugation, treated with 100 µg/ml RNAse A for 30 mins at 37°C and stained with 50 µg/ml propidium iodide in PBS. The analysis was performed on cells using the BD FACSCanto flow cytometer (FACS) in the Laboratory of Prof. Herman Favoreel (Laboratory of Immunology, Department of Parasitology, Virology & Immunology, Gent University). Analyses were done by Dr. Korneel Grauwet.

2.3.7. Establishment of primary cell cultures from cotton leafworm midgut

Primary cultures of midgut cells were prepared by Dr. Silvia Caccia and ir. Kaat Capelle from actively feeding last instars of cotton leafworm. Dissected midguts were obtained as described in Cermenati et al. (2007) and cells dissociated for 1.5 h with 2 mg/ml of collagenase (Type I-AS; Sigma) in Insect Physiological Solution (IPS). This buffer contained 47 mM KCl, 20.5 mM MgCl2, 20 mM MgSO4, 5.6 mM KH2PO4, 5.3 mM K2HPO4, 1 mM CaCl2 and 75 mM glucose, which mimics the osmolarity, pH and salt composition of hemolymph of lepidopteran larvae.

2.3.8. Analysis of lectin interactions with larval midgut cells

Midgut columnar cells and stem cells were incubated for 1 h with 0.85 µM of FITC-labeled lectins (Table 2.1.). Control cells were incubated with equal amounts of PBS. Additionally, one preparation of cells was incubated with FITC-BSA, for the sake of control of non-glycan based interactions between proteins and cells. After incubation, cells were fixed for 15 min with 4% paraformaldehyde in PBS. After 3 rinses with PBS, samples were mounted in Vectashield Mounting Medium (Vector Laboratories). Image acquisition settings were identical as described in Section 2.3.4. with the exception that pixel size was 0.08 µm.

Using ImageJ software mean pixel intensities were measured in manually selected microvillar zone, basal and lateral part of the membrane of columnar cells as well as in the cell membrane (perimeter) of stem cells. The signal intensities in the given region were individually measured for each imaged cell and divided by background signal in a given image to reduce the impact of inconsistencies of

background fluorescence between images. The differences in relative binding between the zones were analyzed using t-tests or ANOVA in SPSS 22 Statistics 22 (IBM). A non-parametric Mann-Withney U test for median was used to confirm t-test results in case of non-normally distributed data.

2.3.9. Verification of lectin binding specificity

In this series of experiments, FITC-labeled lectins (HHA, RSA and SNA-I) were pre-incubated for 30 min with a specific competing sugar: 2 mg/ml of yeast mannans (Sigma) for HHA, 20 and 100 mM of GalNAc for RSA, 20 mM of α2,6-sialyllactosamine (Carbosynth) for SNA-I. The IPS buffer used to maintain insect midgut cells contained 75 mM glucose therefore non-specific sugar inhibition could be generally excluded. However, in case of testing of RSA specificity a non-specific sugar GlcNAc was used at 100 mM as an additional control to check for impact of elevated sugar concentration on binding specificity. Subsequently midgut cell cultures were incubated with these mixtures for 1 h, washed with PBS, mounted on glass slides and imaged under a confocal laser scanning microscope as mentioned above.

For each cell 5 to 8 z-sections were taken at 2 µm-spacing. The microvillar pole of the cells was manually selected in each picture and the average pixel intensity was measured using ImageJ. The ratio of fluorescence intensity in the microvillar pole over the background was calculated to the reduce influence of potential inconsistencies between pictures. The impact of the addition of specific competing sugar on the lectin binding was analyzed using independent-samples t-test in SPSS Statistics 22 (IBM).

2.4. Results

2.4.1. SNA-I and MAA reduce viability of cultured insect cells

To analyze lectin effects on the viability on insect cell lines, the CF-203 cells and S2 cells were incubated with SNA-I and MAA at wide range of concentrations (0.1 – 10000 nM). CF-203 cells are derived from midgut and thus serve as a model for lectin activity in the digestive tract, while S2 cells are established from embryos (macrophage-like cells) and thus can be helpful to infer potential lectin effects on tissues other than gut. MAA and SNA-I reduced viability of both cell lines which indicated a broad toxic potential of the two lectins (Fig. 2.1).

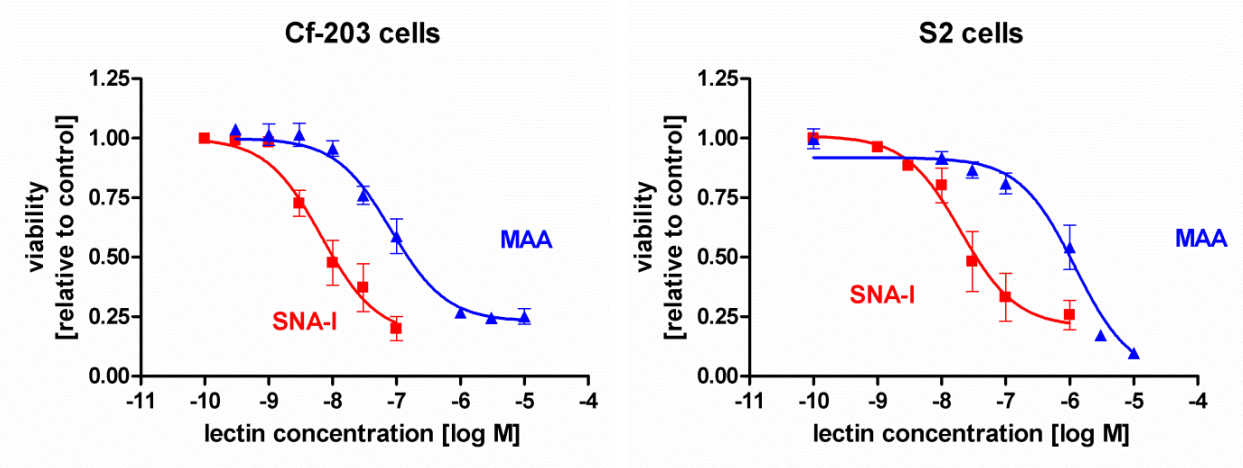


Fig. 2.1. SNA-I and MAA are toxic to insect cells. Cell viability was measured using the MTT assay after 4 days incubation with range of lectin concentrations. Each experiment was performed in duplicate with at least 4 technical replications per concentration.

However, analysis of the EC_{50} (effective concentration reducing the viability by 50%) indicated significant differences in the entomotoxic potential between lectins and in susceptibility between the cell lines (Table 2.2.). SNA-I was more potent lectin than MAA. The former induced considerable reduction of viable cell numbers at low nanomolar concentrations (EC_{50} = 6.7 nM in CF-203 cells) while the latter was one to two orders of magnitude less effective.

Table 2.2. Comparison of half maximal effective concentration values (EC_{50}) between lectins and cells. SNA-I is significantly more toxic than MAA towards both cell lines and the midgut cell line CF-203 is significantly more susceptible than the S2 cell line to both lectins.

	SNA-I EC_{50} (95% C. I.)		**MAA EC_{50}** (95% C. I.)		**p-value SNA-I vs. MAA**
Cell line	nM	µg/ml	nM	µg/ml	
CF-203	**6.7** (2.7-16.5)	**1.6** (0.7-4.0)	**81.8** (51.8-129.4)	**10.6** (6.7-16.8)	**<0.0001**
S2	**19.9** (7.5-52.7)	**4.8** (1.8-12.7)	**1205.0** (552.1-2624.2)	**156.7** (71.8-341.1)	**<0.0001**
p-value S2 vs. CF-203	<0.0074		<0.0001		

95% C. I. – 95% confidence intervals. p-values were calculated using F-test for comparison of nonlinear regression parameters (log EC_{50}) in GraphPad Prism 4.0.

CF-203 midgut cell line was more susceptible to both lectins than S2 cells. EC_{50} of SNA-I was approximately 3- fold lower in CF-203 cells compared to S2 cells (p=0.0074). The EC_{50} of MAA in S2 cells was rather high (0.5-2.5 µM) and its activity was approximately 15-fold lower against S2 cells compared to CF-203 cells (p<0.0001).

2.4.2. SNA-I and MAA are internalized in both insect cells lines

To verify if the tested lectins can be internalized by insect cells, the cells were incubated with lectins labeled with a fluorescent tag (fluorescein isothiocyanate - FITC) and imaged using confocal microscopy. Both lectins were able to penetrate into S2 and CF-203 cells although MAA appeared to be internalized with a relatively lower efficiency (Fig. 2.2. and Fig. 2.3.). Both lectins localized in the discrete puncta inside the cells, likely in organelles such as lysosomes, endosomes, endoplasmic reticulum or the Golgi apparatus. There was a relatively low intensity of fluorescence at the level of the cell membrane which suggested rather weak binding and/or rapid internalization.

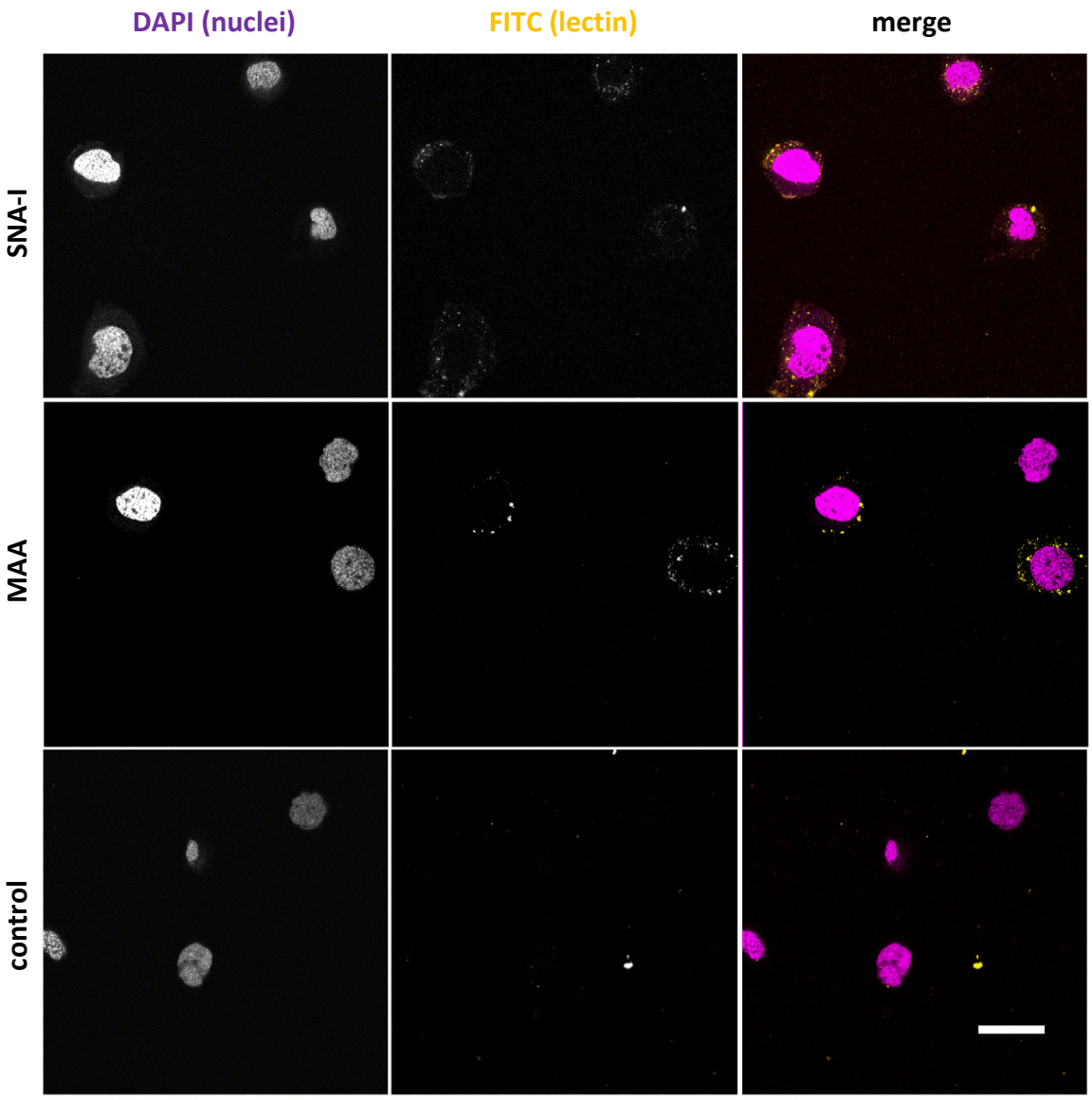


Fig. 2.2. SNA-I and MAA are internalized in CF-203 cells. Cells were incubated with FITC-labeled lectin for 1 hr and imaged using confocal microscope. Scale bar is 40 µm.

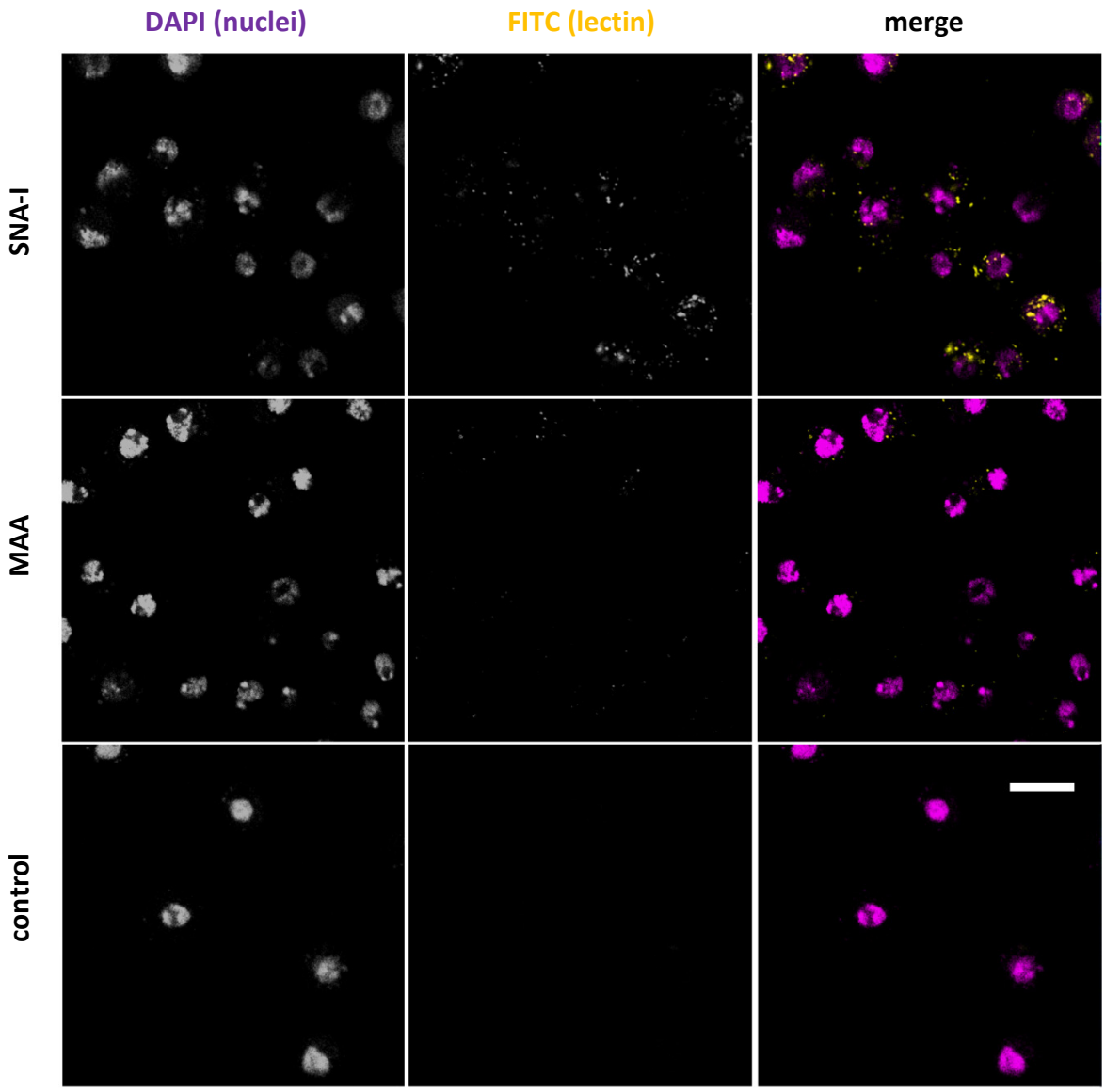


Fig. 2.3. SNA-I and MAA are internalized in S2 cells. Cells were incubated with FITC-labeled lectin for 1 hr and imaged using confocal microscope. Scale bar is 20 µm

To compare the localization of SNA-I and MAA the cells were incubated with both lectins labeled with two different fluorophores (MAA-TRITC and SNA-I-FITC). This showed that both lectins have nearly identical subcellular distribution. Imaging of single cells at high magnification revealed that both lectins accumulated in S2 and CF-203 cells at one side proximally to the nucleus.

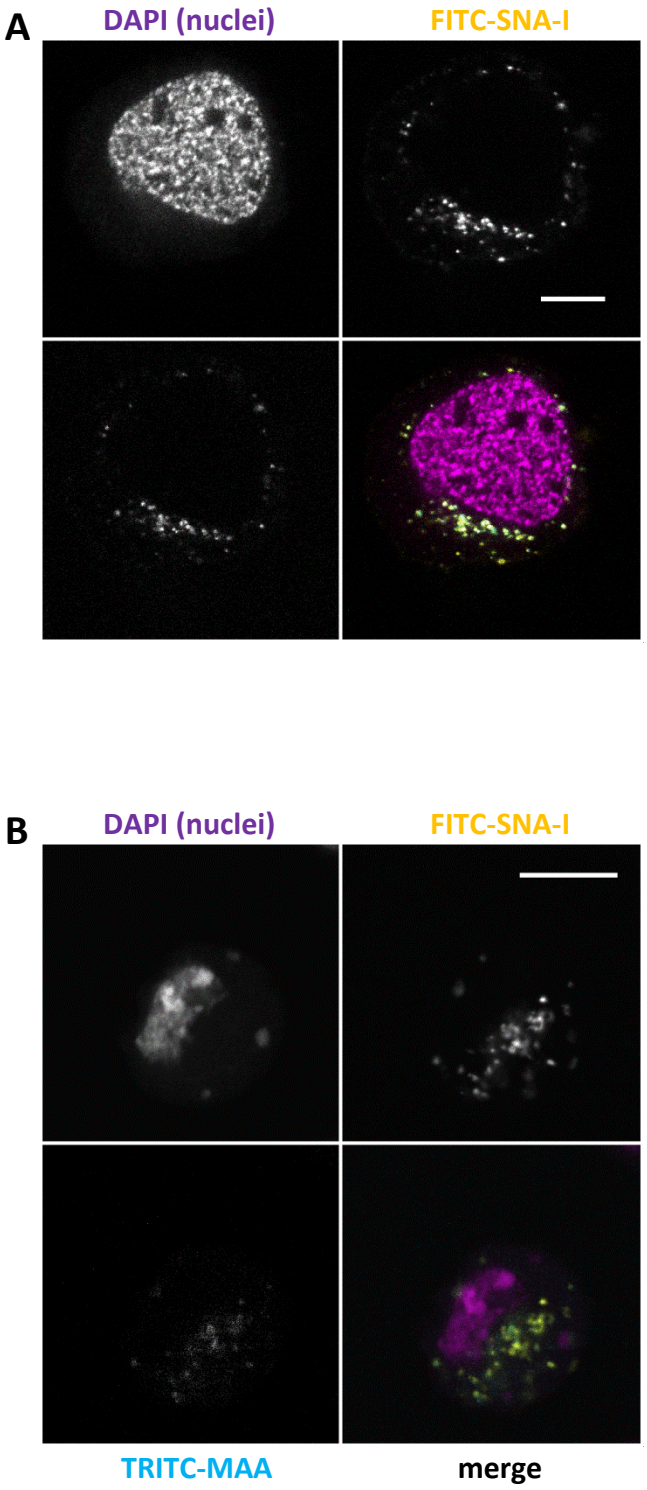


Fig. 2.4. SNA-I and MAA co-localize in insect cells. Cells were incubated with a mixture of FITC-labeled SNA-I and TRITC-labeled MAA, and imaged using confocal microscopy. (A) CF-203 cell. (B) S2 cell. Scale bars are 10 µm.

2.4.3. Lectins induce apoptotic-like morphology

The incubation of CF-203 cells with SNA-I and MAA induced cell shrinkage, blebbing and formation of vesicles reminiscent of apoptotic bodies. Incubation with SNA-I at 10 nM was enough to induce morphological changes in the majority of the cells, while after incubation with 100 nM SNA-I nearly no cells with normal morphology were observed (Figs. 2.5. and 2.6.). This indicated that reduction of viable cell number shown in Fig. 2.1. was caused not only by growth inhibition but also by induction of cell death.

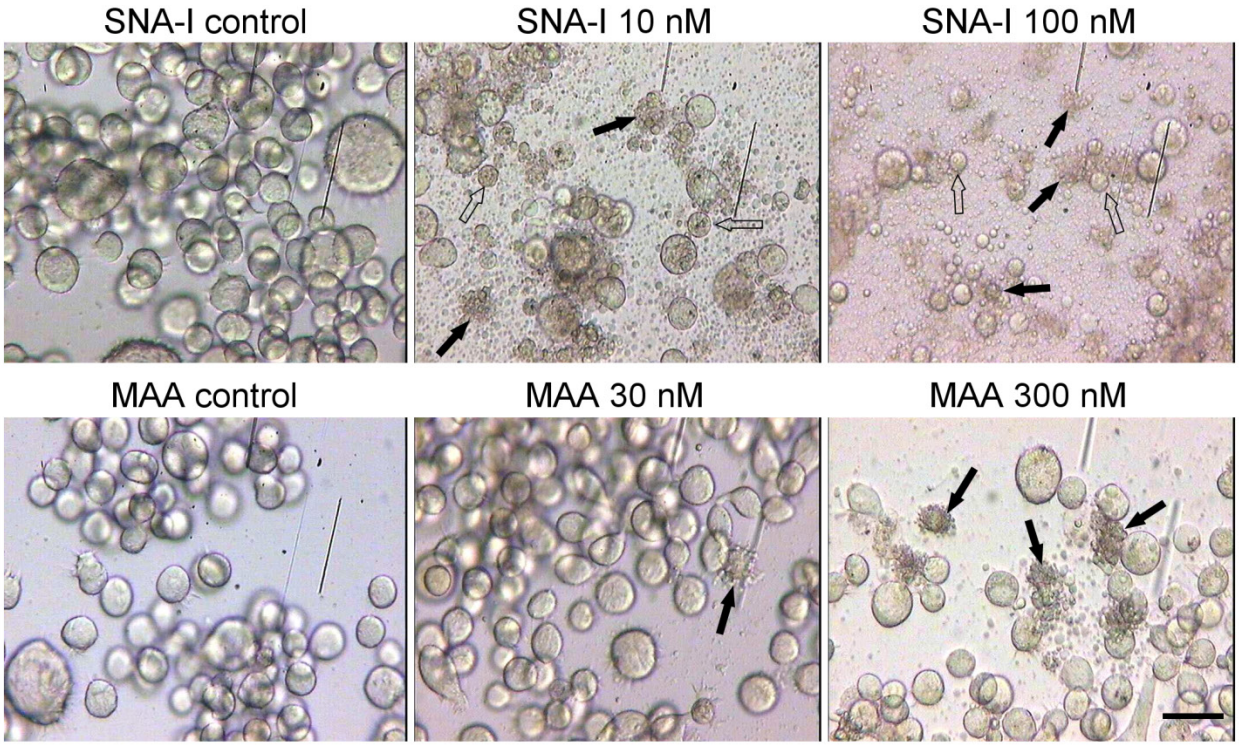


Fig. 2.5. Effects of SNA-I and MAA on insect cell morphology of CF-203 cells. Images were obtained using optical transmission microscope Empty arrows indicate shrunk cells. Black arrows indicate blebbing cells surrounded by putative apoptotic bodies. Scale bar is 50 µm.

MAA also caused similar morphological changes in CF-203 cells. However, this protein was clearly less potent than SNA-I. After incubation with 30 nM MAA only single cells with membrane blebbing were visible. More cells with apoptotic-like morphology were observed after incubation with 300 nM MAA (~10%) but still much fewer compared to SNA-I treatments.

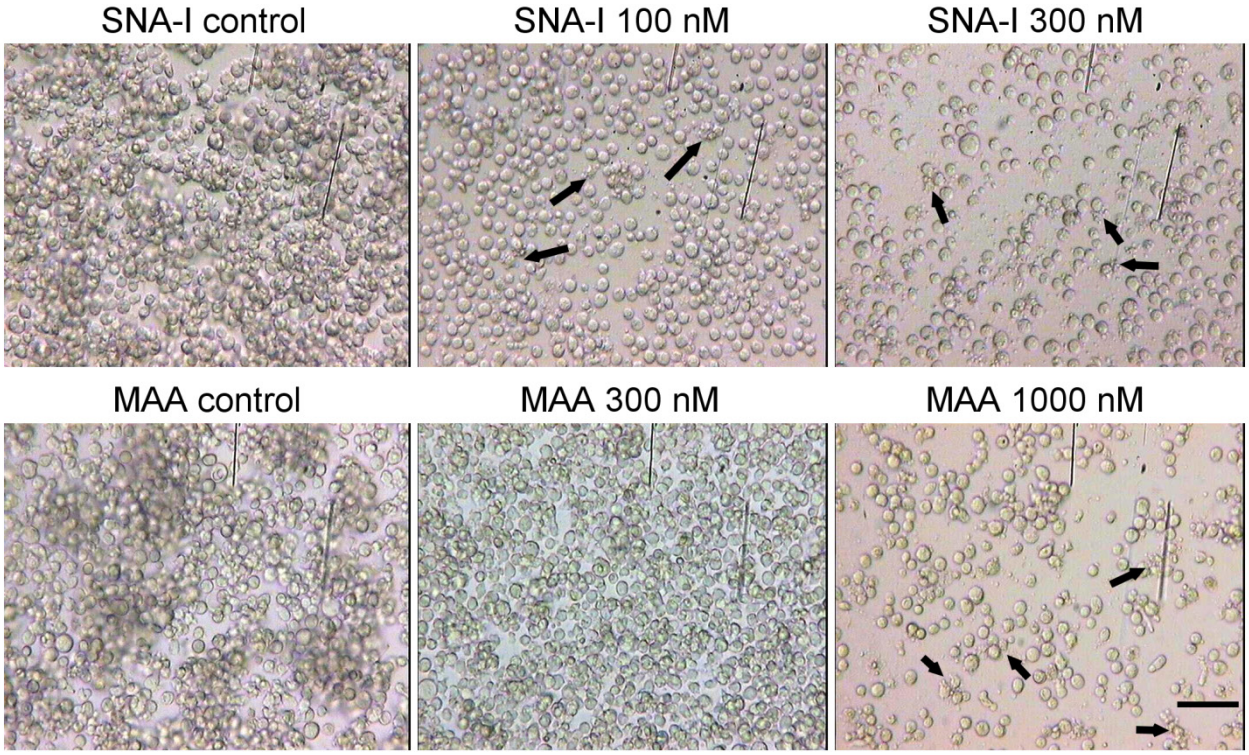


Fig. 2.6. Effects of SNA-I and MAA on insect cell morphology of S2 cells. Images were obtained using optical transmission microscope. Black arrows indicate blebbing cells surrounded by putative apoptotic bodies. Scale bar is 50 µm.

In S2 cells treated with both lectins (Fig. 2.6) similar structures were also present but at clearly lower incidence compared to CF-203 cells. Since S2 cells are much smaller than CF-203 cells a clear observation of membrane blebbing and apoptotic bodies was rather difficult.

2.4.4. Incubation with lectins stimulates DNA fragmentation

One of the hallmarks of apoptosis is ordered internucleosomal DNA fragmentation. This can be observed as a characteristic ladder pattern of genomic DNA resolved in agarose electrophoresis. This approach was used to verify if incubation with lectins induced apoptosis in insect cells. Treatment with SNA-I at 30 nM for 24 hrs was enough to cause a clear laddering of DNA in CF-203 cells (Fig. 2.7.) similar to this caused by Actionomycin D (ActD) which has been shown previously to induce apoptosis in insect cells (Palli et al., 1996). MAA did not produce such an effect even at 10 fold higher concentration although it caused cell membrane blebbing in a fraction of the cells. Most likely this was due to timing – clear blebbing and mortality was observed only after 4 days of incubation with MAA while the DNA was isolated already after 24 hrs.

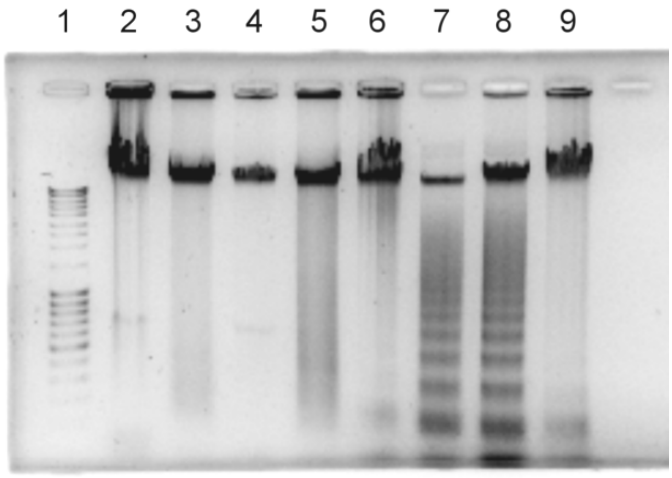
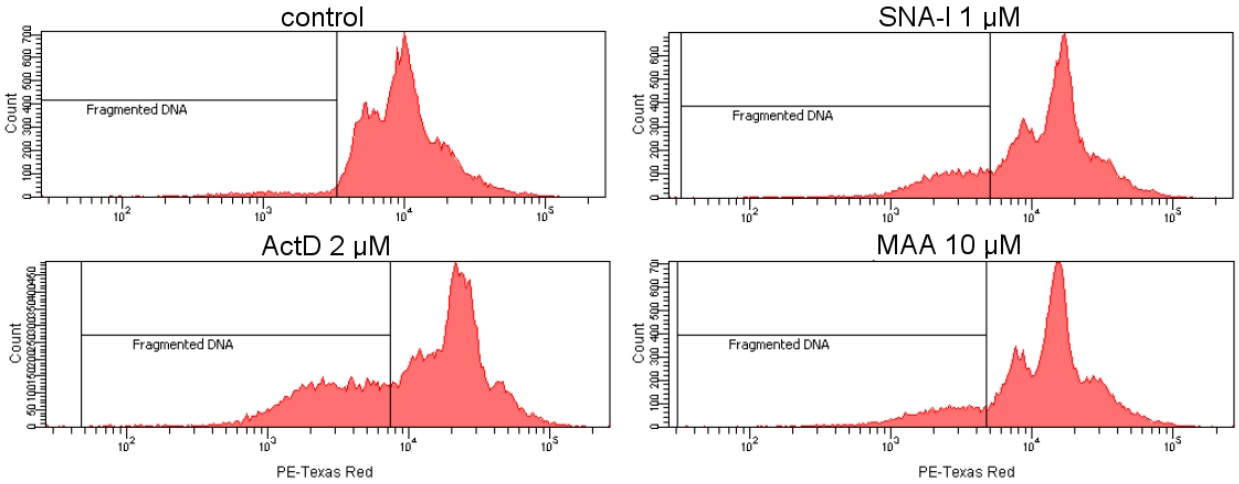


Fig. 2.7. SNA-I induced internucleosomal DNA fragmentation in CF-203 cells. DNA isolated from treated cells was resolved by agarose gel electrophoresis. Picture Lanes: 1. Marker, 2. S2 cells control, 3. S2 treated with 300 nm SNA-I, 4. S2 treated with 3000 nM MAA, 5. S2 treated with 100 nM actinomycin D, 6. CF-203 cells control, 7. CF-203 treated with 30 nM SNA-I, 8. CF-203 treated with 8 nM actinomycin D 9. CF-203 treated with 300 nM MAA.

None of the lectins treatments caused any DNA laddering in *Drosophila* S2 cells and surprisingly, actinomycin D also did not induce this effect. Yet in the latter case and in the treatment with SNA-I the DNA smearing was visible, which might be indicative of nonspecific DNA degradation. There are no published reports showing clear DNA laddering in S2 cells, thus it appeared likely that cell death in these cells might be associated with this kind of random fragmentation (Didenko et al., 2003)

To verify this assumption we analyzed DNA fragmentation in the S2 cells by flow cytometry. This method is based on the observation that fixation and permeabilization allows fragmented DNA to diffuse out of the cells. This leads to reduction of the DNA content in cells which after labeling with a fluorescent stain (propidium iodide) can be measured using a fluorescence detector. To ensure that full extend of DNA fragmentation can be observed the concentration of ActD was incrased to 1 and 2 µM. In these treatments DNA fragmentation was detected in approx. one third of the S2 cells (Fig. 2.8). To explore the full potential of MAA and SNA-I the concentrations were also increased to 10 µM in case of MAA (10-fold higher than EC_{50}) and 1 µM for SNA-I (50-fold higher than EC_{50}). These treatments caused DNA fragmentation in only up to 25% of the cells and again SNA-I appeared to be slighlty more effective. This experiment indicated that the two lectins do not induce cell death effectively in S2 cells, even when the highest lectin concentrations were used for treatment.



Treatment	% cells with fragmented DNA	SD
Control	3.8	0.4
MAA 3 µM	7.6	0.3
MAA 10 µM	16.4	1.5
SNA 0.3 µM	10.6	0.8
SNA 1 µM	21.4	5.0
ActD 1 µM	28.1	7.9
ActD 2 µM	35.3	2.7

Fig. 2.8. Analysis of induction of DNA fragmentation in S2 cells. Upper panel shows typical readout of flow cytometry experiments. Table in lower panel shows a summary of the results. SD is standard deviation (n=2).

2.4.5. Lectin-binding sites are distributed asymmetrically in the midgut epithelium cells

To analyze the distribution of different glycans in the midgut epithelium the midgut cells isolated from midgut of the cotton leafworm larvae were incubated with a selection of FITC-labeled lectins (Table 2.3.). The chosen lectins were specific to mannose oligomers (GNA and HHA), GalNAc/Gal (RSA and SSA), GlcNAc (WGA and Nictaba), Siaα2-6Gal/GalNAc (SNA-I) and Galβ1,4GlcNAc, Galβ1,3GalNAc including α2,3-sialylated or sulphated forms (MAA). The carbohydrate binding specificity of the selected lectins covers most of the glycan motifs that may be present in insect cells. Each fluorescently labeled lectin tested bound to the microvilli of the columnar cells. Measured fluorescence intensities in the microvillar region of the cell were significantly higher than the levels of autofluorescence ($p<0.05$), except for the treatment with MAA ($p=0.054$). To reveal spatial differences in the type of glycans present on the gut

columnar cells lectin binding to the basal pole and the lateral membranes was quantified. Subsequently, the relative lectin binding to the basal, lateral and microvillar (apical) zones of the cell membrane was calculated for each individual cell (Fig. 2.9 and Table 2.3).

Table 2.3. Lectin binding to different regions of midgut columnar cells and to stem cells. Values are average ratios of fluorescence intensities in indicated regions of midgut cells relative to background fluorescence ± standard deviations.

			Columnar cells			Stem cells	
Lectin	**Specificity**	**Measured cells**	**Microvilli**	**Lateral**	**Basal**	**Measured cells**	**perimeter**
BSA	none	14	1.2±0.2	1.2±0.1	1.2±0.1	-	n.d.
SNA-I	Siaα2,6GalNAc/Gal	19	5.0±2.7	2.0±0.8	1.9±0.5	10	3.2±0.8
MAA	Siaα2,3GalNAc/Gal	24	1.4±0.3	1.1±0.1	1.1±0.3	7	1.3±0.1
WGA	GlcNAc	15	25.9±22.7	4.1±1.5	6.3±3.7	6	6.8±2.3
Nictaba	GlcNAc	9	4.6±1.8	5.3±2.3	7.6±4.8	10	11.9±5.2
RSA	GalNAc/Gal	26	20.3±9.4	6.2±4.1	8.9±5.4	7	20.9±8.1
SSA	GalNAc/Gal	21	75.0±25.8	14.5±8.5	17.5±10.7	11	14.2±5.6
HHA	Mannose	22	27.1±10.0	28.1±14.6	56.6±34.1	8	20.3±5.9
GNA	Mannose	19	2.3±0.9	6.2±2.6	14.4±7.5	12	11.3±8.3

Four lectins bound more readily to the apical brush border microvilli than to the basal pole, WGA by 1.8 fold ($p=0.001$) SNA-I and RSA by 2.1 fold and SSA by 4.1 fold (all three $p<0.001$). In contrast, GNA, HHA and Nictaba bound preferentially to the basal pole compared to the apical microvilli by 6.7 fold ($p<0.001$), 2.5 fold ($p<0.001$) and 2.0 fold ($p=0.049$), respectively. Furthermore SNA-I, RSA, SSA and WGA showed a 2.1, 2.6, 3.0 and 4.6 fold higher (all four $p<0.001$) binding to the microvilli compared to the lateral membranes, respectively. Only in the case of GNA the fluorescence intensity was significantly higher (2.8 fold, $p<0.001$) in the lateral zone compared to the apical brush border membrane. Furthermore, as shown in Fig. 2.9.B for three lectins GNA, HHA and WGA binding was higher in the basal pole compared to the lateral membranes ($p<0.001$, 0.018, 0.027, respectively).

To verify the specificity of the lectin binding primary midgut cells were treated with lectins, HHA, RSA and SNA-I, preincubated with their competing sugars: yeast mannans, GalNAc and α2,6-sialyllactosamine (6'SiaLacNAc), respectively. As shown below (Table 2.4. and Fig. 2.10.) incubation of the

cells in the presence of the complementary sugars resulted in a significant reduction (40-70%) of the lectin binding to the microvilli, indicating that lectin binding was glycan-dependent.

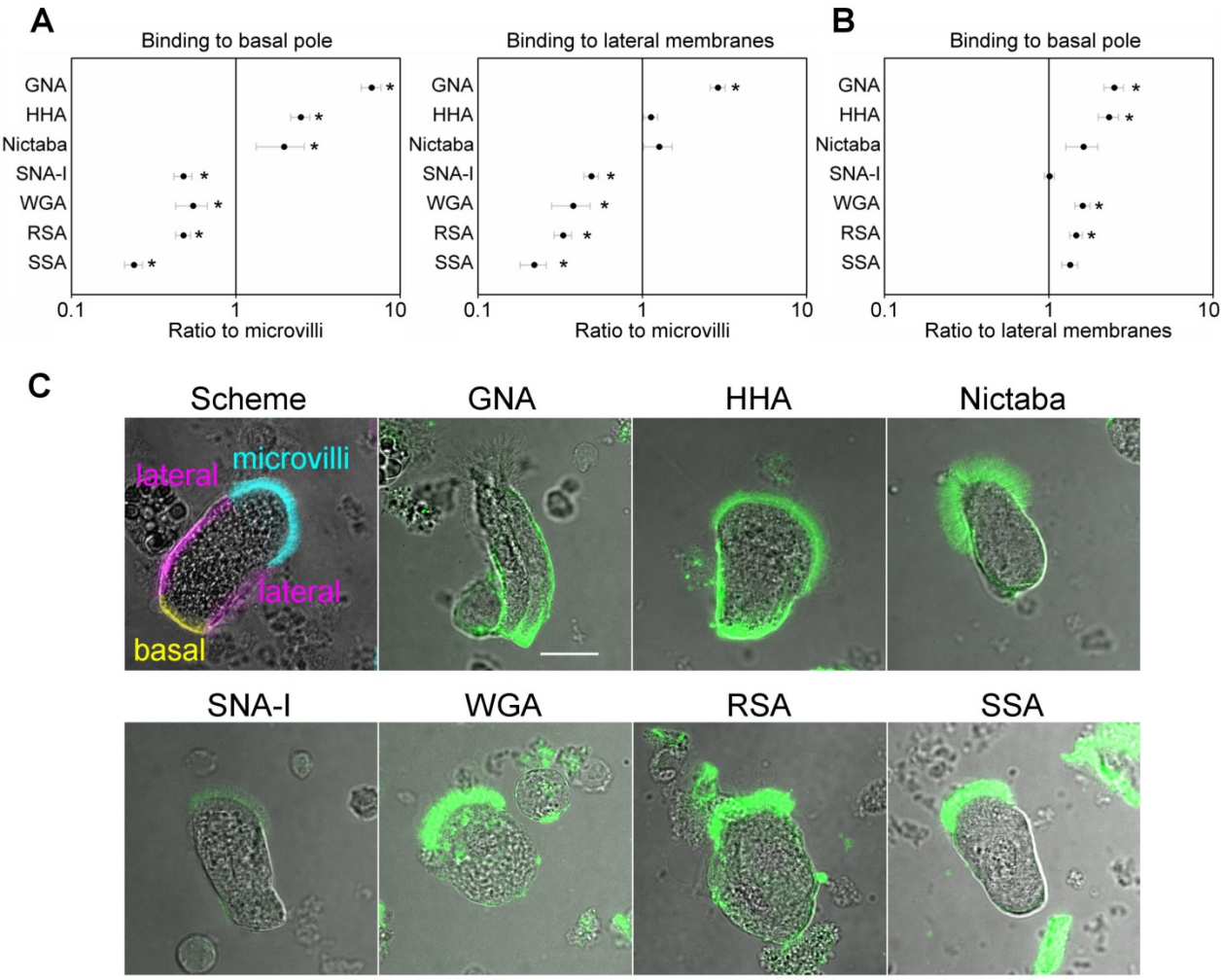


Fig. 2.9. Lectin binding to different regions of midgut columnar cells. (A) Ratio of lectin binding to basal or lateral zones over the binding intensities to microvilli suggest that glycans recognized by SSA, RSA, WGA and SNA-I are more abundant at the microvillar zone while those recognized by Nictaba, HHA and GNA are more abundant at the basal poles. Asterisks indicate statistically significant differences ($p<0.05$, independent sample t-test). (B) Comparison between the basal and lateral parts of the cell membranes indicates differential binding for WGA, RSA, HHA and GNA. Data are presented as average values ± SEM. Asterisks indicate statistically significant differences ($p<0.05$, independent sample t-test). (C) Scheme indicating three different zones of measurement and representative confocal images of midgut columnar cells incubated with lectins. Scale bar is 20 µm.

RSA was also incubated with high concentration of GlcNAc (100 mM) to test the specificity of the sugar inhibition. GlcNAc is not recognized by RSA, so it should not affect the interaction between the midgut cells and the lectin. As expected, GlcNAc caused no reduction in RSA binding, in contrast to GalNAc, which allowed us to exclude potential non-specific inhibition.

Table 2.4. Reduction of lectin binding to microvilli of columnar cells after incubation with complementary sugars.

	Fluorescence in the microvillar pole			
	n	Mean ± SD	Binding inhibition	p-value
SNA-I	6	2.85 ± 1.00	**49.8 ± 9.5%**	**0.008**
SNA-I + 20 mM 6'SiaLacNAc	6	1.43 ± 0.27		
SNA-I	12	3.96 ± 0.76	**36.1 ± 24.5%**	**0.001**
SNA-I + 20 mM 6'SiaLacNAc	13	2.53 ± 0.97		
RSA	5	6.41 ± 0.88	**36.9 ± 20.6%**	**0.001**
RSA + 20 mM GalNAc	9	4.05 ± 1.32		
RSA + 100 mM GlcNAc	12	8.30 ± 3.30	**42.1 ± 20.0%**	**0.002**
RSA + 100 mM GalNAc	13	4.81 ± 1.66		
HHA	9	3.90 ± 1.18	**62.1 ± 11.5%**	**0.000**
HHA + 2 mg/ml yeast mannans	8	1.48 ± 0.45		

The experiment with SNA-I was repeated twice. For RSA a second repetition of the experiment was done using higher concentrations of GalNAc or GlcNAc to check for non-specific inhibition of the lectin binding. Mean values are ratios of fluorescence relative to background. SD is standard deviation. p-values were calculated using independent sample t-test.

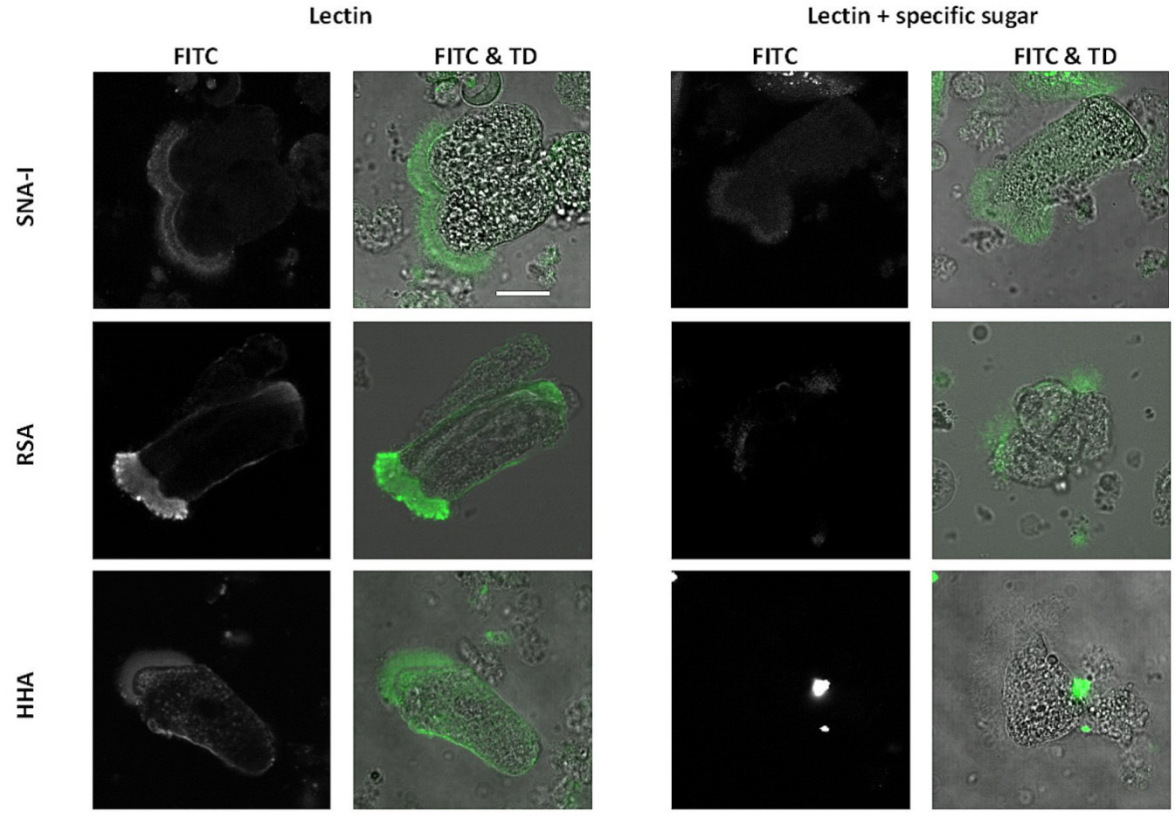


Fig. 2.10. Lectin binding in the presence and absence of lectin-specific sugars. Confocal images of lectin binding to midgut columnar cells after pre-incubation with competing sugars. Scale bar is 20 µm.

2.5. Discussion

The lectins SNA-I and MAA showed toxicity towards the CF-203 midgut cell line already at nanomolar concentrations which indicated high insecticidal activity and suggested that these lectins might be promising candidates for insect pest control. A similar level of activity of SNA-I towards CF-203 cells was previously reported but the authors of the same study observed no negative effects on the S2 cell line (Shahidi-Noghabi et al., 2010a). In contrast the results presented in this chapter indicate that SNA-I was active against S2 cell line while MAA induced considerable toxicity only at the highest concentrations tested (above 1 μM). Since S2 cells are derived from embryonic hemocyte-like cells they could be considered as a model for general lectin toxicity (Schneider, 1972). Thus these results imply that the both lectins are potentially effective midgut toxins. In addition, SNA-I might also induce systemic toxicity if it passes through gut to the hemolymph.

Both SNA-I and MAA induced apoptotic-like changes in the morphology of insect midgut cells which indicated that these lectins not only inhibit cell divisions but actively trigger programmed cell death in those cells. In agarose gel electrophoresis DNA of S2 cells treated with SNA-I appeared as a smear which often is a hallmark of the necrotic cell death (Sata et al., 1997). However, it was demonstrated by flow cytometry that non-apoptotic DNA fragmentation occurred in only a minor fraction of lectin-treated S2 cells, indicating that lectins are not potent death inducers in s2 cells. It has been shown previously that fungal lectins caused apoptosis in CF-203 cells but only growth inhibition in *Drosophila* cells (Hamshou et al., 2013; Hamshou et al., 2012). Therefore, in line with these findings the mode of toxic action of lectins might also depend on the target cell line.

SNA-I was approximately 12-60 fold more toxic to both cell lines under study compared to MAA. One of the reasons causing such disparity might be the structure of these lectins. SNA-I is built of 4 heterodimers of A and B subunits. The B subunit is responsible for sugar binding, while the A domain has ribosome inactivating (RIP) activity (Van Damme et al., 1996; Van Damme et al., 1997b). The inactivation of ribosomes can lead to cessation of protein translation and, in turn, may result in cell death (Shang et al., 2015). MAA, in contrast, contains only lectin subunits and has no RIP activity (Van Damme et al., 1997a) which might explain its lower potency to induce the insect cell death. Nonetheless, as indicated for other toxic lectins including RSA or ricin, the internalization and inhibition of translation is not necessary for their toxicicity. These lectin may trigger cell death solely by binding to cell membrane glycans (Audi et al., 2005; Hamshou et al., 2013; Hamshou et al., 2012). Thus differences in activity

between the two lectins can also depend on their binding specificity and interaction with cell surface receptors.

SNA-I is specific towards sialic acid linked via α2,6-linkage to galactose or GalNAc (Van Damme et al., 1996; Van Damme et al., 1997b). MAA can bind to glycans containing terminal galactose however this lectin clearly "prefers" sulphated or α2,3-sialylated forms of this sugar (Geisler and Jarvis, 2011). Sulphated oligosaccharides have been found, for instance, in mosquitoes but the SO_4 in those glycans is usually attached to mannose or hexosamine residues rather than to galactose (Kurz et al., 2015). Although glycans with terminal galactose are abundant in insects, no α2,3-sialylated glycans have been identified so far, in contrast to α2,6-sialylated glycans which have been found in *Drosophila melanogaster* embryos and adults (Aoki et al., 2007; Koles et al., 2007). This is in line with that the insect genomes encode single sialyltransferase with strict specificity for the α2,6-linkage (Kajiura et al., 2015; Koles et al., 2004).

This notion appears to be supported by the analysis of lectin binding to primary insect midgut cells. SNA-I clearly bound to midgut epithelium microvilli as well as to non-differentiated cell membranes. On the contrary, the binding of MAA was not significant, which indicated the presence of α2,6-sialylated glycans but not of α2,3-sialylated or sulphated structures. Interestingly, the presence of sialylated glycans in insects has been controversial for years and only few analyses could achieve high enough sensitivity to unambiguously detect sialylated N-glycans (Aoki et al., 2007; Koles et al., 2007; Roth and Kempf, 1992). The low level of sialylated glycans was attributed to the lack of enzymes necessary for the synthesis of ManNAc, a key intermediate for Neu5Ac synthesis. Since insect genomes contain all other enzymes necessary for the process of sialylation, it was hypothesized that the presence of ManNAc or sialic acid among nutrients would enable glycan sialylation (Angata and Varki, 2002; Koles et al., 2009). This phenomenon was observed in cultured insect cell lines (Hollister et al., 2003). The artificial diet used for rearing the caterpillars of *S. littoralis* contained bovine milk κ-casein, which is a sialylated protein (Holland et al., 2005), thus the delivery with the diet might have enhanced the sialylation of proteins in *S. littoralis* gut cells by the mechanism described above (Vancova et al., 2012).

Confocal microscopy indicated a clear asymmetry of lectin binding to the *S. littoralis* midgut epithelial cells. This asymmetry was apparently related to the carbohydrate specificity of the lectins. The mannose-specific lectins (HHA and GNA) bound preferentially to the basal region of the cells. A similar trend was observed for Nictaba which is specific for GlcNAc oligomers. In contrast, the GalNAc-recognizing lectins RSA and SSA, and WGA that binds to terminal GlcNAc residues, bound more readily to

the apical region of the columnar midgut cells. Furthermore, for WGA, RSA, HHA and GNA, there were significant differences between binding to the basal pole and the lateral membrane.

It is well established that mannose residues are found in the majority of the insect *N*-glycans (Aoki et al., 2007; Aoki and Tiemeyer, 2010; Dojima et al., 2009; Rendić et al., 2007), glycophosphatidylinositol anchors (Varki and Lowe, 2009) and a minor fraction of *O*-glycans (Aoki et al., 2008). GlcNAc oligomers are present in chitin or in the chitobiose core of all *N*-glycans, while terminal GlcNAc can be found in glycosphingolipids, a minor fraction of the complex and hybrid *N*-glycans as well as in *O*-glycans (Aoki et al., 2007; Aoki et al., 2008). GalNAc residues have been reported to be component of insect *O*-glycans, glycosphingolipids (Varki et al., 2009) and complex *N*-glycans (Kurz et al., 2015).

The lectin binding patterns suggest that GalNAc and terminal GlcNAc residues (most likely on *O*-glycans and/or glycosphingolipids) are more abundant in the apical region of the midgut cells, whereas mannose residues (most likely on high- and oligomannose *N*-glycans) appear to be more common in the basal region of the cells. This is in line with previous reports implying that different glycans might regulate the sorting of proteins to apical or basal poles of the epithelia (Huet et al., 2003; Potter et al., 2006).

Lectin binding studies in *D. melanogaster* also indicated that GalNAc moieties are enriched on the apical/luminal regions of the gut and showed that this modification is crucial for the gut function(Tian and Ten Hagen, 2007b). In line with that, the attachment of O-linked GalNAc of *Drosophila* protein Tango1 was shown to be required for secretion at the apical pole of the midgut cells (Zhang et al., 2014b). Syed et al. (2012) reported that the luminal deposition of putatively *O*-glycosylated proteins is essential for proper development and growth of the *D. melanogaster* hindgut. Moreover, gut expression of *pgant5* coding for polypeptide GalNAc-transferase 5 was essential for the fruit fly viability (Tran et al., 2012).

On top of that, the interaction with glycans on the insect midgut cell surface appears to be a prerequisite for lectin entomotoxicity (Coelho et al., 2007; Hamshou et al., 2013; Macedo et al., 2015), therefore the observed prominent binding of RSA, SSA and SNA-I to midgut microvilli further substantiate the use of lectins specific to GalNAc/Gal and sialic acid for insect pest control.

Chapter 3

Insecticidal activity of lectins against the red flour beetle, *Tribolium castaneum*

Part of this chapter was published in:

Walski, T., Van Damme, E. J.M., & Smagghe, G. (2014). Penetration through the peritrophic matrix is a key to lectin toxicity against *Tribolium castaneum*. Journal of Insect Physiology, *70*, 94-101.

3.1. Abstract

It was previously shown that the lectins SNA-I and MAA are toxic to insect cell lines and that lectins specific to GalNAc/Gal and complex glycans show strong interaction with brush border microvilli of primary midgut cells. These features make the lectins promising candidates for insect control agents. However, insecticidal activity of these proteins against beetles has not been analyzed so far. Therefore, this chapter describes the efforts to evaluate the potential of GalNAc/Gal and complex-glycan binding lectins to control pest beetles which is one of the main goals of this thesis.

Initially, the activity of SNA-I, MAA and two GalNAc/Gal specific lectins, SNA-II and RSA was assessed using a TcA cell line isolated from the red flour beetle, *Tribolium castaneum*, a model beetle and an important pest. Subsequently, lectin effects on *Tribolium* larvae were verified in feeding assays. Additionally, factors affecting lectin toxicity in vivo including the stability in the larval gut or efficiency in passing through the peritrophic matrix (PM) were analyzed.

MAA had no effect on the TcA cells, whereas the other three lectins were highly toxic to this cell line. Their effectiveness in vitro was in decreasing order SNA-II > SNA-I > RSA (EC_{50} = 0.1, 0.5 and 3.6 µg/ml, respectively). These lectins caused DNA fragmentation and induced various morphological changes in TcA cells including cell detachment, shrinkage, vacuolization, swelling and blebbing. Conversely to cell assays RSA had the strongest effect of the three lectins in feeding assays. None of the lectins caused acute mortality but growth of the larvae on diets containing RSA, SNA-II or SNA-I at 2% (w/w) was reduced by 93%, 64% and 20%, respectively. RSA and SNA-II were resistant to larval gut proteases, while SNA-I was susceptible to digestion. Moreover, confocal microscopy revealed that RSA passed through the PM more efficiently than SNA-II. In another set of experiments, lectin injections directly into the larval hemolymph caused acute mortality at doses that were 3-4 orders of magnitude lower than those in the feeding tests. Similarly to the cell assays, SNA-II was the most toxic lectin in injection tests, followed by SNA-I and RSA.

In conclusion, our data suggested that the lectin stability in the midgut and passing through the PM and the gut into the hemolymph is crucial for a high toxic effect. The ability to pass the PM might be affected mainly by the protein molecule dimensions and the size of the PM pores. Ultimately, the development of methods enhancing lectin delivery into the hemolymph might allow exploiting their full insecticidal potential.

3.2. Introduction

In recent years a lot of research has been devoted to the discovery of novel insecticidal proteins with a potential to replace, or synergize with, the currently used *Bacillus thuringiensis* toxins (Carlini and Grossi-de-Sá, 2002; Gatehouse, 2008; Maqbool et al., 2001). Diverse proteins have been shown as promising candidates for that purpose, for instance, inhibitors of digestive enzymes (Alvarez-Alfageme et al., 2011; Christeller et al., 2005), spider venoms (King and Hardy, 2013), cathepsin (Li et al., 2008) and *Photorhabdus* toxin (Blackburn et al., 2005). Also lectins or carbohydrate binding proteins received particular attention for their potential insecticidal properties (Van Damme et al., 1998a). Lectins are found in every kingdom of life (Varki and Lowe, 2009) and represent a very heterogeneous group of proteins in terms of three-dimensional structure, glycan specificity, function and toxicity (Van Damme et al., 2008). A number of them have been proven toxic to insects owing to their carbohydrate binding properties, e.g. snowdrop lectin (GNA) showed activity against brown planthoppers (Foissac et al., 2000) and tomato moths (Fitches et al., 1997). Garlic lectin (ASAL) was toxic to cotton leafworm (Sadeghi et al., 2008) and to a number of aphid species (Upadhyay and Singh, 2012). Other examples of lectins with pest-control potential include the *Moringa oleifera* lectin with mosquitocidal activity (Coelho et al., 2009) or the lectin from *Bauhinia monandra* leaves that was effective against flour moth, cowpea weevil and Mexican bean weevil (Macedo et al., 2007).

Experiments described in the previous chapter indicated that two lectins that bind to complex glycans SNA-I and MAA might block the growth and kill insect cells. Additionally, GalNAc/Gal lectins have been shown to strongly interact with the brush border epithelium of insect midgut cells. These features put the complex-glycan and GalNAc/Gal binding lectins forward as interesting candidates for insect control agents. Indeed, a few other reports showed that lectins specific to sialic acid or GalNAc/Gal can be orally toxic to several moth (Lepidoptera) and aphid (Hemiptera) species (Hamshou et al., 2010; Hamshou et al., 2013; Shahidi-Noghabi et al., 2010b). However, the activity of these lectins has not been tested so far on beetles (Coleoptera).

SNA-I and SNA-II are lectins from the bark of elderberry (*Sambucus nigra*), while RSA is found in the fungus *Rhizoctonia solani*. These three lectins are structurally related and contain domains similar to ricin B chains, although they differ in the size of the protein. SNA-I is a ~240 kDa octameric protein composed of four A chains and four B chains (Van Damme et al., 1996) and exhibits specificity towards oligosaccharides with the Neu5Acα2,6GalNAc/Gal motif (Shang and Van Damme, 2014). RSA and SNA-II are both homodimeric proteins composed of two ~16 and ~32 kDa units, respectively.

These two lectins also have similar glycan binding specificity towards GalNAc/Gal containing glycans (Candy et al., 2001; Kaku et al., 1990; Maveyraud et al., 2009; Skamnaki et al., 2013). MAA, isolated from *Maackia amurensis* bark, specifically recognizes the Neu5Acα2,3Galβ1,4GlcNAc/Glc oligosaccharide which represents a unique specificity among plant lectins. This protein has a molecular weight of approx. 130 kDa, it is a heterotetrameric protein built of ~32 and ~37 kDa subunits. It belongs to legume lectin group and is structurally unrelated to other three lectins (Van Damme et al., 1997a).

Efforts focused on studying the mode of insecticidal action of lectins, revealed a number of features contributing to their toxicity, e.g. glycan mediated binding to epithelial proteins (Du et al., 2000), stability in the gut as well as transport of the lectin to the hemolymph and other tissues (Fitches et al., 2008). However, lectin receptors like aminopeptidase N and ferritin are expressed ubiquitously in guts of insects belonging to various orders, and glycan structures present on insect glycoproteins are very similar, with only minor species specific differences (Aoki et al., 2007; Dojima et al., 2009; Hakim et al., 2010; Rendic et al., 2008). In line with these data, Harper et al. (1995) observed that binding to the gut epithelium contributes to the toxicity but the extent of lectin binding does not correlate with its potency and species/order selectivity. Similarly, resistance to proteolysis is not a factor entirely explaining the different activity of lectins towards insects of different orders, as shown for GNA, which is stable in the gut of aphids, caterpillars and beetles but by far less effective against insects of two latter orders (Fitches et al., 2010; Hogervorst et al., 2006). Therefore, despite the progress in the field, the factors determining lectin effectiveness and selectivity of insecticidal properties remain largely unknown.

This chapter aims to address one of the main goals of this thesis that is evaluation of the potential use of GalNAc/Gal and complex-glycan binding lectins to control the pest beetles. Additionally, it describes the efforts to understand the insecticidal effects and mechanisms underlying the differential activity of lectins, MAA, RSA, SNA-I and SNA-II, against a model and pest insect, the red flour beetle, *Tribolium castaneum.* For that goal, activity of these lectins against the *T. castaneum* cell line was analyzed first and complemented with tests of insecticidal activity against *T. castaneum* larvae. Additionally, resistance of the ingested lectins to proteolytic degradation was studied and confocal microscopy was used to investigate whether the toxicity of the lectins can be linked to their efficiency in traversing the peritrophic matrix (PM) in the larval gut. Finally, lectins were injected directly into the larval hemolymph to test the lectin toxicity to larvae without impact of the midgut

defense mechanisms. Our results indicate that passing of the orally administered lectins through the PM might be one of the crucial events determining the insecticidal efficiency.

3.3. Materials and methods

3.3.1. TcA cell line and insect culture

In all the experiments the red flour beetle, *Tribolium castaneum* GA-1 strain was used. Beetles were reared in a diet composed of a wheat flour supplemented with 5% yeast, at 30 °C and 60% humidity, in darkness. Every month adult beetles were transferred to a jar with a fresh diet. Under these conditions *T. castaneum* has 5-6 larval stages, after which it develops into pupa and then into adult. The whole life cycle takes roughly 1 month. To synchronize the larvae, adult beetles were put to a fresh diet and allowed to lay eggs. After 24 hrs adults and eggs were separated using a 700 µm sieve. Eggs were isolated from flour using a 200 µm mesh sieve and used to start a synchronized culture.

The red flour beetle cell line BCIRL-TcA-CLG1 (TcA) was established and kindly donated by Dr. Cynthia L. Goodman (Agricultural Research Service, Biological Control of Insects Research Laboratory, USDA). The TcA cell line was maintained in EX-CELL® 420 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum in 25 ml cell culture flasks at 27 °C (Goodman et al., 2012). Cells were subcultured weekly at 1:5 dilution with fresh medium.

3.3.2. Lectin purification and fluorescent labeling

The lectins used were RSA, an agglutinin from the sclerotes of the phytopathogenic fungus *Rhizoctonia solani*, SNA-I and SNA-II agglutinins from the bark of elderberry (*Sambucus nigra*) and MAA, a agglutinin from the *Maackia amurensis* bark. The lectins were isolated using ion exchange and affinity chromatography according to previous reports as described earlier (Van Damme et al., 1998b) and in Chapter 2. For localization experiments lectins were fluorescently labeled with FITC (fluorescein isothiocyanate) or TRITC (tetramethylrhodamine isothiocyanate) according to manufacturer instructions (Thermo Fisher Scientific) essentially as described in (Hamshou et al., 2012) and in Chapter 2. The labelling efficiency or FITC-to-protein ratios (F/P) were calculated based on absorbance measurements at 280 nm and 488 nm using a Nanodrop 2000c spectrophotometer. The F/P values were 7.9 for SNA-I, 4.2 for SNA-II and 2.6 for RSA (equivalent to 0.03, 0.07, 0.09 µmol of FITC per mg of protein, respectively). Efficiency of TRITC labeling (T/P) was calculated based on absorbance

measurements at 280 nm and 555 nm. The T/P values were 2.19 and 1.09 for SNA-II and RSA, respectively (equivalent to ~0.035 µmol of TRITC per mg of protein in the both cases).

3.3.3. Tests of lectin toxicity towards TcA cell line

TcA cells were collected at logarithmic phase of growth (2-3 days after subculturing) and diluted twice with a fresh medium. Then lectin stock solutions (in PBS) were added to the cells at 1:10 ratio to give final lectin concentrations of 0.1-1000 nM. Afterwards, 100 µl aliquots of cell suspensions with added lectins (or PBS in case of control) were transferred to flat bottomed, transparent 96-well plates. Lectin effect on TcA cell morphology was documented after 2 days. Cells were imaged directly in 96 well plates with a Nikon A1R confocal microscope, using a 488 nm laser and a transmitted light detector (objective: S Plan Fluor ELWD 40x, NA=0.6, pinhole radius = 44.7 µm, pixel size = 0.33 µm).

For measurement of lectin impact on cell viability the initial set-up was the same except that cells were incubated for 4 days with lectins. After that period cells were transferred to black bottomed 96-well plates (to reduce the measurement background) and mixed with fluorescent PrestoBlue® cell viability reagent (Invitrogen) at 1:10 ratio. This reagent is rapidly metabolized by living cells to a fluorescent compound in proportion to the number of viable cells. After 20 mins of incubation at 27 °C fluorescence was measured using a Tecan (Männedorf) plate reader (560 nm excitation and 600 nm emission wavelengths). Proportion of viable cells was calculated based on measurement of serial dilutions of the control cells treated with buffer instead of lectins. For each lectin 3 independent biological replications were performed, comprising 4–5 technical replications for each concentration. Half-maximal effective concentrations (EC_{50}) for lectin toxicity tests were estimated using a sigmoidal curve fitting in GraphPad Prism 4 software.

3.3.4. DNA fragmentation assay

To analyze DNA fragmentation 1.5 ml of TcA cells were mixed with lectin or Actinomycin D solution and transferred into 96-well plate (100 µl per well). After 48 hrs of incubation cells were transferred into tubes, centrifuged, washed with PBS and disrupted by freeze-thawing. Cells were further homogenized in DNA extraction buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA-NaOH, 0.1% SDS, pH 8.0) using pestle. The extract was digested with RNase A (20 µg/ml) for 30 min at 37°C and proteinase K (100 µg/ml) for 60 min at 50°C. Finally DNA was purified using a standard phenol-

chloroform method and the yield was estimated with Nanodrop spectrophotometer. 3 µg of DNA were resolved in a 1.5% agarose gel at 100 V and visualized by staining with ethidium bromide.

3.3.5. Localization of lectin in TcA cells

For confocal microscopy TcA cells were diluted (1:5) with fresh EX-CELL® 420 medium without FBS and seeded on sterilized cover glasses (No. 1 thickness) for 40 mins. Subsequently the cells were washed with medium to remove non-attached cells and incubated with fluorescent-labeled lectins (0.3 µM) for 1 hour in darkness. Then cells were washed 3 times using PBS (1 min), fixed with 3.7% formaldehyde in PBS (5 mins), washed 3 times (1 min) and stained with nuclear dye Hoechst 33258 at 1 µg/ml in PBS (15 mins) and washed 3 times (1 min). Cover glasses were mounted on microscope slides using Vectashield medium (Vector Labs) and imaged using a confocal microscope Nikon A1R. The following set up was used: 60x oil immersion Plan Apo objective (1.4 NA). A 405.3 nm laser and 450/50 nm filter was used for Hoechst 33258, 488.0 nm laser and 525/50 nm bandpass filter was used for FITC-labeled lectins. Pixel size was 0.08 µm. Thickness of the optical section was set to 1 µm. Obtained images were edited for brightness and contrast in FiJi software (Schindelin et al., 2012).

3.3.6. Lectin effect on larvae – feeding assay

To examine the insecticidal effects of the lectins against the red flour beetle larvae, lectin powder was mixed with a standard diet and homogenized using a 200 µm sieve. Individual 1st instar larvae were placed in separate tubes containing 19–21 mg of diet. Fifteen to twenty larvae were examined per treatment and each experiment was repeated twice.

To analyze the impact of the glycan binding specificity or lectin charge on the toxic effect against larvae the setup was identical as above except that diets were mixed with lectins and GalNAc, GlcNAc or $CaCl_2$ powders. The effect of lectins on *T. castaneum* was monitored based on mortality, larval length, weight and time of development from egg to adult. Darkened cuticle and immobility were treated as indication of larval death. Statistical analysis was done using ANOVA and a post-hoc Tukey test in SPSS 22 (IBM).

3.3.7. Evaluation of lectin impact on larval feeding parameters – flour disk assay

Lectin effect on larval feeding and growth were analyzed using flour disk assay as described in (Contreras et al., 2013a; Liu and Ho, 1999). To prepare the flour disks presieved full grain wheat flour was mixed with water or lectin solution at 1:9 ratio (w/v, e.g. 200 mg flour and 1.8 ml of water). This suspension was constantly mixed and aliquoted in 60 µl spots on the bottom side of a 96-well plate cover. These were dried overnight in the vertical flow cabinet to form the disks. Two week old larvae were weighed and divided into 5 groups containing 10 larvae each (50 larvae in total) per treatment. 6 flour disks were weighed and given as food for each group. After 3 days of incubation larvae and flour disks were weighed again to calculate the growth and diet consumption. The following factors were calculated: (1) relative growth = larval growth / larval weight at start, (2) relative consumption = diet consumption / larval weight at start, (3) Food conversion efficiency = larval growth / diet consumption, (4) feeding deterrence index = consumption of disks containing lectins / consumption of control disks. Differences between treatments and control were analyzed using t-test for independent samples in SPSS Statistics 22 (IBM).

3.3.8. Assessment of lectin resistance to larval digestive enzymes

Flour disks containing FITC-labeled lectins were prepared essentially as described above. The concentrations of the FITC-labeled lectins in the disks were 2.5 mg/g for RSA, 3.5 mg/g for SNA-II and 7.5 mg/g for SNA-I. These concentrations were chosen based on a measured protein labeling efficiency (see Section 3.2.2) in order to achieve the same number of FITC molecules in each disk and therefore a similar detection sensitivity despite differences in lectin labeling efficiency. Twenty to thirty third-instar larvae were allowed to feed on the disks containing FITC-lectins for 24 hrs. Afterwards, larvae were removed from the diet, blown with the stream of air to remove disk traces and washed in phosphate buffered saline (PBS). Then, larvae were anesthetized on ice and submerged in a drop of PBS. Heads and posterior abdomen ends were cut off, and guts were pulled out using fine forceps. Guts were homogenized with a pestle in 25 µl of extraction buffer containing 0.1% SDS, 0.1 M Tris–HCl, pH 7.5 and 1 mM PMSF (protease inhibitor), and centrifuged at 16000g for 10 min. Remaining discs containing FITC-lectins were also extracted with the same buffer. Afterwards, fractions of extracts were diluted with PBS for measurement of total fluorescence using Tecan plate reader. The extracts were separated by SDS–PAGE and the measured total fluorescence values were used to ensure equal

well loading. FITC-lectins in gel were detected with the Fluor Imager FLA-5100 (Fuji), using a 473 nm laser and FITC filter.

For the analysis of lectin excretion with feces, larvae were removed from the diet containing FITC-lectins and transferred to a clean Petri dish without diet for 8 h. After this period, larvae were removed and the feces were collected using forceps, suspended in 25 µl of extraction buffer (same as above) centrifuged (16000g for 10 min), and the supernatant was loaded on gel.

For time course analysis of lectin resistance to larval gut proteases, 20 guts of mid-sized larvae were dissected (as described above) and homogenized in 100 µl phosphate buffer pH 6.6 (pH of the median midgut is between 6 and 7). The sample was then centrifuged at 16000g for 10 mins and the supernatant containing the gut juice was collected. The extraction was repeated to ensure efficient recovery of the gut contents. 5 of µg FITC-lectins were incubated at 30°C with a volume of gut juice containing approximately 20 µg of gut protein (i.e., on average equal to the amount of protein from one gut). This ratio was chosen to reflect the amount of FITC-protein consumed by the larvae feeding on diet containing 1% FITC-protein (w/w) for 24 h. In the positive control, FITC-albumin (Sigma) was used. Samples of 10 µl were collected immediately after mixing (0 h) and after 1, 4 and 8 hrs, then mixed with 1 µl of 200 mM leupeptin to inhibit further proteolysis and frozen immediately. The sample collection times were based on the time course of food passage through the gut of *T. castaneum* (Krishna and Saxena, 1962). The digests were separated by SDS–PAGE, and FITC-lectins were detected with the Fluor Imager FLA-5100 (Fuji), using a 473 nm laser and FITC filter. Amounts of the proteins were estimated by densitometry using FiJi software (Schindelin et al., 2012). The experiment was repeated twice.

3.3.9. Lectin localization in larval midgut

The guts of third-instar larvae fed with FITC-lectin were prepared as above and washed in PBS, incubated for 10 mins in 25 µg/ml Hoechst 33258 to counterstain the nuclei and finally mounted on the microscope slides with Fluoroshield (Sigma) in order to prevent bleaching of fluorescence and tissue shrinkage. No fixatives were used to avoid introduction of artifacts in the PM ultrastructure, and imaging time was also restricted to 30 mins post-staining to preclude any damage to the tissue. Images were acquired using a confocal microscope A1R (Nikon), a 405 nm laser and 450/50 emission filter for Hoechst 33258 and a 488 nm laser and 525/50 nm emission filter for FITC-labeled lectins. The following acquisition settings were used: 1.24 µm pixel size (0.21 µm for zoomed pictures), 17.9 µm

pinhole and Plan Apo λ 20x (NA = 0.75) objective. The same acquisition settings were used to compare different lectins. Raw images were minimally edited for brightness and contrast using FiJi software.

For direct comparison of efficiency of lectin passing through the peritrophic membrane third-instar larvae were fed with flour disks containing a mix of TRITC–RSA with FITC–SNA-II or FITC–RSA with TRITC–SNA-II to allow direct comparison of both lectins in the same larva. The 'dye-swap' was included to eliminate potential bias in observation of lectin fate caused by different chemical and spectral properties of TRITC and FITC. Dissected guts were prepared and imaged as described above, except that we used a 488 nm laser and a 515/30 nm emission filter for FITC and a 561 nm laser, a 585/65 nm emission filter for TRITC. Average fluorescence intensity values for each channel were measured using FiJi software in manually selected endoperitrophic (F_{endo}) and ectoperitrophic spaces together with midgut epithelium (F_{ecto}). The penetration efficiency was calculated for each channel as F_{endo}/F_{ecto}. Differences between the two lectins were analyzed with independent-samples t-test in SPSS Statistics 22 (IBM).

3.3.10. Injection of lectins to the larval hemolymph

For lectin injections two-week old larvae weighing 1.2-1.8 mg were selected. Larvae were anesthetized for 4 mins using diethyl ether and injected with 200-400 nl of lectin solutions (1-3000 µg/ml in PBS) by capillary needle attached to FemtoJet® injector (Eppendorf). Injection needles were pulled using Narashige PC-10 microelectrode puller and opened to a sharp tip using fine forceps. At least 12 larvae were injected with each lectin concentration and the whole experiment was repeated thrice. Following the injection larvae were allowed to recover for 1 hour and were transferred to individual wells of 24-well plate to prevent cannibalism. On the next morning any dead larvae or those with visible cuticle tanning were considered as mechanically damaged by injection (usually <10%) and discarded from the experiment. The rest of the larvae were given flour to feed. Mortality was scored daily for 10 days. Not moving blackened larvae were considered dead. Fractions of dead larvae were used to estimate EC_{50} values by sigmoidal dose-response curve fitting in GraphPad Prism 4.0.

3.4. Results

3.4.1. SNA-I, SNA-II and RSA but not MAA are toxic to TcA cell line from *T. castaneum*

For the initial analysis of lectin activity towards the red flour beetle, the TcA cell line derived from this insect was incubated with MAA, SNA-I, SNA-II and RSA at 1000 nM for 48 hrs and effects on the cells were observed with the inverted microscope. The TcA cell line is a heterogenous mixture of two types of cells: semi-adherent round cells that do not detach after division but form clumps and spindle like cells attached to the vessel surface. SNA-I, SNA-II and RSA clearly reduced cell numbers compared to control and induced diverse changes in cell morphology (Fig. 3.1). The most frequently observed phenotypes included cell detachment and disappearance of spindle-shaped cells, as well as shrinkage and vacuolization characteristic for cells undergoing autophagy. Interestingly, in case of each lectin some cells were swollen, enlarged resembling the oncotic phenotype but only few cells releasing apoptotic bodies or with membrane blebbing could be observed.

Incubation with MAA did not cause any visible reduction of the cell numbers or any of the morphological changes described for the other three lectins, suggesting the lack of toxicity towards TcA cells. To verify this assumption the experiment was repeated and TcA cells were incubated with 1 µM MAA for 4 days after which the cell viability was assessed using PrestoBlue® reagent. This assay indicated no significant difference in cell viability between the MAA treatment and the control confirming that this lectin is not toxic to TcA cells (Fig. 3.1B).

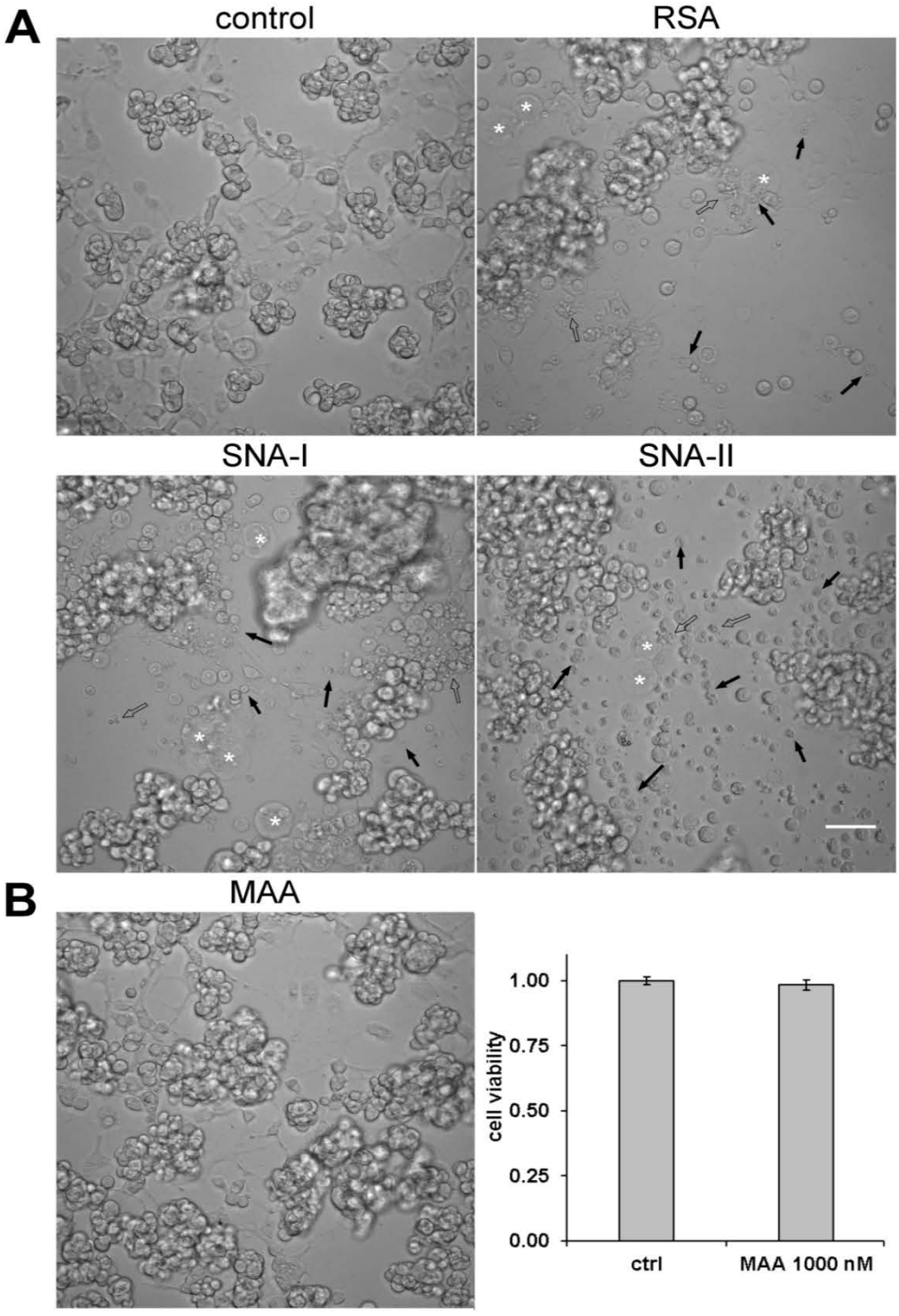


Fig. 3.1. Lectin effects on morphology of TcA cells. (A) RSA, SNA-I and SNA-II induced diverse changes in the appearance of the TcA cell line derived from *Tribolium castaneum.* Black arrows – shrunk and vacuolized cells, open arrows – blebbing cells and apoptotic body-like vesicles, asterisks - swollen cells. (B) MAA had no effect on cell morphology and did not reduce the cell viability. Cells were incubated with 1 µM lectins and imaged after 2 days. Cell viability was analyzed after 4 days of incubation. Bars indicate mean viability relative to control, error bars are standard deviations (n=5). Scale bar is 40 µm.

3.4.2. SNA-II is the most toxic out of the three lectins towards TcA cells

To look more closely at the insecticidal potency of RSA, SNA-I and SNA-II a viability of TcA cells was monitored after incubation with the lectins in a range of concentrations (0.1-1000 nM) for 4 days. Each of the lectins caused a dose dependent reduction of cell viability (Fig. 3.2). SNA-II was the most potent of the three lectins. It caused a significant reduction of cell viability already at sub nanomolar concentrations and the EC_{50} calculated (Table 3.1) for this lectin was 0.9 nM (0.06 µg/ml). SNA-I was also highly active although less than SNA-II, the EC_{50} for SNA-I was 2.0 nM (0.48 µg/ml). The fungal lectin RSA was the least active of the three lectins as the EC_{50} was estimated at 112.8 nM (3.61 µg/ml).

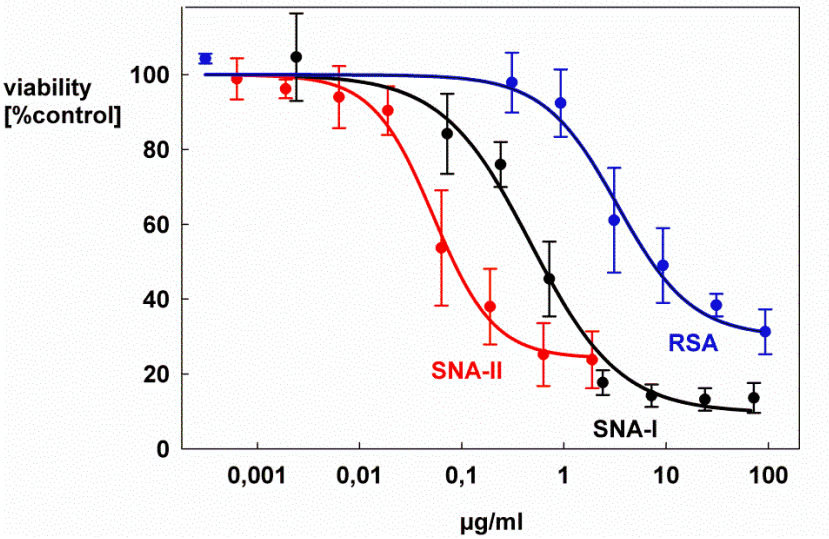


Fig. 3.2. Lectins are toxic towards the *Tribolium castaneum* cell line. Sigmoidal dose-response curves indicate that SNA-II is the most potent out of the three lectins tested. Cell viability was measured after incubation with lectins for 96 h. Points are means, error bars are standard deviations (n=3).

Table 3.1. Comparison of half maximal effective concentration values (EC_{50}) between lectins.

	EC_{50} (95% C. I.)		
Cell line	nM	µg/ml	R^2
RSA	**112.8** (89.7-142.5)	**3.61** (2.87-4.56)	0.97
SNA-I	**2.0** (1.7-2.3)	**0.48** (0.41-0.56)	0.98
SNA-II	**0.9** (0.8-1.3)	**0.06** (0.05-0.08)	0.98

EC_{50} and its 95% confidence intervals (95% C. I.) were calculated using sigmoidal dose-response curve fitting in GraphPad Prism 4.0.

3.4.3. SNA-I, SNA-II and RSA induce DNA fragmentation in TcA cells

To gain insight into the mode of action of lectins in TcA cells, DNA fragmentation, which can be indicative for induction of cell death, was analyzed. For that purpose lectins were incubated with the highest possible lectin concentrations (limited by lectin solubility and experimental setup) being 1 µM, 4 µM and 10 µM in case of SNA-I, SNA-II and RSA, respectively. After 48 hrs of incubation all three lectins caused some level of DNA fragmentation (Fig. 3.3A). Some ladder-like pattern (characteristic for apoptosis) was visible in the DNA resolved in the agarose gel. However, the majority of fragmented DNA appeared as a smear (characteristic for necrosis), also in the case of the positive control Actinomycin D (1 µM). Additionally, 40-60% of DNA remained intact as estimated by semi-quantitative gel densitometry. The presence of DNA laddering, smearing and intact DNA is consistent with non-uniform effects on cell morphology triggered by lectins (Section 3.3.1). At lower concentrations, that is 10 times over EC_{50} (~1 µM for RSA, 10 nM for SNA-II, 20 nM for SNA-I) clear DNA fragmentation was visible only in case of SNA-II and still it appeared as a combination of laddering and smearing (Fig. 3.3B).

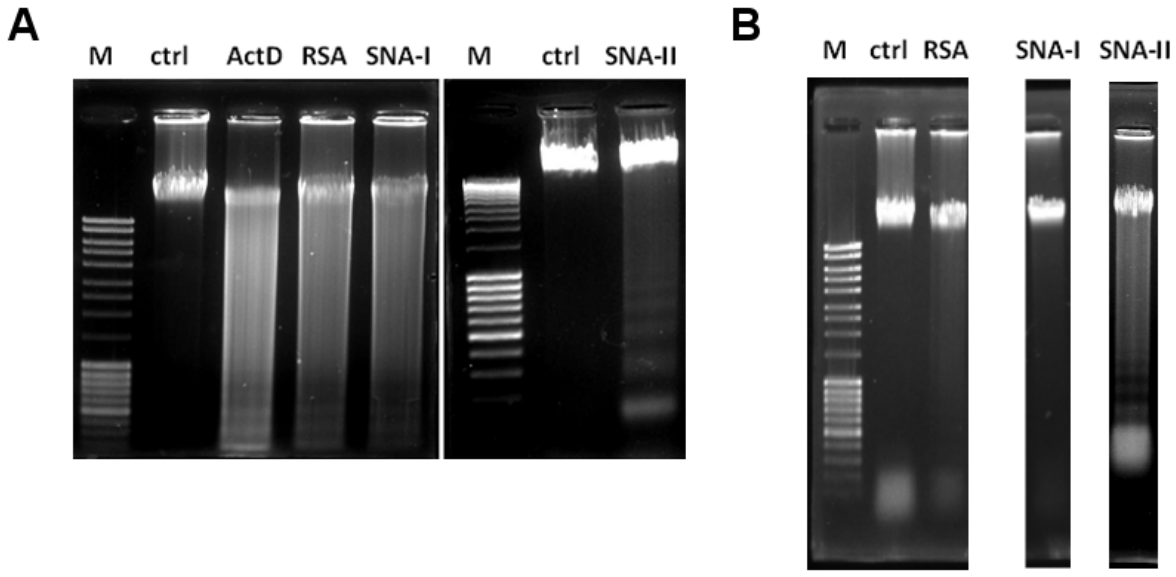


Fig. 3.3. DNA fragmentation induced by lectins in TcA cells. Cells were incubated with lectins for 48 hrs, then DNA was isolated and resolved in 1.5% agarose gel electrophoresis. (A) Incubation with high lectin concentrations induced mainly random DNA fragmentation visible as a smear in the agarose gel, but some DNA laddering could also be observed in the lower parts of the pictures. (B) At lower concentrations (10-fold EC_{50}) only SNA-II caused DNA fragmentation.

3.4.4. SNA-I, SNA-II and RSA are internalized in TcA cells

Lectins were conjugated with fluorescent labels and incubated with TcA cells to analyze their fate. All three lectins visibly bound to the plasma membrane of TcA cells and were internalized (Fig. 3.4). RSA showed apparently the strongest binding to the cell membrane although it was the least toxic of the three tested lectins. Interestingly, in case of FITC-SNA-I there was a higher fluorescence in regions of the cell membrane attached to other cells compared to sections of membrane exposed to the medium. Most of the internalized lectin was localized in large 0.5-2 um vesicles. Unfortunately, the nature of these vesicles could not be studied due to limited availability of organelle markers for other than *Drosophila* cells. Among the available stains BSA-Ceramide-Texas Red was used to test if the lectins localize to the Golgi apparatus. However, this compound did not label this organelle selectively in the TcA cells.

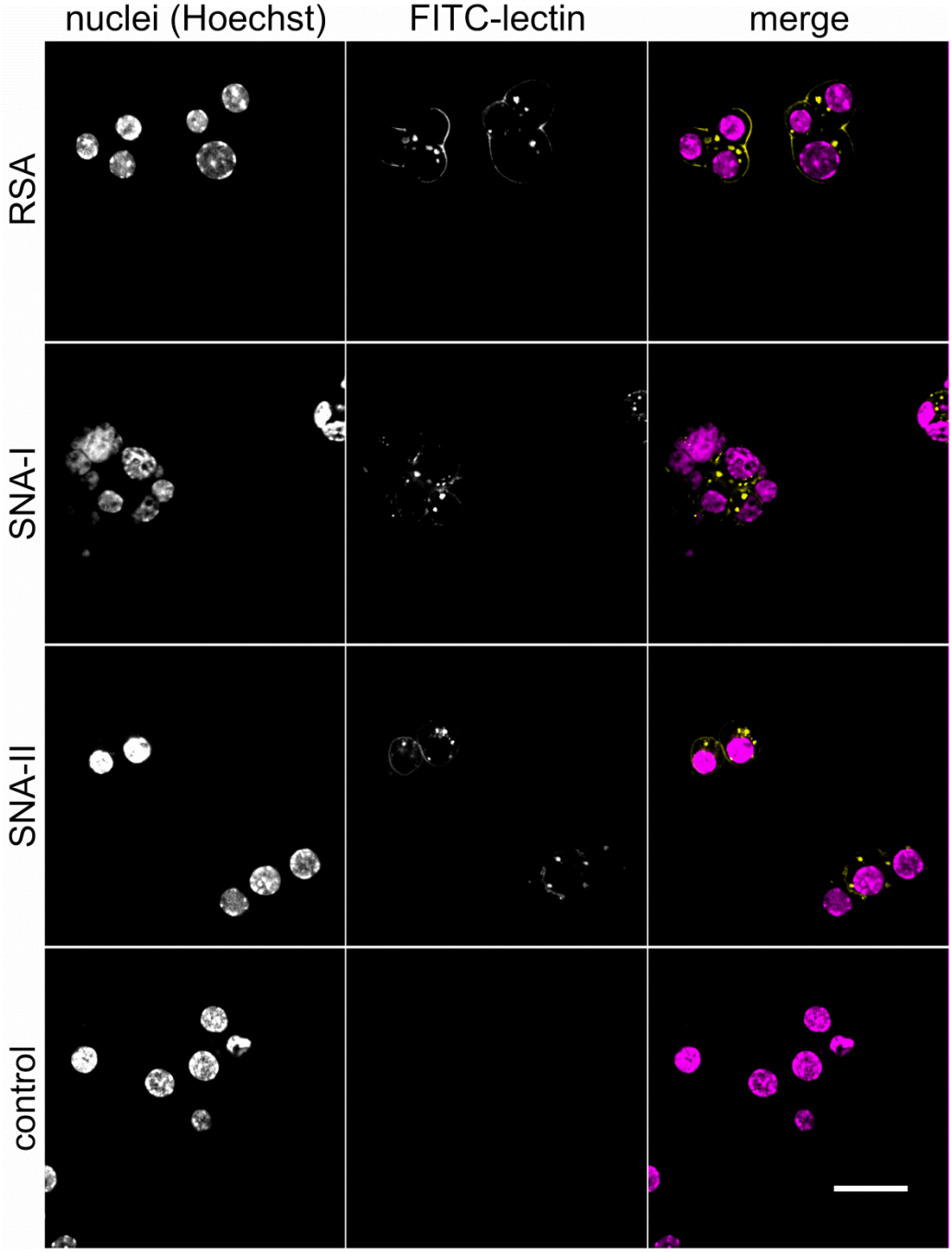


Fig. 3.4. Localization of FITC-labeled lectins in TcA cells. All three lectins bind to the cell membrane and are internalized. Cells were incubated for 1 hr with FITC-labeled lectin and imaged using a confocal microscope. In merged images FITC-lectins are shown in yellow, Hoechst in magenta. Scale bar is 20 µm.

3.4.5. RSA has the strongest effect against *T. castaneum* larvae

Following the tests on *Tribolium* cell line, the toxicity of lectins in vivo was assessed using a feeding assay. The addition of lectins to the diet of first-instar *T. castaneum* larvae did not result in acute mortality but caused severe developmental retardation (Fig. 3.5). Yet the strong effects were visible only at high lectin concentrations (1-2% w/w). The fungal lectin RSA showed the highest activity towards larvae. This lectin at 2% (w/w) almost entirely blocked larval growth (up to 95%) within the first 16 days of the experiment (Table 3.2).

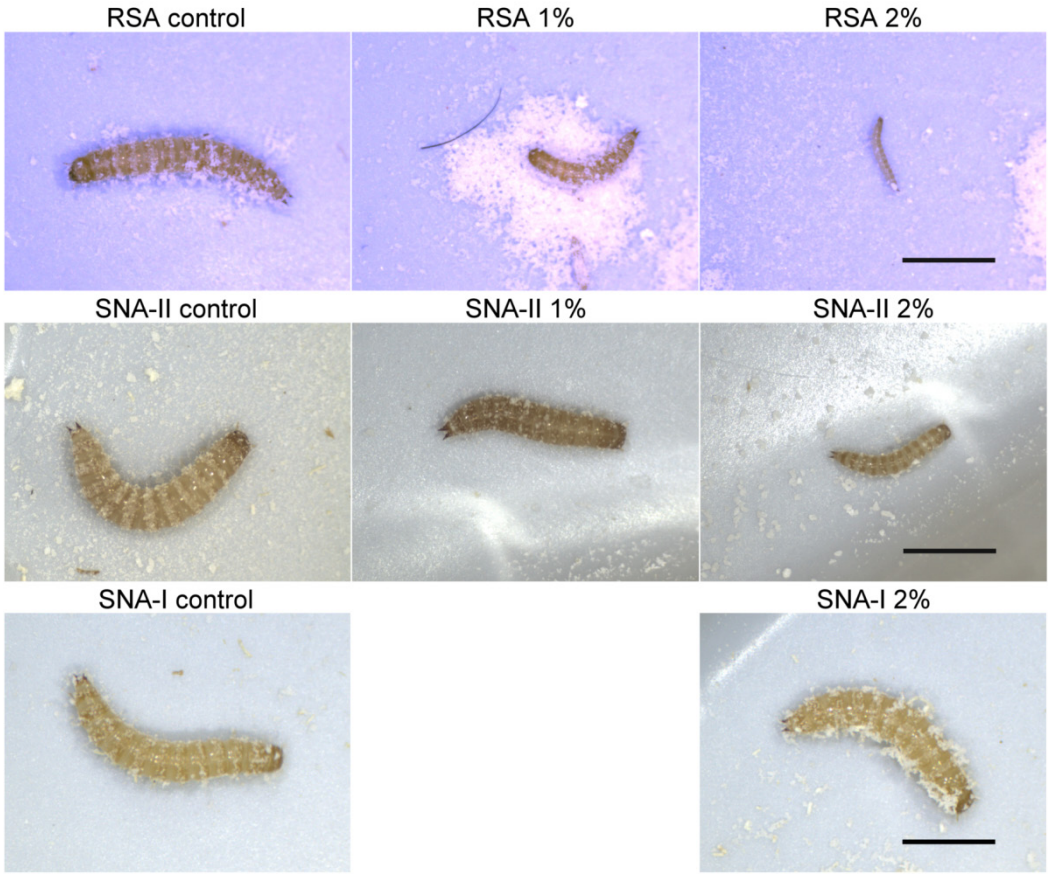


Fig. 3.5. Size of larvae after 16 days of feeding on diets containing RSA, SNA-I and SNA-II. Larvae that were fed on diets containing RSA and SNA-II were visibly smaller than the respective controls. There was no such effect in case of SNA-I. Scale bars are 2 mm.

Table 3.2. Effect of dietary lectins on growth and development of larvae of T. *castaneum*. Out of the tested lectins RSA had the highest impact on the larval mass gain and the time needed to develop from the first instar to the adult stage.

Lectin	Dose	Larval weight at day 16 (mg)	Time of adult eclosion	% adult eclosed
RSA	0%	2.12 ± 0.53**a**	28.9 ± 4.3**a**	100 ± 0.0**a**
	1%	0.34 ± 0.22**b**	55.9 ± 14.3**b**	70.5 ± 17.3**b**
	2%	0.14 ± 0.07**c**	88.0 ± 27.3**c**	21.7 ± 29.3**c**
SNA-II	0%	1.88 ± 0.52**a**	30.6 ± 5.5**a**	98.3 ± 2.9**a**
	1%	1.06 ± 0.55**d**	36.4 ± 4.3**d**	69.6 ± 0.5**a**
	2%	0.67 ± 0.44**d**	54.0± 19.7**b**	62.8 ± 14.9**a**
SNA-I	0%	2.4 ± 0.29**e**	27.0 ± 2.5**a**	100
	2%	1.89 ± 0.38**a**	31.9 ± 2.7**ad**	90.9[§]

Values are means ± standard deviations (experiments were repeated twice with at least 20 larvae in each treatment). Same letters indicate no statistically significant differences at 0.05 level, ANOVA, Tukey post-hoc test. [§] in case of SNA-I adult eclosion was scored for one repetition only.

Furthermore, RSA reduced the percentage of beetles reaching the adulthood by up to 80% and increased the time to adult eclosion by up to 3-fold. 2% SNA-II was less effective in slowing down the larval growth than RSA. Larvae that fed on the diet containing SNA-II weighted significantly more than those treated with RSA and the time to adult eclosion was increased by up to 1.8-fold. SNA-I feeding showed only little impact on *T. castaneum* larvae contrary to its strong effects on TcA cells. 2% (w/w) SNA-I only slightly affected larval weight at day 16 (1.89 ± 0.38 mg compared to 2.40 ± 0.29 mg in controls) and the time to adult eclosion (31.9 ± 2.7 days compared to 27.0 ± 2.5 days for control larvae). The relative order of lectin toxicity against larvae was RSA > SNAII > SNA-I.

3.4.6. Lectins differ in their resistance to larval proteolytic enzymes

The resistance of the lectins to proteolysis (or lack thereof) could be one of the reasons why RSA showed the relatively weakest toxicity against cultured *Tribolium* cells, but yielded the highest impact on larvae. Potentially, this might explain the generally low insecticidal activity of all the three lectins against the larvae. To evaluate this factor guts of larvae fed with food containing FITC-labeled lectins were extracted and analyzed by SDS-PAGE (Fig. 3.6A).

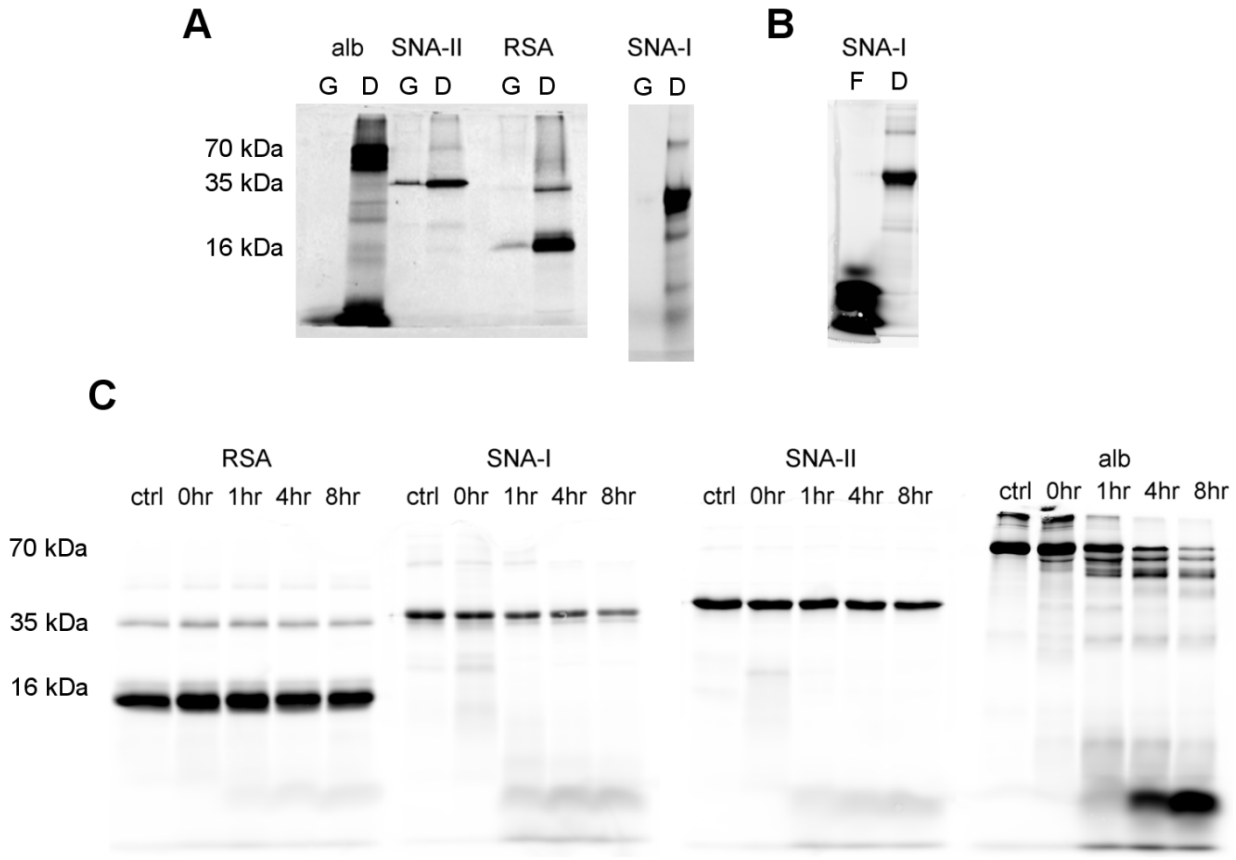


Fig. 3.6. Resistance of lectins to larval gut proteases. RSA and SNA-II are more resistant to digestion by the digestive enzymes of *T. castaneum* larvae than SNA-I. (A) Fluorescence images of SDS–PAGE resolved gut and disc extracts, lane G – gut extracts from larvae fed with FITC-labeled proteins , lane D – extracts from diets containing undigested lectins. Sizes of major bands are consistent with SNA-II (32 kDa) and RSA (16 kDa) monomers, indicating that these proteins were not digested. No band of approximately 32 kDa was present in case of FITC-SNA-I-fed larvae. alb – FITC-labeled albumin used for control. (B) lane F – SDS–PAGE of feces produced by FITC-SNA-I-fed larvae shows major fluorescent bands of low molecular weight, lane D – extracts from diet containing SNA-I. (C) SDS–PAGE of FITC-labeled proteins incubated with gut juice. RSA and SNA-II appear more stable than SNA-I. Albumin is extensively digested by gut proteases thus FITC-albumin was used as a positive digestion control. Ctrl – FITC-labeled protein not-incubated with the gut juice, 0, 1, 4 and 8 hr indicate duration of incubation.

SNA-I and SNA-II are composed of approx. 32 kDa subunits, while RSA is built of approx. 16 kDa monomers. Fluorescent polypeptides of 32 kDa were observed in the gut extracts of larvae fed with FITC-labeled SNA-II and at 16 kDa in the case of FITC–RSA fed larvae. Thus it is evident that at least fraction of these lectins remained undigested in the larval gut. In contrast, no visible bands of right size were detectable in the gut extract of larvae fed with FITC-SNA-I and FITC-albumin used as a positive control. In the extract from feces (Fig. 3.6B), a very faint band of similar size to SNA-I monomer (32 kDa) was detected, however bands of lower molecular weight were much more pronounced. This observation suggests that most of SNA-I was degraded in the gut and excreted with the feces.

For a more in depth comparison of proteolytic resistance, lectins were incubated with larval midgut extract. This approach (Fig. 3.6C) confirmed the high stability of RSA and SNA-II. The amount of intact RSA remaining in the sample was 89.7% ± 10.9% after 1 h, 91.8% ± 10.1% after 4 h and 87.4% ± 3.2% after 8 h. In case of SNA-II, there was still 83.0% ± 6.8%, 85.2% ± 11.5% and 78.1% ± 15.3% of the starting amount of lectin after the respective incubation times. SNA-I was the least stable of the three lectins tested as 72.2% ± 2.5% of the native protein remained undigested in the extract after a 1 h incubation, which further decreased to 67.7% ± 0.6% after 4 h and 54.0% ± 5.3% after 8 h.

3.4.7. RSA passes more effectively through the peritrophic matrix than SNA-II

Insect midguts are lined with the peritrophic matrix (PM) which can restrict diffusion of gut contents and lectins need to pass through this structure in order to interact with midgut cells and induce toxicity. To test if lectins can pass through the PM, larvae were fed with FITC-lectins and confocal microscopy was used on dissected larval guts. For all lectins the majority of the fluorescence was detected within the endoperitrophic space (the space enclosed by the PM) indicating that transport of the lectins through the PM might be restricted (Fig. 3.7). However, multiple fluorescent spots were also detected beyond the endoperitrophic space and on the surface of the gut epithelium of each larva fed with FITC-labeled RSA. This indicated that the lectin could pass through the PM and interact with the epithelial cells throughout the entire length of midgut. In the case of SNA-II, apparently less fluorescent signal was detected beyond the endoperitrophic space and on the surface of the midgut cells. Even though the majority of SNA-I was found to be digested, it was not observed to pass the PM effectively. The fluorescence signal in the guts of larvae fed with FITC-SNA-I was low compared to the other two lectins, which supports the idea that SNA-I is quickly excreted from the gut.

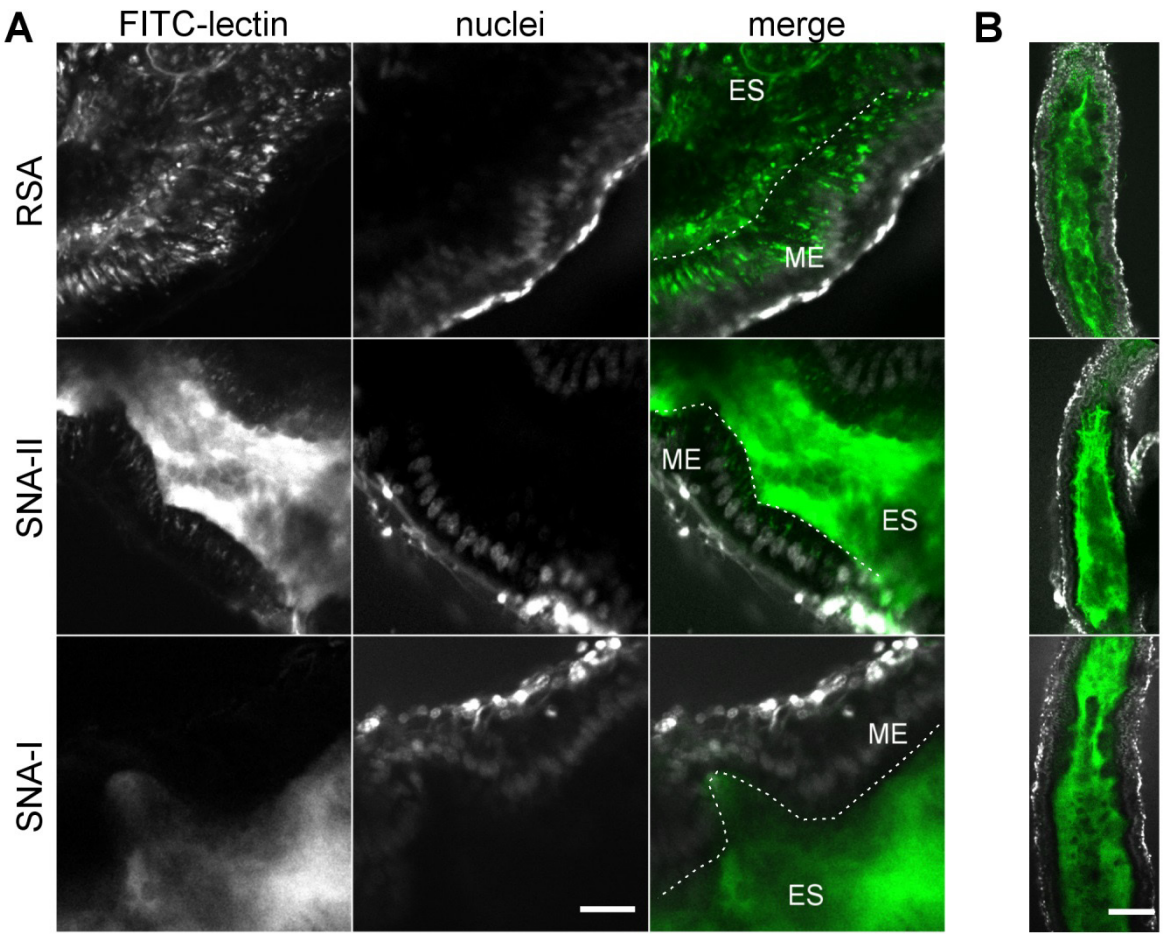


Fig. 3.7. Confocal images of guts from larvae fed with FITC-labeled lectins. Guts of larvae fed with FITC-RSA display a more intensive fluorescence signal in the ectoperitrophic space and at the surface of epithelial cells compared to SNA-II. Almost no SNA-I was observed to traverse the PM. Nuclei were stained with Hoechst 32358. (A) Zoomed images, ES – endoperitrophic space, ME – ectoperitrophic space and midgut epithelium, bar = 20 µm. (B) Lower magnification showing a greater part of the midgut, bar = 100 µm.

To verify the apparent difference between RSA and SNA-II in the efficiency of penetration through the PM larvae were fed with a mixture of both lectins labeled with two different dyes (TRIC-SNA-II with FITC-RSA and FITC-SNA-II and TRITC-RSA). This approach allowed to directly compare lectin transport in the same regions of the same guts (Fig. 3.8). The penetration efficiency (ratio of fluorescence in the ectoperitrophic space over the endoperitrophic space) was 0.36 ± 0.17 for RSA and 0.16 ± 0.07 for SNA-II ($P = 0.019$), thus clearly indicating a higher permeation of RSA through the PM.

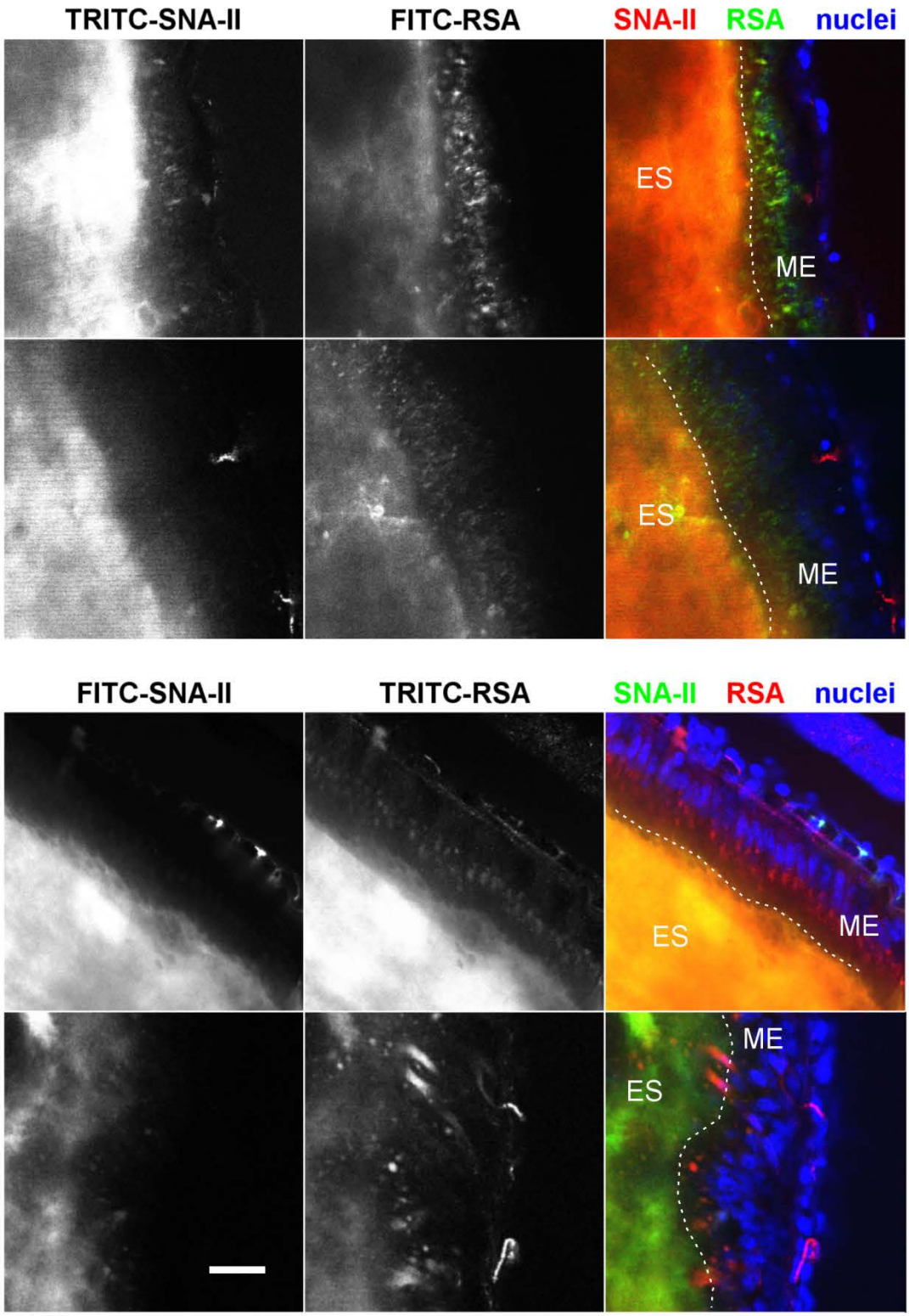


Fig. 3.8. Direct comparison of lectin passing through the peritrophic matrix. Larvae were fed with a mixture of both lectins labeled with either TRITC or FITC. Confocal images of midgut demonstrate that, regardless of dye combination, RSA passes through the PM more efficiently than SNA-II. Nuclei were stained with Hoechst 32358. Dashed line denotes border of PM. ES – endoperitrophic space, ME – ectoperitrophic space and midgut epithelium. Bar = 20 µm.

3.4.8. Impact of lectin charge and sugar binding on toxicity towards larvae is unclear

Lectin toxicity is believed to be related to their ability to interact with glycoproteins present in the midgut lumen and epithelium cells. However, both SNA-II and RSA could bind to mucin-type proteins modified with GalNAc sugars present in the peritrophic matrix (PM) which could diminish the amounts of lectins that pass to the midgut cells. If binding to the PM is a key factor responsible for trapping lectins within the endoperitrophic space then inhibition of binding would increase the lectin toxicity. On the other hand if lectin binding to the midgut cells is the key for lectin toxicity then inhibition of binding would reduce their negative effects.

To test those hypotheses larvae were fed on diets containing the RSA at 2% (w/w) and specific monosaccharide GalNAc also at 2% (w/w). This was approximately 150-fold molar excess compared to the lectin and was the highest sugar concentration that did not affect the larvae growth (Table 3.3). After feeding for 16 days larvae fed on RSA only weighed 0.12 ± 0.12 mg, while those feeding on RSA and inhibiting sugar weighed 0.15 ± 0.07 mg (P = 0.221, t-test), compared to approx. 2 mg in untreated larvae. Therefore, the lectin effect was neither enhanced nor diminished by the addition of the carbohydrate.

Table 3.3. Effect of competing sugar (GalNAc) on RSA toxicity towards larvae.

	Larval weight at day 16 (mg)	Time of adult eclosion	% adult eclosed	% dead larvae
Control	1.98 ± 0.48	28.4 ± 3.8	100	0
Control + 2% GalNAc	2.07 ± 0.29	27.3 ± 3.6	100	0
	p = 0.310	**p = 0.238**		
2% RSA	0.12 ± 0.12	-	0[§]	53
2% RSA + 2% GalNAc	0.15 ± 0.07	114.5 ± 13.4	22.2[§]	56
	p = 0.221			

Values are means ± standard deviations (n=20). [§] Experiment was terminated after 158 days. p-values were calculated using one sided t-test.

A similar test was performed for SNA-II. However, to increase the threshold of potential negative or positive effects of the sugar competition the lectin concentration was reduced to 1% (w/w) while the GalNAc concentration was kept at 2% (w/w) which was approx. 580-fold molar excess of sugar (Table 3.4). Additionally, to exclude the potentially non-specific effects of sugar supplementation an additional control was added containing 1% SNA-II and 2% GlcNAc which is not recognized by this lectin. SNA-II alone reduced the larval weight at day 16 to 1.04 ± 0.36 mg, compared to 0.78 ± 0.30 mg in case of SNA-II + GalNAc and 0.86 ± 0.44 mg in case of SNA-II + GlcNAc. ANOVA and post hoc Tukey test did not indicate any significant differences. Yet, an independent sample t-test between lectin alone and lectin with competing sugar yielded P = 0.048 for SNA-II + GalNAc and 0.180 for SNA-II + GlcNAc. However, there were no significant differences between GalNAc and GlcNAc effect (P = 0.322).

Table 3.4. Effect of specific sugar (GalNAc), non-specific sugar (GlcNAc) and calcium chloride on SNA-II toxicity towards larvae.

	Larval weight at day 16 (mg)	Time of adult eclosion	% adult eclosed	% dead larvae
control	1.74 ± 0.46 **a**	28.3 ± 1.6 **a**	90	0
control + 1% $CaCl_2$	1.82 ± 0.50 **a**	28.7 ± 2.2 **a**	90	0
1% SNA-II	1.04 ± 0.36 **b**	36.3 ± 2.6 **b**	60	0
1% SNA-II + 1% $CaCl_2$	0.78 ± 0.35 **b**	37.6 ± 6.4 **b**	50	0
1% SNA-II + 2% GalNAc	0.78 ± 0.30 **b**	35.4 ± 3.4 **b**	70	0
1% SNA-II + 2% GlcNAc	0.86 ± 0.44 **b**	33.5 ± 3.3 **ab**	40	0

Values are means ± standard deviations (n=10). Same letters indicate no significant differences at 0.05 level, ANOVA, Tukey post-hoc test.

Due to the isoelectric point of SNA-II (pI = 6.4) and the pH in the *Tribolium* larvae gut lumen (up to 7.5; Vinokurov et al., 2009) this lectin could be negatively charged after ingestion. The PM is also negatively charged, therefore, electrostatic repulsion could further restrict SNA-II passing through this structure. To verify this assumption SNA-II was used together with calcium chloride ($CaCl_2$) which was shown before (Miller and Lehane, 1993) to reduce such electrostatic interactions and increase the permeability of the PM for negatively charged compounds (Table 3.4). The weight of larvae fed with

SNA-II and $CaCl_2$ was 0.78 ± 0.35 and was not significantly different from weight of larvae fed on SNA-II alone (P = 0.062, t-test).

3.4.9. RSA acts through induction of feeding deterrence

As shown in the paragraph 3.3.5 neither of the tested lectins induced rapid mortality of treated larvae but rather growth inhibition and development retardation. This observation raised the question whether this growth inhibition was due to direct damage to the larvae or due to decreased consumption of the diet containing lectins. To address this problem the flour disk assay was performed to evaluate the lectin effect on diet consumption, larval growth and efficiency of food to biomass conversion (Table 3.5). This experiment indicated that RSA indeed decreased the diet consumption and larval growth by up to 70% compared to the control (both P < 0.001, t-test).

Table 3.5. Impact of dietary lectins on growth and feeding of T. *castaneum* larvae.

		Relative growth (mg/mg)	Relative consumption (mg/mg)	Efficiency of food conversion [%]	Feeding deterrence index [%]
RSA	0%	0.26 ± 0.08	1.48 ± 0.24	0.18 ± 0.04	0 ± 16.0
	1%	0.18 ± 0.06*	0.79 ± 0.16***	0.23 ± 0.07	46.8 ± 11.1***
	2%	0.11 ± 0.04***	0.56 ± 0.12***	0.21 ± 0.09	62.3 ± 8.4***
SNA-I	0%	0.30 ± 0.03	1.56 ± 0.15	0.19 ± 0.02	0 ± 9.6
	2%	0.31 ± 0.04	1.29 ± 0.14*	0.24 ± 0.01***	17.1 ± 9.1*
	5%	0.30 ± 0.05	1.03 ± 0.19***	0.29 ± 0.03***	33.6 ± 12.4***

Values are means ± standard deviation (each treatment contained 5 groups of 10 larvae and was repeated twice). Relative growth is the ratio of larval weight gain to starting larval weight. Relative consumption is the ratio of diet consumption to starting larval weight. Efficiency of food conversion is ratio of weight gain to consumption. The feeding deterrence index is the % reduction of diet consumption. Asterisks indicate statistically significant differences compared to control – 0% lectin. (* - P < 0.05, ** - P < 0.01, *** - P < 0.001, one-sided, independent sample t-test).

However, the efficiency of food to biomass conversion (or the ratio of growth to consumption) was not affected and equaled 0.19 ± 0.04 in the control and 0.21± 0.09 in the treatment with 2% RSA

(P = 0.143) indicating that growth inhibition was most likely caused by the feeding deterrent activity of the lectin. For the sake of control a similar experiment was performed using SNA-I which was found in section 3.3.5 to have a negligible effect on larval growth and development. The flour disk assay confirmed the low activity of SNA-I as this lectin had no effect on larval growth (P = 0.480), even at concentration as high as 5% (w/w). Surprisingly, SNA-I reduced the relative diet consumption from 1.56 ± 0.15 in the control to 1.03 ± 0.19 mg/mg (P < 0.001). Thus dietary SNA-I did not reduce but actually enhanced the food conversion efficiency.

3.4.10. Delivery of lectins into hemolymph dramatically increases their toxicity

Experiments described earlier in this chapter indicated that lectins are toxic to *T. castaneum* insect cells but their activity after ingestion by larvae appears to be restricted by proteolysis in the gut and limited penetration through the peritrophic membrane. To further support this notion the lectins were delivered directly into larval hemolymph through microinjection to evaluate the insecticidal potential of the lectins without the impact of the protective effects of the gut environment and the peritrophic matrix. Injected lectins showed markedly higher larval toxicity compared to the delivery of lectins by feeding. All three lectins caused a significant larval mortality already one week after injection (Fig. 3.8)

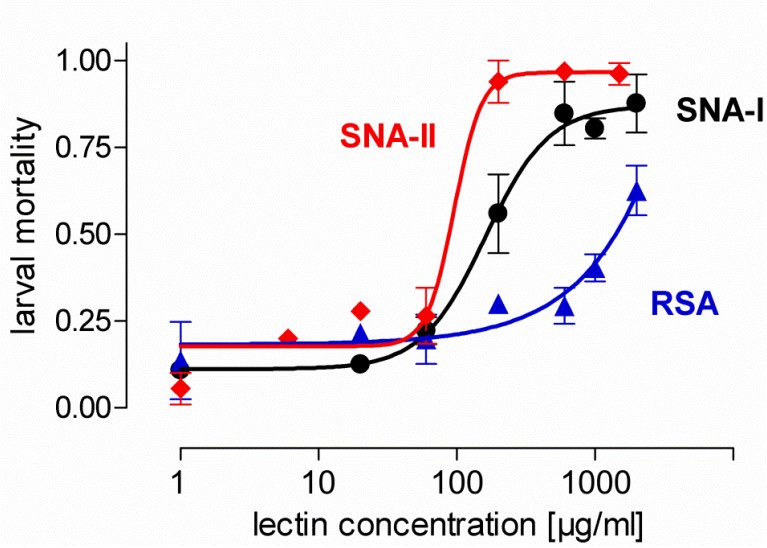


Fig. 3.9. Lectins injected into the hemolymph display a high insecticidal activity. Approximately 200-400 nl of lectin solutions were injected per larvae. Values are means ± standard deviations (n=3). At least 12 larvae were injected per concentration in each of the repetitions.

. Relative toxicity of the injected lectins was completely different compared to feeding assays and similar to the in vitro tests on the TcA cell line. SNA-II was the most toxic lectin (EC_{50} = 95.7 µg/ml) and caused almost complete mortality already at 200 µg/ml (Table 3.6). SNA-I was also highly toxic but was less active than SNA-II. It required concentrations of at least 600 µg/ml to kill most of the larvae (EC_{50} = 160.5 µg/ml). RSA in turn was the least active of the three lectins. The highest concentration tested, 3000 µg/ml, caused only up to ~60% larval mortality.

Table 3.6. Comparison of EC_{50} values of injected lectins.

	EC_{50} (95% C. I.) µg/ml	R^2
RSA	**~2000** (nd)	0.99
SNA-I	**160.5** (145.3-177.3)	0.95
SNA-II	**95.7** (77.2-136.2)	0.97

EC50 and its 95% confidence intervals (95% C. I.) were calculated based on mortality caused be lectin injection using sigmoidal dose-response curve fitting. In case of RSA no precise estimation of EC50 could be obtained because even the injections with highest concentrations of this lectin lead to max. ~60% mortality.

Table 3.7. Comparison of estimated lectin doses that caused clear negative effects on T. *castaneum* in feeding, injection and in vitro assays.

	Estimated effective doses		
	Injection into larvae[A] (ng lectin / mg larvae)	In vitro treatment[B] (ng lectin / mg TcA cells)	Feeding assay[C] (ng lectin / mg larvae)
RSA	>222.2	998.8-8709.0	60000-160000
SNA-I	16.1-59.1	142.7-1069.5	>240000
SNA-II	8.6-45.4	17.4-152.8	60000-160000

Highest and lowest estimates of doses are shown which were obtained using following parameters: for **A** injection volume = 200-400 nl, larval weight = 1.2-1.8 mg and EC_{50} values from Table 3.6. For **B**: cell radius = 5-7 µm, cell density in vitro experiments = 1-2 M cells/ml, lectin EC_{50} values in TcA cells were from Table 3.1. For **C**: daily consumption =0.2-0.5 mg/mg larvae, 2% (w/w) lectin content for RSA and SNA-II, 5% for SNA-I and 16 day long feeding.

In this experiment 300 nl of lectin solutions were injected per larvae. Taken this into account the average lectin doses that caused 50% mortality were 26.5 and 37.6 ng per mg of larvae for SNA-II and SNA-I, respectively. These doses are are at least three orders of magnitude lower than those ingested by larvae in feeding assay (~100 µg per mg larvae) and are in a similar range to those effective against TcA cells (Table 3.7). Altogether, these data confirmed the high toxic potential of lectins and underlined the importance of lectin passing through peritrophic matrix and midgut cells for the maximum toxicity.

3.5. Discussion

Our experiments with the *T. castaneum* cell line (TcA) indicated a high cytotoxicity for RSA, SNA-I and SNA-II but not for MAA which apparently had no effect on the growth, viability or morphology of the TcA cells. Similarly to the situation described for MAA activity towards CF-203 and S2 cells in the previous chapter (2.5. Discussion) the lack of MAA toxicity might be linked to the absence of glycans in the TcA cells that match the strict binding specificity of this lectin (Aoki et al., 2007). Among the three other lectins SNA-II was the most toxic while SNA-I and RSA were approximately 8-fold and 60-fold less active, respectively.

All three lectins induced various morphological changes in TcA cells including cell detachment, shrinkage, vacuolization but also swelling, membrane blebbing and formation of apoptotic-body like vesicles. This suggest that lectins might trigger various modes of cell death – cell shrinkage and vacuolization is indicative of autophagy, membrane blebbing and apoptotic bodies are hallmarks of apoptosis, while cell swelling and vacuolization is characteristic for oncosis that eventually leads to necrosis (Fink and Cookson, 2005). High doses of RSA, SNA-I and SNA-II induced DNA fragmentation in TcA cells. Some of the fragmented DNA was visible as ladder pattern typical for apoptosis but the majority of the DNA was fragmented randomly which is typical for necrosis (Fink and Cookson 2005). This suggests that the lectin effect on TcA cell does not involve a selective and efficient induction of apoptosis. Obviously, further studies of biochemical markers of apoptosis, autophagy and necrosis are required to fully characterize the mode of action of lectins in *T. castaneum* cells.

This is in line with other observations from insect cell lines indicating that some stimuli (e.g. oligomycin A) can induce various modes of cell death (Tettamanti et al., 2006). Cells can respond to toxic stimuli first by autophagy which may result in repair of the damage and cell rescue. Failure to compensate for the damage may lead to apoptosis characterized by organized DNA fragmentation.

Otherwise, if the damage is too severe and too rapid (e.g. in case of prolonged exposure or high dose of the toxin) oncosis/necrosis with uncontrolled cell disruption and DNA fragmentation may occur (Wu et al., 2011).. However, other data indicated that *Sambucus nigra* lectins induce caspase activity and apoptosis in lepidopteran cells while RSA did not cause cell death but growth inhibition in S2 cells (Hamshou et al., 2012). These observations suggest that lectin effects and modes of action might be cell-type specific.

RSA, SNA-II and SNA-I were internalized in TcA cells. However, a fraction of these lectins remained bound to the plasma membrane. It has been shown previously that multiple lectins can interfere with protein translation and induce autophagy after internalization (Shang et al., 2015). Some other lectins can trigger cell death or inhibition of proliferation by binding to the cellular receptors (Audi et al., 2005; Hamshou et al., 2013; Lichtenstein and Rabinovich, 2013). Therefore, it is possible that binding and internalization affect the cells differently and thus lead to the diverse observed effects on TcA cells.

When the same lectins were introduced into the diet of *T. castaneum* larvae, they did not kill the larvae but retarded larval growth and development. Surprisingly, RSA, that was the least active lectin against TcA cells in vitro, produced the highest toxic effect against the larvae of *T. castaneum* in vivo. The two other lectins, namely SNA-II and SNA-I, were remarkably less detrimental towards the beetle larvae despite their high toxicity against the TcA cell line. To understand those unexpected differences in lectin toxicity, we first investigated the lectin stability in the larval guts. These experiments demonstrated that most of the ingested SNA-I was effectively proteolyzed and excreted with the feces, which explains lack of toxicity of this lectin towards larvae. In contrast, both RSA and SNA-II were resistant to proteolytic cleavage, indicating another mechanism reducing their toxicity. These stability studies were performed using lectins that were labeled with FITC. This fluorescent tag attaches to side chains of lysine and arginine. Interestingly, carboxyl sides of these enzymes are trypsin cleavage sites, thus, extensive labeling of those amino acids with FITC could potentially reduce trypsin digestion efficiency. However, lectins contain from 30 to 160 of these amino acids but only 2-8 FITC molecules were linked, thus less than 10% of the cleavage sites could be protected this way.

Midguts of many insects, including beetles, contain a protective porous structure, called the peritrophic matrix. The PM pores usually range between 4 and 10 nm in diameter (Lehane and Billingsley, 2012). The size of the PM pores results in a selective permeability of the PM (Terra, 2001) and provides protection of the gut epithelium cells by physical sequestration of large particles within

the gut lumen (Hegedus et al., 2009). Recently, Agrawal et al. (2014) demonstrated that the permeability limit of *T. castaneum* larval PM for FITC-dextran decreases from 2 MDa molecules in the anterior midgut through 150-70 kDa in the median midgut and 4kDa in the posterior parts. This suggests that the PM pore diameters are between 54 and 3 nm. Because RSA and SNA-II differ in protein dimensions we hypothesized that the lectin toxicity may correlate with the efficiency of passing through the pores of the PM. In line with this assumption, we observed that the RSA lectin (32 kDa), which is the most toxic of the two proteins, passed through the PM efficiently throughout the length of the midgut. In contrast, the permeation through the PM of the larger protein, namely SNA-II (64 kDa), was apparently restricted.

RSA is a dimer consisting of two identical 15.5 kDa subunits, with molecule dimensions of approximately 3 x 3 x 6 nm (Skamnaki et al., 2013). In a gel filtration experiment (Vranken et al., 1987) this protein coeluted with chymotrypsinogen (25 kDa), indicating that RSA has equivalent spherical diameter of approximately 4 nm. Both measures of RSA dimensions imply that RSA may pass through the small pores of the PM, exactly as we observed.

Analysis of the SNA-II structure showed that the lectin exists in solution as a mixture of monomers and homodimers with maximum dimensions of 7 and 10 nm, respectively (Maveyraud et al., 2009). Coexistence of monomeric and oligomeric SNA-II may explain the observed partial permeability of the PM for that lectin. It seems likely that the size of the monomeric SNA-II (32 kDa) allows it to traverse the PM, while the oligomers with larger dimensions than the pore opening are retained in the endoperitrophic space.

Our data indicated that this phenomenon might be essential for lectin toxicity in *T. castaneum*. Even in the case of RSA, which is the lectin that passed through the PM most efficiently, the bulk of the lectin still remained in the endoperitrophic space. This might explain the fact that negative effects were observed only at relatively high concentrations of the lectins (1-2% w/w). The low permeability of the PM in *T. castaneum* could be responsible for the generally low susceptibility of this species to toxic proteins, as was also evident in studies with *Bacillus thurnigensis* toxins (Contreras et al., 2013a; Contreras et al., 2013c; Oppert et al., 2010; Oppert et al., 2011). These works demonstrated that *T. castaneum* larvae are resistant to Cry3Aa, Cry3Ca and Cry34Ab/Cry35Ab and susceptible only to high concentrations of Cry3Ba and Cry23Aa/Cry37Aa. Crystal structures of both Cry3Aa and Cry3Ba have been solved, indicating approximately 8 nm as the maximum molecule dimension (Li et al., 1991). Thus, it seems likely that, similar to SNA-II, Cry3 molecules are too big to pass the PM and hence

cannot cause high toxicity. Finally, a similar supposition could be drawn from the work of Hernández-Rodríguez et al. (2013) in which they showed a higher toxicity of Cry1 toxins to *Ostrinia nubilalis* with a PM pore diameter of approximately 40 nm (Harper and Hopkins, 1997) compared to *Spodoptera frugiperda* with 7-8 nm wide PM pores (Ferreira et al., 1994). In conclusion, our results are in line with several previous studies which proposed that binding or/and physical interaction with the PM will affect the efficiency of several toxins (Granados et al., 2001).

In the gut of *T. castaneum* larvae with a pH of 5.6-7.5 (Vinokurov et al., 2009), RSA (pI = 9.0) is always positively charged, while the charge of SNA-II (pI = 6.4) might change to negative as it moves along the midgut. This would further restrict SNA-II from passing through the PM due to the electrostatic interaction with the negatively charged proteoglycans (Barbehenn, 2001). FITC attaches to proteins through side chains of lysine and arginine, which are positively charged. This in turn might increase the negative charge of proteins and decrease the permeability of these proteins through PM. Nonetheless, suppression of such interaction did not increase the toxicity of SNA-II which suggests that repulsion between the lectin and the negatively charged PM is not a major reason for its restricted penetration through the PM and low toxicity. Additionally, Agrawal et al. 2014 observed that FITC-dextrans, which are negatively charged in gut physiological conditions, could freely pass through peritrophic matrix.

Binding of the lectins to the PM may also contribute to their detrimental effects on insects, as in the case of WGA (Harper et al., 1998) or, on the contrary, diminish the amount of lectin interacting with the gut epithelium. The conducted experiments with competing sugars did not clarify the impact of sugar binding on the lectin toxicity. One possible reason is that binding inhibition reduced both lectin sequesteration by the PM as well as interaction with the midgut epithelium. Additional difficulty in the interpretation of the results of this experiment comes from that N-acetylaminosugars like GalNAc and GlcNAc can be involved in signaling (e.g. circadian rhythm, Notch or insulin pathways). Therefore, supplementation of the diet with these sugars might have had an impact not only on the lectin interaction with insect gut but also on many other biological phenomena (Sekine et al. 2010, Sakaidani et al. 2011). Although, we were not able to determine if the above hypotheses are true for RSA and SNA-II, we assume that binding to the PM does not explain differences in the potency between the two proteins since both of them have similar sugar-binding specificities.

Negative effects of dietary RSA on larvae was mostly caused by a reduction of feeding which resulted in retarded growth and eventual death of starvation after prolonged exposure (over 100

days). It is still an open question whether feeding reduction is induced by lectins specifically or whether larvae restrict feeding as a kind of generic defensive reaction aimed to reduce the intake of the toxin.

In contrast, RSA, SNA-I and SNA-II injections caused acute larval mortality at doses similar to those effectively killing cultured TcA cells which confirms that those lectins indeed have high insecticidal properties. However, the efficiency of lectins delivered through the diet is largely reduced due to a combination of multiple phenomena. (i) Reduction of feeding diminishes the dose of lectin in the insect gut. (ii) Most of the lectin molecules cannot pass through the PM and are retained in the gut lumen. This makes them more available to (iii) activity of proteolytic enzymes and exposes them for (iv) excretion. Comparison of the doses that were toxic through injection with those in the feeding assays indicated that less than 0.1% of the lectin ingested by larvae bypasses those lines of defense and enters the hemolymph.

Previous reports point out that the three lectins of this study and several other ones, including ConA, garlic and snowdrop agglutinin, are much more effective against aphids than against other insects (Bandyopadhyay et al., 2001; Fitches et al., 1997; Gatehouse et al., 1999; Hamshou et al., 2013; Shahidi-Noghabi et al., 2010b; Upadhyay et al., 2010). Aphids do not produce a PM in their gut, therefore it is likely that lectins may freely interact with the gut epithelium and reveal their full insecticidal potential.

In conclusion, this chapter along with a number of other publications indicates that the protein ability to pass through the PM is one of the key features determining their insecticidal effect. Since protein and PM pore size dimensions may influence this phenomenon, it seems justified to use these criteria to select for the most promising insecticidal proteins. Further research on methods to enhance lectin delivery into the hemolymph may contribute to the rational design of more effective insecticides.

Chapter 4

Analysis of glycan composition in *Tribolium castaneum*

Results shown in this chapter are included in the manuscript in preparation:

Walski, T., Van Damme, E. J. M., Smargiasso, N., Christiaens, O., De Pauw, E., & Smagghe, G. Differential N-glycan processing is required for successful metamorphosis in the pest beetle *Tribolium castaneum.*

4.1. Abstract

N-glycans are involved in numerous biological phenomena including receptor functions, transport or cell adhesion. They also play important roles in immunity and the central nervous system. Yet, most of the knowledge on N-glycosylation comes from the mammalian or nematode models. The limited data on insect N-glycans comes mainly from studies on the fruit fly, *Drosophila melanogaster* or on insect cell lines and thus lack relevance in the context of pest insect control. In this chapter we studied the composition of N-glycans in the red flour beetle, *Tribolium castaneum*, which is a first step towards deciphering functions of N-glycosylation in this pest and model insect.

There are no standardized protocols to study insect N-glycosylation. Therefore, to find the best set-up several analytical methods including high pressure liquid chromatography (HPLC), matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) and liquid chromatography coupled with mass spectrometry (LC-MS) and glycan labeling strategies using 2-aminopyridine, 2-aminobezamide or permethylation have been used.

The most successful approach included on-filter tryptic digestion, release of the N-glycans using PNGase A, 2-AB labeling and glycan analysis using MALDI-MS. Using these methods, similar sets of more than 20 N-glycan structures could be identified in larval and adult samples of the beetle *T. castaneum*. The identified glycans were mostly of the high mannose and paucimannose type but several complex N-glycans were also identified. Interestingly, there were clear differences in the relative content of the N-glycans between larvae and adults indicating that insect metamorphosis and adult development is associated with a shift in the N-glycan profile towards more processed forms. Additionally, both larval and adult N-glycan compositions were markedly different from that of the fruit fly. Especially the content of difucosylated N-glycans was much higher in *Tribolium* than in *Drosophila*.

Altogether data obtained in this chapter indicated that N-glycosylation might play a role in insect metamorphosis and confirmed that *D. melanogaster* as a model has a limited validity for pest insects supporting the need of glycomic studies on particular pest species.

4.2. Introduction

In previous chapters it was shown that lectins can recognize glycans present on insect cells and can be toxic towards the red flour beetle, *Tribolium castaneum*. The next objective of this thesis is to study the role(s) of N-glycans in the red flour beetle and to explore possibilities to utilize the information on the glycome of this pest insect for crop protection purposes. The majority of studies dealing with the physiological functions of N-glycans have been performed in vertebrates. In this subphylum of the animal kingdom N-glycosylation was found to be involved in protein folding, stability and targeting to appropriate cellular compartments (Roth et al., 2010). It was also shown that N-glycans are implicated in diverse biological phenomena such as cell adhesion, receptor-ligand binding, cancer development or immune response (Marth and Grewal, 2008; Saremba et al., 2008; Zhao et al., 2008). Relatively little data are available concerning N-glycosylation functions in insects and the few available studies focused mainly on the fruit fly *Drosophila melanogaster* as a model. In this insect N-glycosylation was investigated mainly in the context of immunity and biology of the nervous system (Katoh and Tiemeyer, 2013). There is even less information on the importance of N-glycosylation in agricultural pest insects, although such knowledge would be invaluable in terms of developing novel pest control strategies.

Analysis of N-glycans produced by fruit flies, mosquitoes, silkworms or various insect cell lines indicated that insect N-glycomes are composed mainly of high mannose ($Man_{5-9}GlcNAc_2$) and paucimannose N-glycans (short structures with possible core fucoses), while complex oligosaccharides (glycans containing at least one GlcNAc attached to $Man_3GlcNAc_2$) are found in rather low amounts (Aoki et al., 2007; Dojima et al., 2009; Kim et al., 2009; Kurz et al., 2015). Nevertheless, despite the similar repertoire of N-glycans the abundances of particular oligosaccharide structures appear to be species specific, which implies that data obtained for model organisms cannot simply be translated to pest insects.

Previous studies showed that insects contain more than 20 major N-glycan structures and even triple of that number of low abundant N-glycans. This raises several important questions. Are all the N-glycans equally important or are some of these N-glycans expendable? Does each of these N-glycans have a specific role? These questions are not fully answered yet, even in model organisms. Therefore, as the first step towards uncovering of the roles of N-glycosylation in *Tribolium castaneum* we characterized the profile of the N-glycans produced by this insect.

Multiple methods are used for the analysis of the N-glycans in biological samples and no golden standards exist. Most of the methods have been optimized to study mammalian N-glycans, which are markedly different from the insect ones. Thus not of all these techniques are ideally suited for studying insect N-glycosylation. The native N-glycans are polar and non-volatile which makes them difficult to ionize and analyze by mass spectrometry (MS). The common solution to that problem is labeling of the glycans with tags as 2-aminopyridine (PA) or 2-aminobenzamide (2-AB), which increases ionization efficiency of the glycans and facilitates interpretation of the spectra obtained with MS (Lamari et al., 2003). Additionally, such derivatizations enable the analysis of N-glycans by high pressure liquid chromatography.

PA-labeling normal phase (NP)-HPLC, which separates N-glycans by size, was used to study high mannose N-glycan processing in lepidopteran Sf9 cells (Kawar et al., 2000). Other studies used a combination of strategies including NP-HPLC and MS to detect N-glycans in *D. melanogaster* embryos or *Bombyx mori* larvae (Aoki et al., 2007; Kajiura et al., 2015). The tagging of N-glycans with 2-AB was reported to have a higher labeling efficiency (~90%), to label all N-glycans uniformly and to be compatible with sialylated N-glycan analysis contrary to PA-labeling (Bigge et al., 1995). Several studies used this method and HPLC or MS to analyze the N-glycans in *D. melanogaster* larvae (Roberts et al., 1998) or cultured cells (Kim et al., 2009).

Permethylation followed by MS is another approach especially preferred for the analysis of vertebrate N-glycomes containing sialylated N-glycans. This method improves detection of these negatively charged sugars and together with the analysis of N-glycan fragmentation allows determination of isomeric glycans (North et al., 2009). With this technique numerous low abundant N-glycans were detected in mosquito larvae (Kurz et al. 2015) and fruit fly embryos (Aoki et al., 2007; North et al., 2006).

A specific first goal of this chapter was to test the above approaches to find the optimal methodology for studying N-glycosylation in *Tribolium.* A *s*econd objective was an in depth characterization of the N-glycosylation potential of the red flour beetle. The final aim was to compare the N-glycan profiles from the larvae, adults, guts and heads of the red flour beetle to identify possible life stage- and tissue-specific characteristics.

4.3. Materials and methods

4.3.1. Insect sample collection, protein and N-glycan purification

The red flour beetles *Tribolium castaneum*, were cultured (as described in Section 3.3.1) in whole grain wheat flour supplemented with 5% yeast, at 27°C and 60% relative humidity. Approximately two-week-old larvae and mature adults were isolated from flour using a sieve. Wheat flour also contains glycosylated proteins therefore to reduce the carry-over of these proteins the collected insects were blown with a stream of air and starved for 24 hrs. One sample of beetles was frozen at -80°C for later preparation of proteins and N-glycans from whole insects, while the other sample was chilled on ice to sedate insects before dissection. To dissect guts larvae were placed in a drop of phosphate buffered saline, pH 7.6 (PBS), and the anterior and posterior ends of the larvae were cut off using fine scissors. Subsequently, the guts were pulled using forceps, placed directly in protein extraction buffer composed of 4% SDS, 0.1M DTT in 0.1M Tris/HCl pH 7.4 and ground using a pestle. Adult heads were cut off using fine scissors, placed in an Eppendorf tube and frozen at -80°C. All the frozen samples were ground in liquid nitrogen using pestle and extracted with extraction buffer added at 1:10 (w/v) ratio. Proteins were extracted according to Zielinska et al. (2010). To ensure good protein extraction all samples were vortexed for 5 mins, incubated in a boiling water bath for 5 mins, followed by incubation in an ultrasonic bath for 10 mins and vortexing for 5 mins. Next, extracts were clarified by centrifugation at 5000g for 15 mins. Supernatants were collected and the protein content was estimated by measuring the absorbance at 280 nm, assuming that a 1 mg/ml protein solution has an absorbance of 1.1. Protein extracts were mixed with 200 µl of 8M urea in Tris/Cl pH 8.5 (UT) per 500 µg protein and placed on top of an Amicon Ultra-0.5 mL centrifugal filters (30 kDa cut off) and centrifuged at 14 000 g for 15 mins. Protein extracts were cleaned by another 3 rounds of washes with UT and centrifugation. Subsequently, protein thiol groups were carboxymethylated by incubation of samples with 0.05 M iodoacetamide in UT for 20 mins in dark. Next proteins were washed twice with UT and twice with 40 mM ammonium bicarbonate. Protein extracts were collected by centrifugation (1500 g, 1 min) of filters placed upside-down in the new collection tubes and the protein yield was measured by the absorbance at 280 nm. In total, approximately 2 mg of proteins were obtained from 400 heads (40 mg), 300 guts, 40 beetles (80 mg), 60 larvae (90 mg).

Approximately 0.5 mg of protein extract was treated with MS grade porcine trypsin (Sigma) at a ratio of 1:100 (5 µg trypsin per sample) and the samples were placed at 37°C for 16 hrs. The reaction

was stopped by boiling the samples for 5 mins and the samples were lyophilized. Another 0.5 mg of protein extract was used for the release of glycans using PNGase F (5U per sample) in 40 mM ammonium bicarbonate for at 37°C for 16 hrs. Released glycans were cleaned on SepPak C18 cartridges (Waters). Cartridges attached to a 10 ml syringe were conditioned by eluting with methanol (5 ml), 5% acetic acid (5 ml), propan-1-ol (5 ml) and 5% acetic acid (3 x 5 ml). Samples were acidified with 20 µl of 5% acetic acid and applied on the cartridges. The N-glycans were collected from the run-through and elution with 5% acetic acid (4 ml). The N-glycans and the remaining protein extracts (1 mg) were lyophilized.

4.3.2. Glycan hydrolysis and fluorescent labeling of monosaccharides.

Initial glycan analyses described in points 4.3.2 – 4.3.6 were performed in cooperation with Prof. Erika Staudacher of the Glycobiology Division, Division of Biochemistry, Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria.

Monosaccharide analysis was performed essentially as described in Stepan and Staudacher (2011). To hydrolyze glycans into monosaccharides protein samples (200 µg) were dissolved in 300 µl of 4M trifluoroacetic acid (TFA) and incubated at 115°C for 2 hrs. Afterwards the samples were dried in a rotary evaporator. Subsequently, 500 µl of 30% (v/v) methanol was added and samples were dried again. This step was repeated three times to ensure the complete evaporation of TFA. The dried samples were dissolved in 20 µl of 1M sodium acetate and vortexed for 5 mins. Then 40 µl of a solution containing 3 mg/ml of anthranilic acid (AA), 30 mg/ml of sodium cyanoborohydride in 2% (w/v) boric acid in methanol was added and samples were incubated at 80°C for 1 hr. The samples were analyzed by HPLC on an Agilent 1200 LC (liquid chromatography) system with a reversed phase C18 column (ODS Hypersil, 5 µm, 250x4 mm, Thermo Scientific, part no. 30105- 254030). Solvent A was 1.0% tetrahydrofuran, 0.5% phosphoric acid, and 0.2% 1-butylamine in water. Solvent B was 50% acetonitrile in solvent A. A linear gradient of 5–25% B was applied for the first 10 mins followed by a 25-100% B gradient over 6 mins. The column was washed with 100% solvent B for 6 mins and reequilibrated by 100-5% B gradient over 1 min. A constant flow of 1ml/min was used. Approx. 5 µl of each of the sample was manually injected. Fluorescence detection was done using 360 nm excitation and 425 nm emission wavelengths. Monosaccharides were identified based on the elution times of AA-labeled standards: glucosamine, galactosamine, galactose, mannose and fucose. The relative quantitation of the sugars was based on the areas of the identified peaks.

4.3.3. Sialic acid detection

Sialic acid detection was based on the protocol described by Burgmayr et al. (2001).To release the sialic acids the protein samples (300 µg) were dissolved in 2M acetic acid, incubated for 2 hrs at 80°C to release sialic acids and dried in a rotary evaporator to remove acetic acid. Dried samples were dissolved in 50 mM Tris/HCl pH 7.5. Half of the sample was incubated with 25 mU of sialic acid aldolase (Sigma) and 1 mM NADH for 1hr at 37°C. The other half was treated the same way but 1 µl of water was added instead of the enzyme. Sialic acid aldolase converts N-acetylneuraminic (Neu5Ac) acid to N-actylmannosamine and pyruvate. Therefore, incubation with the enzyme would reduce the amount of detected sialic compared to the untreated samples and thus would unambiguously prove the presence of Neu5Ac. After that the samples were dried, dissolved in 25 mM H_2SO_4 (20 µl) mixed with 100 µl of 1,2-diamino-4,5-methylenedioxybenzene (DMB) labeling solution (15.75 mg of DMB, 697 µl of 2-mercaptoethanol, 17.1 mg of $Na_2S_2O_5$ in 10 ml water) and incubated for 2.5 hrs at 60°C. The reaction was stopped by chilling the samples on ice. Labeled samples were analyzed on same HPLC system as described in section 4.3.2. The mobile phase was methanol:acetonitrile:water 7:9:84 (v/v/v). The flow rate was 1 ml/min. Fluorescence detection was done using 373 nm excitation and 448 nm emission wavelengths. 10 µl of sample was applied by manual injection. DMB labeled N-acetylneuraminic acid and N-glycolylneuraminic acid were used for calibration. The relative abundance of each sialic acid was calculated as the ratio of the area of a given peak to the area of the DMB reagent peak.

4.3.4. MALDI-TOF mass spectrometry

1 µl of glycan sample was spotted on the target plate and dried under reduced pressure. Next 1 µl of matrix (2% 2,5-dihydroxybenzoic acid in 50% acetonitrile in water) was spotted on the dried glycans and transferred to the desiccator to dry. Spectra were acquired using DYNAMO linear MALDI-TOF (Matrix-Assisted Laser Desorption Ionization with Time Of Flight detector) mass spectrometer (Thermo BioAnalysis) operated with a dynamic extraction setting of 0.1. The external mass calibration was performed with N-glycan standards derived from bovine fibrin. About 20 of individual laser shots were summed. For N-glycan assignment 0.5 m/z (mass to charge) difference was allowed between the theoretical values and those of detected peaks.

4.3.5. PNGase A release and PA-labeling of N-glycans

To release all the N-glycans, including those containing core α1,3 fucose, tryptic peptides (500 µg) were dissolved in 0.1 M citrate phosphate buffer pH 5.0 and incubated with 0.1 mU of peptide:N-glycosidase A (PNGase A, Roche) at 37°C for 18 hrs. After the incubation glycans were separated from the peptides on a Sephadex G15 column using 1% acetic acid as the eluent and lyophilized. For labeling with 2-aminopyridine (PA) 1g of PA was dissolved in 760 µl of 37% HCl and then diluted three times in water to prepare the working solution. Lyophilized glycans were dissolved in 80 µl of working solution and incubated for 30 mins in a boiling water bath. 10 mg of sodium cyanoborohydride, $NaBH_3CN$ was dissolved in 20 ul of working solution and diluted with 30 µl water. 4µl of that solution was added to glycans previously dissolved inworking solution and incubated for 16 hrs at 90°C. Labeled glycans were purified on a Sephadex G15 column using 10 mM ammonium acetate pH 6.0 (Gutternigg et al., 2004).

4.3.6. HPLC analysis of N-glycans

PA-labeled glycans (10 µl) were analyzed on a HPLC system as described in previous sections on a normal phase Palpak type N column (5µm, 250x4.6 mm) to separate N-glycans by size. Solvent A was a 65:25:10 (v/v/v) mixture of acetonitrile:water:TEA buffer. TEA buffer contained 0.1% trimethylamine and 0.2 M acetic acid, pH 7.3. Solvent B was 50:40:10 mixture of the same components. The program included a 0-100% linear gradient of solvent B over 50 minutes, followed by a wash and a reequilibration at 0% solvent B for 15 minutes. The flow was set to 1 ml/min. Column temperature was set to 40°C, fluorescence detection was done using 310 nm excitation and 380 nm emission wavelengths. Column was calibrated using maltose oligosaccharides (3-15 glucose units) and authentic PA-labeled N-glycans. Separate peaks were collected manually into glass vials and dried using rotary evaporator and analyzed by MALDI as described above (Gutternigg et al., 2004).

4.3.7. Improved protocol for N-glycan preparation and analysis

Improved protocol for preparation and analysis of N-glycans was established and performed in cooperation with Mass Spectrometry Laboratory, Department of Chemistry, University of Liège directed by Prof. Edwin De Pauw. N-glycan labeling, clean-up as well as MS analysis was done by Dr. Nicolas Smargiasso.

The initial steps of improved protocol were similar to that in section 4.3.1 except that MS grade porcine trypsin (Thermo Scientific) was added directly to proteins cleaned up on Amicon Ultra-

0.5 ml centrifugal filters (30 kDa cut off) at 1:100 w/w ratio (5µg trypsin per 500µg peptides per filter) and digestion was performed for 16 hrs at 37°C. Tryptic peptides were collected by centrifugation which allowed separation of high molecular mass contaminants. Subsequently, peptides were subjected to an additional clean up step using Sep-Pak C18 cartridges (Waters) and the 1-propanol/5% acetic acid system. The method was similar to that described in section 4.3.1 except that the adsorbed glycopeptides were washed three times with 5 ml of 5% acetic acid and subsequently eluted with 5 ml of 20% 1-propanol in 5% acetic acid and 5 ml of 40% propanol in 5% acetic acid. Subsequently peptides were dried using a rotary evaporator and resuspended in an appropriate buffer for glycan release. The peptide content was estimated based on measurement of the absorbance at 280 nm. Approx. 4 mg of tryptic peptides were used to for glycan release using either 0.4 mU glycerol-free PNGase A from almonds (ProGlycAn, Vienna, Austria) in 0.1M phosphate-citrate buffer pH 5 or 5 U of PNGase F from *Elizabethkingia meningoseptica* (Sigma-Aldrich) in 40 mM ammonium bicarbonate pH 8 for 16 hrs. Released glycans were separated from peptides on Sep-Pak C18 cartridges (Waters) using 1-propanol/5% acetic acid system, as described in 4.3.1, using elution with 5% acetic acid. Samples were dried, resuspended in water and desalted using Glycoclean H-cartridge (Prozyme) according to manufacturer's protocol and then evaporated to dryness. Glycans were resuspended in labeling solution (750 mM $NaBH_3CN$, 175 mM 2-aminobenzamide in DMSO/acetic acid at 10:3 ratio) and incubated at 65°C for two hours. After further purification on a Glycoclean S-cartridge (Prozyme) according to the manufacturer's protocol, glycans were resuspended in 50% acetonitrile in water for MS analysis. MALDI-TOF analyses of larva and adult samples were performed on an UltraFlex II (Bruker) mass spectrometer (4000 laser shots per spectrum). The matrix was 2,5-DHB prepared at 20 mg/mL in 50% acetonitrile, 0.1% formic acid. Samples were prepared in four biological replications and three mass spectra were recorded per replication. Laser intensity was optimized for every sample depending on the signal obtained. UPLC-MS was performed using BEH Glycan (Waters) column coupled to a Q Exactive mass spectrometer (Thermo) operating in the positive ion mode. 100 mM ammonium formate, pH 4.5 and acetronitrile were used as solvents A and B, respectively. For each spectrum, the intensities of the glycan peaks were normalized on the total glycan signal allowing relative proportions to be determined. N-glycan permethylation was preformed according to Mechref et al. (2009). For that, dried glycans were resuspended in 200 µl of dimethylsulfoxide (DMSO). Subsequently 4 µl of MiliQ water and 74 µl of methyl iodide (ICH_3) was added. The mixture was applied on a spin column packed with sodium hydroxide beads and centrifuged at 400 g for 30 s to draw the solution through the column. This step was repeated eight times, after this the column was

washed twice with 100 µl of DMSO to ensure complete collection of the permethylated (perMe) glycans. To clean up the perMe N-glycans 200 µl of chloroform and 200 µl were added and vortexed for 3 mins. Then the sample was centrifuged at 3000 g and the aqueous phase was removed. That step was repeated five times. The remaining chloroform phase was dried in a rotary evaporator. PerMe N-glycans were re-dissolved in 200 µl of 50% methanol in water and purified on C18 cartridges conditioned with methanol, water, acetonitrile and once more with water. Glycans adsorbed on the cartridge were washed with water and 10 % acetonitrile (5 and 2 ml, respectively) and were finally eluted in four 0.5 ml fractions of 80% acetonitrile and evaporated to dryness.

Detected peaks were annotated using the GlycoWorkbench (Ceroni et al., 2008) based on N-glycans described in *Drosophila*. Ambiguous peaks were annotated based on MS/MS fragmentation spectra. Differences between the relative glycan content were analyzed using one sided t-test in SPSS 22.

4.4. Results

4.4.1. Analysis of monosaccharide composition of *T. castaneum* glycans

To gain some first insight into the glycosylation profile of *Tribolium castaneum* we looked at the monosaccharide composition. For that purpose proteins extracted from whole larvae, whole adults as well as from larval guts and adult heads were treated with trifluoreoacetic acid (TFA) to release the glycans and to hydrolyze oligosaccharides into monosaccharides. Subsequently, monosaccharides were labeled with a fluorescent tag (anthranilic acid) and their composition was analyzed using reversed phase high pressure liquid chromatography (RP-HPLC) on a C18 column. Five main monosaccharides building N- and O-glycans, in particular galactose, N-acetylgalactosamine, N-acetylglucosamine, fucose and mannose were identified based on the retention times of the standards (Table 4.1.). Note that TFA hydrolysis removes N-acetylgroups therefore to indentify GlcNAc and GalNAc glucosamine (GlcN) and galactosamine (GalN) were used as standards, respectively.

In each of the samples mannose was the most abundant monosaccharide, yet there were clear differences in the monosaccharide content between the analyzed life stages and tissues (Fig. 4.1.). Since both mannose (Man) and GlcNAc are mostly found among N-glycans, which usually contain 2-4 GlcNAc units and 2-9 mannoses. Therefore, a high ratio between the two (Man:GlcNAc) might indicate the prevalence of high mannose glycans, while lower ratio might indicate that most of the N-glycans

are more processed. The lowest Man:GlcNAc ratio was observed in samples from adult heads (2.47), followed by whole adults (2.94) and the highest in whole larvae (4.56). This suggests that adult heads contain the highest share of the more processed N-glycans. The Man:GlcNAc ratio found for the sample from larval guts was extremely high – 18.84 which might indicate incomplete hydrolysis of the high mannose glycans or the presence of mannose polymers from yeast used as additive to the insect diet.

Similarly, the GalNAc to galactose (GalNAc:Gal) ratio might be indicative of the relative amounts of the two common O-glycans that is T antigen (Galβ1-3GalNAc) and Tn antigen (GalNAc). GalNAc: Gal ratios were 1.16 and 1.54 for whole adult and adult heads, respectively. In contrast, in whole larvae and in larval guts GalNAc: Gal ratios were 0.75 and 0.84, respectively. Therefore, both adult samples contained relatively more of T antigen glycans while in whole larvae and larval guts Tn antigen was apparently more abundant. The fucose content was the highest in the sample from whole adult beetles (7.4%), while the others contained similar levels (5.0-5.6%).

Table 4.1. Relative contents of anthranilic acid-labeled monosaccharides identified by HPLC.

.		Larvae		Adults		Larval guts		Adult heads	
Monosaccharide	**Retention time**	**area**	**%total**	**area**	**%total**	**area**	**%total**	**area**	**%total**
GlcNAc	6.6	155.3	**12.0%**	35.8	**17.0%**	30.0	**3.4%**	50.0	**14.8%**
GalNAc	7.0	174.7	**13.5%**	28.7	**13.6%**	113.9	**12.8%**	87.9	**26.1%**
Galactose	8.3	235.0	**18.1%**	24.6	**11.7%**	135.6	**15.3%**	57.0	**16.9%**
Mannose	9.2	667.7	**51.4%**	105.6	**50.2%**	564.8	**63.5%**	123.6	**36.7%**
Fucose	10.3	65.6	**5.1%**	15.6	**7.4%**	44.5	**5.0%**	18.7	**5.6%**

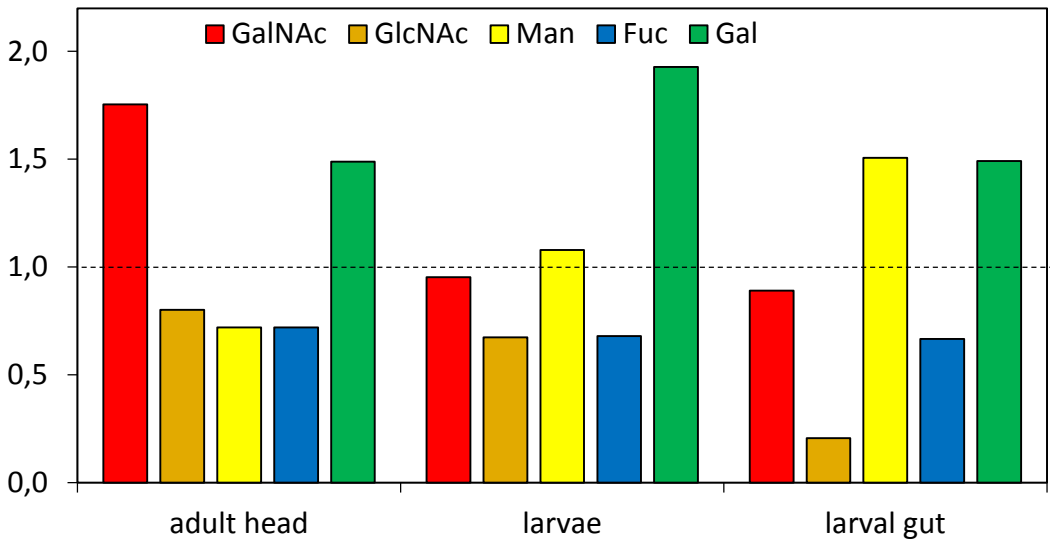


Fig. 4.1. Monosaccharide content in different samples relative to adults (indicated as the reference line).

4.4.2. Identification of sialic acids

The approach described above does not allow to label and detect sialic acid residues which might be present in a subset of insect glycans. Therefore, to identify those sugars the samples were subjected to specific sialic-acid labeling using DMB (1,2-diamino-4, 5-methylenedioxybenzene). The DMB-derivatives were then fractionated by RP-HPLC and the retention times were compared to those of two DMB-labeled sialic acid standards: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (NeuGc).

In all the samples minor peaks were detected at exactly the same retention times as in the case of NeuGc and Neu5Ac standards (6.2 and 7.9 mins, respectively) suggesting that low amounts of sialic acid are present in *T. castaneum.* To confirm that the observed peaks are coming from authentic sialic acids the samples were incubated with sialic acid aldolase (Table 4.2). This enzyme catalyzes the reversible conversion of Neu5Ac to N-acetylmannosamine and pyruvate in the presence of NADPH. Therefore, incubation with the enzyme should reduce the amount of Neu5Ac and thus reduce the peaks detected by RP-HPLC. Surprisingly, the incubation with the enzyme not only did not reduce the peaks but actually increased them. This was potentially due to a contamination in the enzyme batch. Therefore, the presence of the sialic acid could not be undoubtedly confirmed using labeling and HPLC approach and requires further verification using MS.

Table 4. 2. Relative amounts of detected DMB-labeled sialic acids.

	NeuGc		Neu5Ac	
	Native	**Aldolase**	**Native**	**Aldolase**
Larva	0.5%	0.5%	1.5%	3.2%
Adults	1.6%	1.4%	3.1%	7.5%
Larval guts	1.5%	1.4%	5.4%	8.8%
Adult heads	1.9%	1.3%	3.3%	5.2%
water	0.0%	0.4%	0.0%	6.3%

Given values are areas of peaks detected at 6.2 and 7.9 min (NeuGc and Neu5Ac, respectively) relative to peak of unreacted DMB. Aldolase indicates values detected in samples incubated with sialic acid aldolase for 1 hr at 37°C.

4.4.3. Mass spectrometry of *T. castaneum* N-glycans

In an attempt to gain more exhaustive insight into the composition of N-glycans in *Tribolium* we employed MS. For that purpose N-glycans were released from purified beetle proteins using peptide N-glycosidase F (PNGase F) and analyzed using a MALDI-TOF mass spectrometer. However, this approach suffered from rather low detection sensitivity (Fig. 4.2.). In the larval guts only one N-glycan structure was detected, while in both adult samples five structures were found. The best N-glycan profile was obtained for whole larvae, but still only ten unique N-glycans were identified, compared to more than 20 different N-glycans routinely found in other insects (Aoki et al., 2007; Dojima et al., 2009; Kim et al., 2009; Kurz et al., 2015).

Mostly high mannose (5-9 mannoses) and paucimannose (<5 mannoses) N-glycans were detected along with a few fucosylated structures. Complex N-glycans, including sialylated ones, could not be detected. In the used method the sample crystallization on the MALDI plate is crucial for the subsequent MS analysis. In the case of the *Tribolium* samples crystals formed only after 10-fold or higher dilution which indicated that purity of the samples was not high enough. Consequently, in the diluted samples the less prevalent structures could not be identified. Still, the partial N-glycan profiles obtained with this method give some indication on the life stage-specific differences. In both larval guts and whole larvae $Man_9GlcNAc_2$ was by far the most abundant glycan. In whole adults $Man_9GlcNAc_2$ was detected at approximately same levels as $Man_5GlcNAc_2$ and both were lower than

$Man_2GlcNAc_2Fuc$. In adult heads $Man_9GlcNAc_2$ was the minor glycan and the highest MS peak came from $Man_3GlcNAc_2$.

To address the above problems another approach was used. The protein extracts were digested with trypsin and subsequently N-glycans were released from the glycopeptides using peptide N-glycosidase A (PNGase A). In contrast to PNGase F, PNGase A acts only on peptides but can also cleave the glycans modified with core α1,3-fucose.

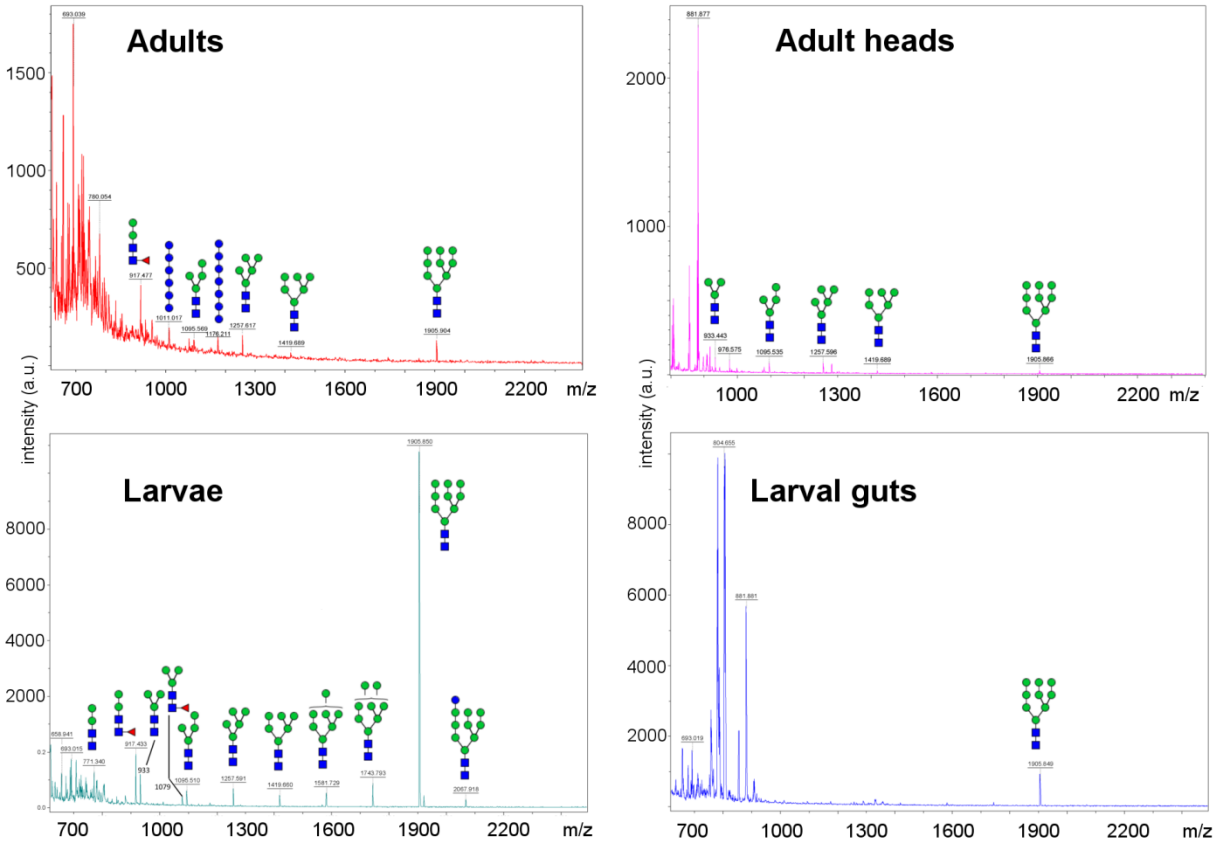


Fig. 4.2. Mass spectra obtained after MALDI-TOF analysis of *Tribolium castaneum* glycans. Glycans were released from the protein extracts using PNGase F. Prominent peaks at low m/z range indicate presence of contaminations in the samples.

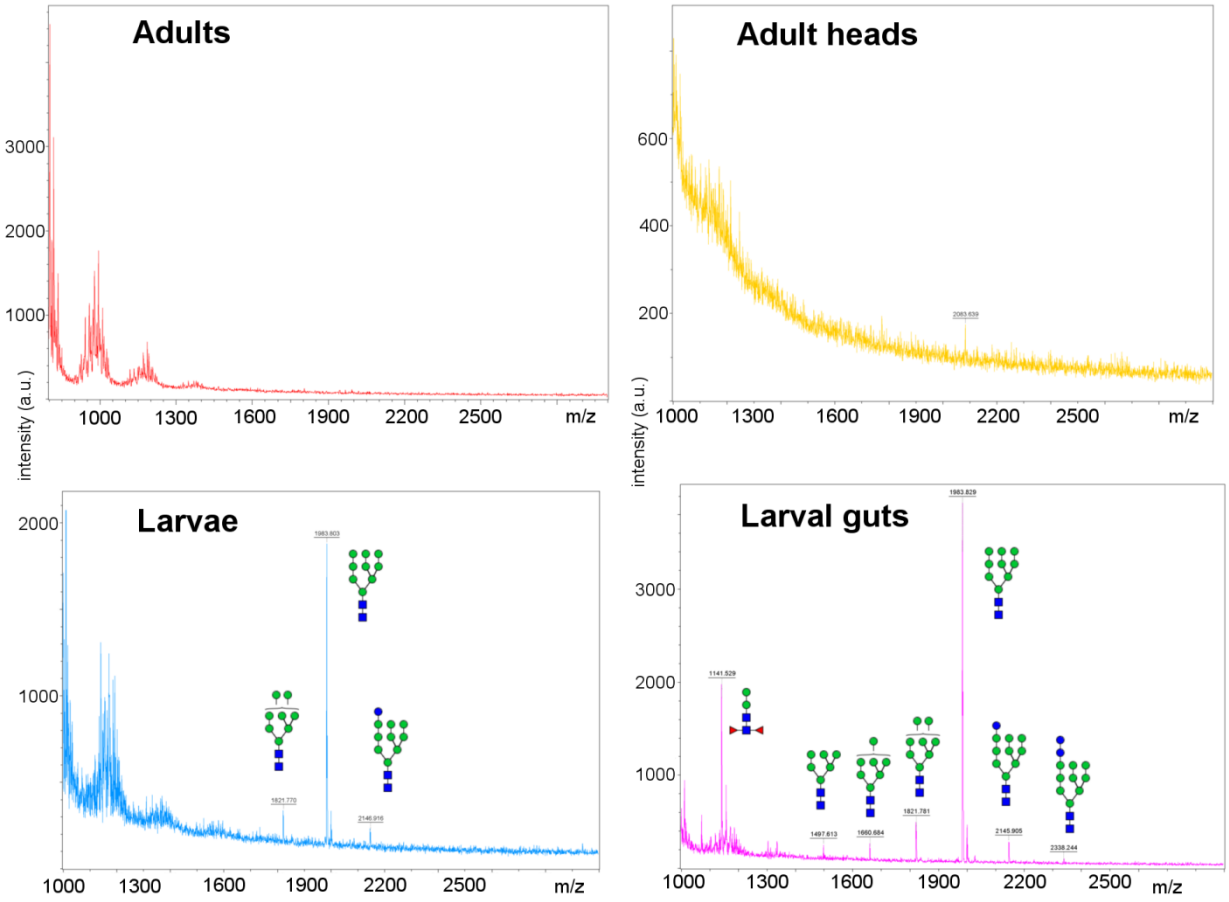


Fig. 4.3. Mass spectra obtained after MALDI-TOF analysis of *Tribolium castaneum* PA-labeled glycans. Glycans were released from the tryptic peptides using PNGase A. The approach was not successful for adult samples.

Therefore, PNGase A was used to expand the coverage of the identified glycans. Released glycans were labeled with 2-aminopyridine to increase the ionization efficiency of the N-glycans and thus to increase the detection sensitivity.

Nevertheless, these efforts were successful only for the sample from larval guts (Fig. 4.3. and Table 4.3.). In line with previous results, the $Man_9GlcNAc_2$ glycan was by far the most abundant in this sample. Interestingly, the difucosylated glycan $Man_2GlcNAc_2Fuc_2$ was also present in high amounts confirming that PNGase A should be used to fully characterize the beetle N-glycan profile. Also in this approach crystallization of the samples was problematic, but this time it was most likely caused by the glycerol which was present in the PNGase A enzyme solution and could not be efficiently removed in the glycan clean-up.

Table 4.3. Summary of the N-glycans identified in native and PA-labeled glycan samples.

Glycan	m/z $[M+Na]^+$		Adults		Adult heads		Larvae		Larval guts	
	Native	PA	Native	PA	Native	PA	Native	PA	Native	PA
M2N2	771.3	849.3	-	-	-	-	++	-	-	-
M2N2F	917.3	995.4	+++	-	-	-	++	-	-	-
M2N2F2	1063.4	1141.4	x	-	x	-	x	-	x	++
M3N2	933.3	1011.4	-	-	+++	-	++	-	-	-
M3N2F	1079.4	1157.4	-	-	-	-	++	-	-	-
M4N2	1095.4	1173.4	++	-	++	-	++	-	-	-
M5N2	1257.4	1335.5	++	-	++	-	++	-	-	-
M6N2	1419.5	1497.5	+	-	+	-	++	-	-	++
M7N2	1581.5	1659.6	-	-	-	-	++	-	-	++
M8N2	1743.6	1821.6	-	-	-	-	++	++	-	++
M9N2	1905.6	1983.7	++	-	+	-	+++	+++	+++	+++
GlcM9N2	2067.7	2145.7	-	-	-	-	+	++	-	++
Glc2M9N2	2229.7	2307.8	-	-	-	-	-	-	-	+

Glycan composition: M – mannose, N – N-acetylglucosamine, F – fucose, Glc – glucose. m/z $[M+Na]+$ indicate theoretical mass to charge ratios of sodiated glycan ions. Native – unlabeled glycans, PA – glycans labeled with 2-aminopyridine. Tolerance of 0.5 m/z was used for assignment of detected ion masses. "-" - indicate that given ion was not detected. "+++" – highest peak in the spectra, "++" – prominent peaks, "+" – low abundant peaks. x – difucosylated glycan M2N2F2 could not be detected in the native samples because they were prepared using PNGase F which does not cleave N-glycans containing α1,3-fucose.

4.4.4. HPLC-analysis of PA-labeled glycans

PA-labeled N-glycans can also be subjected to HPLC analysis, which can give information on the N-glycan profiles. Additionally, separated N-glycan fractions can be collected free of contaminants and subjected to MS. For that, the PA-labeled glycans were subjected to HPLC on PALPAK N Amide column. Peak assignments were based on calibration run with a mixture of isomaltose oligosaccharides (3-15 glucose units) and the elution positions of PA-labeled N-glycan standards.

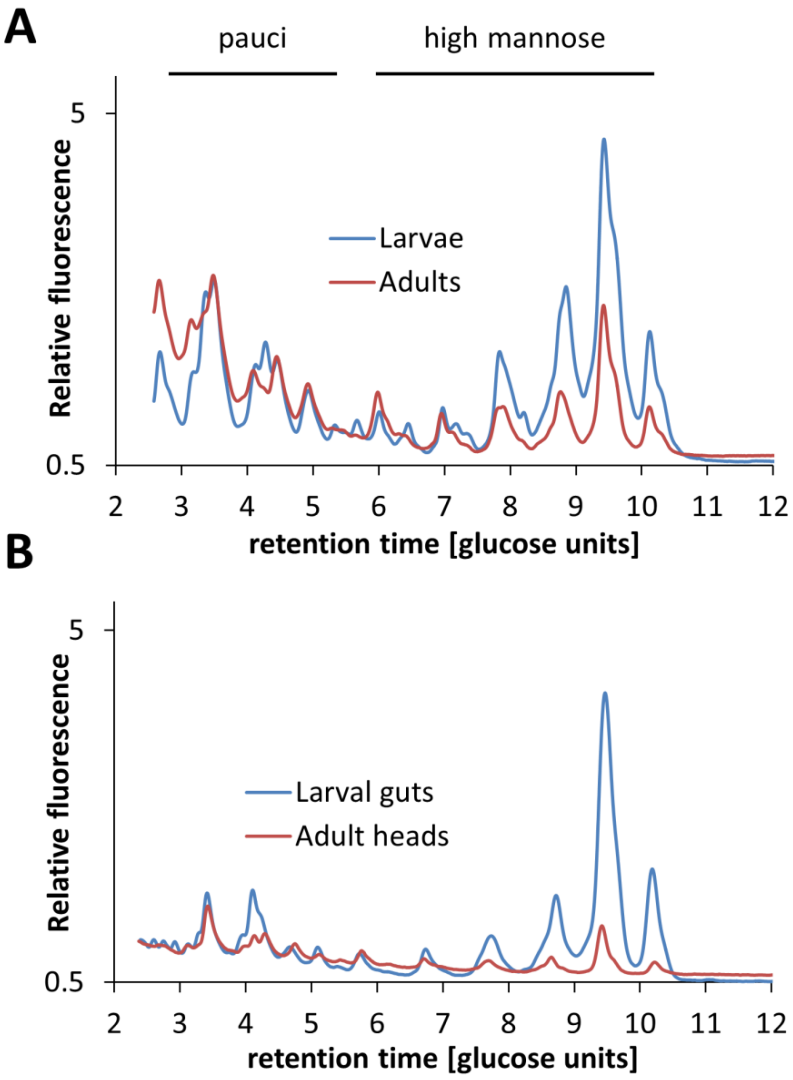


Fig. 4.4. Profiles of PA-labeled glycans separated by HPLC using a Palpak N column. (A) Overlaid chromatograms of whole larval and adult N-glycans. (B) Overlaid chromatograms of N-glycans from larval guts and adult heads. Relative fluorescence is shown in a logarithmic scale.

In this column small paucimannose glycans (containing 2-3 GlcNAc residues, 2-4 mannoses and up to two fucose residues) eluted from the column first while high mannose glycans had longer retention times. Particular high mannose N-glycans could easily be distincted, while for paucimannose glycans a detailed composition can be clearly determined only with the aid of MS (Fig. 4.4., Table 4.4.). Additionally, separation on a Palpak N column allowed assignment of core fucose linkages (α1,3 or α1,6) as α1,3-fucosylated glycans have longer retention times than isomers with α1,6-fucose (Tomiya et al., 1991). Several other peaks that could not be clearly assigned were also observed in some of the chromatograms, potentially coming from minor complex-glycans. But since they were not present in all the samples and did not produce satisfactory signal on MS they were not taken into account for the

quantification of the relative N-glycan content. Analysis of relative contribution of each peak in the total glycan profile confirmed life stage specific differences suggested by the previous approaches.

Table 4.4. Relative amounts of the PA-N-glycans identified by HPLC on Palpak N column in combination with MALDI-TOF.

	GU	glycan	%Total Profile Adults	Adult heads	Larvae	Larval guts
Paucimannose	**3.1**	?	5.7%	1.2%	2.3%	6.5%
	3.4	?	4.0%	-	1.1%	-
	3.6	**M3/M2F^6**	19.0%	23.9%	4.1%	8.5%
	4.1	**M3/M2F^3**	3.6%	7.2%	1.7%	-
	4.4	**M2F2**	5.1%	8.8%	2.6%	1.1%
	4.8	**M3F^6**	3.8%	5.8%	1.8%	1.0%
	5.1	**M3F^3**	0.5%	2.0%	0.3%	-
High mannose	**5.7**	**M5**	5.0%	5.0%	1.0%	1.3%
	6.7	**M6**	3.8%	5.1%	1.4%	2.1%
	7.8	**M7**	7.0%	6.5%	7.0%	4.3%
	8.8	**M8**	10.4%	8.3%	17.0%	9.3%
	9.5	**M9**	26.5%	21.7%	51.1%	55.5%
	10.3	**GlcM9**	5.5%	4.5%	8.5%	10.3%
	Total Paucimannose		41.8%	48.9%	14.0%	17.1%
	High mannose		58.2%	51.1%	86.0%	82.9%

GU – retention time in glucose units. Paucimannose glycans assignments were based on PALPAK N elution coordinates and MALDI-TOF-MS of fractioned N-glycans. M3 was not detected by MS in any of the fractions although it was a prominent glycan in MALDI-TOF spectra of larval and adult N-glycans samples (Fig. 4.2., Table 4.3.). Superscript 3 and 6 digits indicate fucose linkage assignments (α1,3 or α1,6). Glycan content shown was calculated as peak area relative to summarized area of the all identified peaks. ? – uncertain paucimannose N-glycans.

$Man_9GlcNAc_2$ contributed to over half of all the glycans in larvae and larval gut (51.1% and 55.5%, respectively) while in adults and adult heads this glycan constituted 26.5% and 21.7% of the identified profile, respectively. In general the larval samples were enriched in less processed glycans carrying five to nine mannoses. These structures accounted together for over 80% of the total profile, compared to 50-60% in the adult samples.

Only a few paucimannose glycans could be verified using MALDI-TOF on separated HPLC fractions. Those undoubtedly identified were $Man_2GlcNAc_2Fuc$ (995.4 m/z), $Man_2GlcNAc_2Fuc_2$ (1141.4 m/z) and $Man_3GlcNAc_2Fuc$ (1157.4 m/z). Notably, in none of the fractions $Man_3GlcNAc_2$ (1011.4 m/z)

was detected although it was one of the major peaks in the total N-glycan spectra of adult heads and larvae. Clearly, short and fucosylated glycans were much more abundant in both adult samples compared to larval samples. The relative content of the paucimannose glycans was the highest in adult heads in which it reached nearly 50% of all glycans. Prevalence of the paucimannose N-glycans was the lowest in the whole larvae where it accounted for up to 14% of the total glycan profile. Nonetheless, the full characterization of the N-glycomes and comparison between different life stages requires more detailed and confident identification of all glycans using MS.

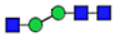
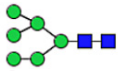
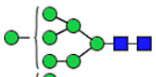
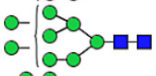
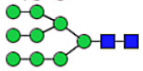
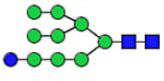
4.4.5. Further optimization of N-glycan analysis by mass spectrometry

Based on the observed drawback of previous approaches a number of improvements have been tested in order to optimize the sample preparation for successful MS analysis of N-glycans. To reduce the number of variables testing of the best setup was done on a sample from adult beetles only. To address the problem of insufficient detection sensitivity the starting amount of sample was increased from 500 µg to 4 mg of tryptic peptides. Additionally, for a higher sample purity tryptic digestion was performed on filters which allowed to get rid of high molecular weight contaminants, after which glycopeptides were purified using solid phase extraction. The glycans were released using glycerol-free N-glycanase. Moreover, two derivatization strategies to increase the detection sensitivity were compared: permethylation and 2-aminobenzamide (2-AB) labeling. Permethylation (perMe) can enhance the detection of sialylated N-glycans, while 2-AB labeling is more compatible with most of standard RP-LC-MS systems. perMe glycans were analysed using MALDI-TOF, while 2-AB labeled glycans were analyzed by LC-MS and by MALDI-TOF.

Clearly, the protocol improvement yielded a more successful N-glycan identification. Using MALDI of perME glycans 16 distinct structures were found including several complex glycans, but no sialylated N-glycans could be detected (Table 4.5.). 2-AB labeling gave even better results, as in LC-MS and MALDI approaches a set of 19 and 18 unique N-glycan structures could be detected, respectively.

However, in the LC-MS run only trace amounts of high mannose N-glycans were detected, which is in contrast to previously obtained results indicating high abundance of these structures. MALDI of 2-AB labeled glycans yielded both a sufficiently high number of detected N-glycans and more reliable quantitative data (compare Tables 4.4. and 4.5.) consequently, this method was used for the final experiment aimed to compare larval and adult N-glycomes.

Table 4.5. Comparison of N-glycans identified by three different analysis approaches.

native m/z [M+Na$^+$]	Glycan content (% total profile) MALDI perMe	LC-MS 2-AB-labeled	MALDI 2-AB-labeled	Deduced structure	Composition
771.3	9.4%	12.9%	6.5%		M2N2
917.3	27.4%	35.8%	21.7%		M2N2F
933.3	6.5%	11.2%	5.2%		M3N2
974.3	nd	0.2%	nd		NM2N2
1079.4	12.6%	15.1%	9.1%		M3N2F
1095.4	5.3%	9.7%	3.6%		M4N2
1136.4	1.8%	1.6%	1.1%		NM3N2
1241.4	0.9%	0.1%	0.7%		M4N2F
1257.4	8.9%	8.0%	5.8%		M5N2
1282.5	3.4%	1.8%	2.1%		NM3N2F
1298.4	nd	0.2%	0.2%		HM3N2
1339.5	0.5%	0.1%	nd		N2M3N2
1403.5	nd	0.2%	0.5%		M5N2F
1419.5	4.9%	2.0%	3.8%		M6N2
1460.5	nd	0.1%	0.3%		NM5N2
1485.5	1.1%	0.2%	0.5%		M3FN2
1581.5	3.8%	0.5%	4.0%		M7N2
1743.6	3.6%	0.1%	5.7%		M8N2
1905.6	9.0%	0.1%	25.0%		M9N2
2067.7	1.5%	nd	4.1%		GlcM9N2
Total identified glycans					
	16	19	18		

Glycan composition: M – mannose, N – N-acetylglucosamine, F – fucose, Glc – glucose, H – undetermined hexose (galactose or mannose). [M+Na]+ indicate mass to charge ratios of sodiated native glycan ions.

4.4.6. Detailed comparison of *Tribolium* larval and adult N-glycomes using MALDI-TOF

Since the improved protocol requires much larger sample amounts it was not feasible to analyze the samples from dissected larval guts and adult heads. However, an in depth statistical comparison of the N-glycan profiles in larvae and adults was possible. For that comparison glycans were isolated from 4th instar (two-week-old) larvae and mature (seven-day-old) beetles. Both larval and adult N-glycomes consisted mainly of high- and paucimannose N-glycans with only a minor fraction of complex glycans (Table. 4.7. and Fig. 4.5.).

In both life stages we could identify over 25 different N-glycans. For the statistical analyses only those glycans detected at average levels above 0.1% of the total amount of N-glycans were selected. In line with the previous indications the composition of the adult N-glycome was shifted towards more trimmed N-glycans. Larval samples were dominated by $Man_9GlcNAc_2$ N-glycan (40.7±7.4% of total profile) and the second most abundant N-glycan was $Man_2GlcNAc_2Fuc$ (11.5±3.1%), while in adults both of these glycans were detected at similar levels (22.0±6.0% and 21.9±5.8% of all N-glycans, respectively). In larvae each of the high mannose structures ($Man_{5-9}GlcNAc_2$) was up to 2 times more abundant compared to adults. In total, almost 2/3 of the larval glycans were of the high mannose type while these glycans constituted less than 40% of the adult sample (P=0.003, Table 4.6.).

Consequently, all types of paucimannose glycans were upregulated in adults compared to larvae. Among them, oligosaccharides carrying a single fucose residue were more abundant in mature adults and accounted for 33.1±6.4% of the total N-glycan pool compared to 17.2±4.0% in larvae (P=0.005). Also non-fucosylated paucimannose glycans were approximately 50% more abundant in adults (P=0.010).

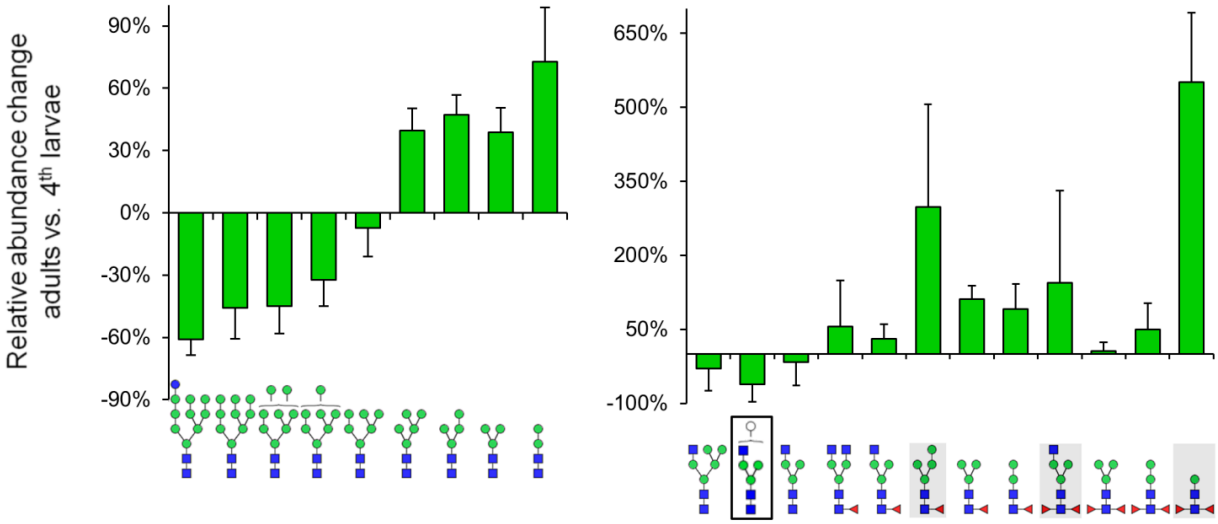


Fig. 4.5. Relative changes of N-glycan abundance in adults compared to 4th instar larvae. Proteins were extracted from whole 4th instar larvae or mature adults. N-glycans were released using PNGaseA, labeled with 2-AB and analyzed using MALDI-MS. Left: mannose series N-glycans, right: insect complex, hybrid and fucosylated N-glycans. Error bars are SD (n=4). Glycan in black outline was below 0.1% of the total pool in adults. Glycans in grey boxes were not detected in 2 or 3 of the 4 samples from larvae.

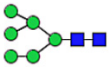
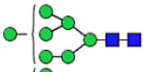
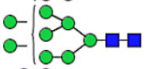
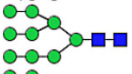
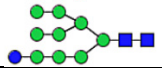
Table 4.6. Comparison of relative contents of different N-glycan groups.

	% total profile (±SD)		
	Larvae	**Adults**	**p-value**
High mannose (M9-M5)	63.3 ± 6.7	37.8 ± 8.3	0.003
Paucimannose (M4-M1)			
non-fucosylated	10.7 ± 1.8	15.3 ± 1.7	0.010
monofucosylated	17.2 ± 4.0	33.1 ± 6.4	0.005
difucosylated	8.8 ± 1.9	13.8 ± 3.3	0.025
Complex	2.9 ± 0.6	3.1 ± 1.2	0.363

Values are means ± standard deviation (n=4). p-values were calculated using independent sample t-test (SPSS 22).

Interestingly both life stages contained high levels of difucosylated N-glycans. Taken together difucosylated N-glycans were significantly more abundant in adults (8.8±1.9% vs. 13.8±3.3%, P=0.025). In this group $Man_2GlcNAc_2Fuc_2$ was consistently detected at levels near 1% of the total N-adult glycans but in larvae it was identified in only one out four biological replications.

Table 4.7. Comparison of the N-glycan profiles of *Tribolium* larvae and adults.

m/z [M+Na$^+$]	Glycan content (% of total) Larva	Adult	p-value	Deduced structure	Composition
891.3	3.5±0.7	6.1±0.9	0.004		M2N2
1021.4	0.2±0.4	1.2±0.2	0.005		MN2F2
1037.4	11.5±3.1	21.9±5.8	0.013		M2N2F
1053.3	3.4±0.7	4.7±0.4	0.015		M3N2
1183.5	7.3±1.3	11±3.8	0.063		M2N2F2
1199.4	4.0±0.8	8.4±1.1	0.001		M3N2F
1215.5	2.4±0.5	3.5±0.2	0.010		M4N2
1256.5	0.8±0.3	0.7±0.4	0.297		NM3N2
1345.5	1.2±0.4	1.2±0.2	0.391		M3N2F2
1361.5	0.2±0.2	0.7±0.3	0.029		M4N2F
1377.5	3.3±0.4	4.5±0.3	0.003		M5N2
1402.5	1.0±0.3	1.4±0.3	0.105		NM3N2F
1418.4	0.3±0.1	0.1±0.1	0.016		HM3N2
1523.5	0.3±0.3	0.4±0.3	0.295		M5N2F
1539.5	2.9±0.3	2.6±0.4	0.231		M6N2
1548.5	0.2±0.2	0.4±0.3	0.139		NM3N2F2
1580.5	0.3±0.1	0.2±0.1	0.161		NM5N2
1605.5	0.2±0.1	0.3±0.2	0.160		M3FN2
1701.6	3.9±0.4	2.6±0.5	0.005		M7N2
1863.6	6.9±0.7	3.8±0.9	0.002		M8N2
2025.7	40.7±7.4	22.0±6.0	0.008		M9N2
2187.7	5.8±1.4	2.2±0.4	0.004		GlcM9N2

Glycan composition: M – mannose, N – N-acetylglucosamine, F – fucose, Glc – glucose, H – hexose (galactose or mannose). [M+Na]+ indicate measured masses of sodiated 2-AB glycan ions. Values are means ± standard deviation (n=4). p-values were calculated using independent sample t-test.

4.5. Discussion

One of the main objectives of this thesis is a study of the functions of the N-glycosylation in the red flour beetle, *Tribolium castaneum.* Investigation of the role(s) of the N-glycosylation requires detailed knowledge on composition of the N-glycans present on *T. castaneum* proteins. Therefore, we used range of analytical techniques to approach this research problem.

Initially, we studied the monosaccharide composition of *Tribolium* protein-linked glycans. These experiments suggested tissue and life stage specific differences in the glycan abundance. Notably, higher levels of mannose were detected in larvae compared to adults. In all samples, but especially in whole larvae and larval guts, high amounts of GalNAc and galactose were found. This confirms a wealth of glycan epitopes that can be recognized by RSA, SSA and SNA-II (GalNAc/Gal specific lectins) and is in line with the observation of lectin binding to insect cells through confocal microscopy (presented in Chapters 2 and 3). In adult samples there was a higher abundance of GalNAc compared to galactose, while in larval samples it was the opposite. This indicates that also O-glycan synthesis might undergo reorganization during *Tribolium* development.

Studies on other species indicate that sialylated glycans are either absent or very low abundant in insects (Kajiura et al., 2015; Aoki et al., 2007; Koles et al., 2007; Roth and Kempf, 1992). In line with those studies, specific labeling followed by HPLC showed that minute amounts of sialic acids might be present in *Tribolium* samples. However, due to technical difficulties it could not be univocally verified whether the detected compounds are truly Neu5Ac and NeuGc.

The first experiments utilizing MALDI-MS for the identification of either native or 2-aminopirydine (PA) labeled N-glycans did not allow a full characterization of *Tribolium* glycosylation profiles. The unsuccessful experiments indicated the utmost importance of sample purification and optimal derivatization for effective N-glycan detection. The HPLC methodology is much less sensitive to contaminations and it was used to compare the global N-glycan profiles between different tissues and life stages. This approach clearly indicated that both whole adults and adult heads are enriched in smaller, more processed, paucimannose glycans. In contrast, whole larvae and larval gut samples were dominated by high mannose N-glycans, which together with the monosaccharide analysis gave evidence that metamorphosis might be associated with a shift in the protein glycosylation. Assignments of HPLC peaks based on their elution times required further confirmation by other methods, e.g. MS. Yet, this was successful for only a handful of N-glycans and further advocated for the need of optimization of sample preparation before MS.

According to some reports the overall yield of PA-labeling of N-glycans might be below 20% (Hase et al., 1981). Thus increased ionization efficiency and higher sample purity achieved by this approach might be counteracted by insufficient yield. Additionally, this procedure requires removal of the excess dye that can lead to loss of short oligosaccharides. Thus the sample processed in this way may be not fully representative (Lamari et al., 2003).

To improve the detection we tested another two derivatization strategies, in particular permethylation and 2-aminobenzamide labeling. The first approach is especially suited for MS of samples containing sialylated glycans. Without derivatization sialic acids are negatively charged which results in a poor detection limit compared to neutral glycans. Additionally, sialic acid groups can be lost during ionization and in the detector thus making identification of low amounts of sialylated N-glycans almost impossible. Those issues can be addressed by permethylation which inhibits negative ion formation and stabilizes the sialic acid residues (Wheeler et al., 2009). Using this method we achieved an improved detection compared to native or PA-labeled N-glycans. Still, we found no masses consistent with sialylated N-glycans in line with previous suggestions of very low abundance of sialylation in insects (Aoki et al., 2007).

Out of the tested approaches, the protocol including on-filter protein trypsinization, release of N-glycans using PNGase A, 2-AB labeling and MALDI-TOF analysis gave the best results in terms of number of identified N-glycans and consistency of glycan quantitation. Using this approach we could confirm that there are statistically significant differences in the composition of the N-glycomes of larvae and adult beetles. In addition to the reduced high mannose N-glycans levels, adult beetle N-glycome exhibited increase in core N-glycan fucosylation.

In a study of fruit fly larval N-glycans Roberts et al. (1998) mainly found high mannose structures ($Man_{5-9}GlcNAc_2$) which were also highly abundant in *Tribolium* larvae. Yet, fucosylated structures represented less than 5% of all *Drosophila* larval N-glycans. That is a markedly lower content compared to beetle larvae in which up to 30% of all N-glycans carried at least one core fucose.

In silkworm, *Bombyx mori* (Lepidoptera) larvae $Man_5GlcNAc_2$ was the most abundant glycan (Kajiura et al. 2015), while $Man_9GlcNAc_2$, the most prevalent glycan in *Tribolium* larvae, made up for less than 10% of all the N-glycans in silkworm. Similar to *Tribolium* larvae *Bombyx* larvae had a high content of fucosylated N-glycans (over 20%) but also contained much higher share of complex N-glycans (nearly 20% compared to approx. 3% in *T. castaneum*). Spectra of N-glycans from larvae of two mosquitoes *Anopheles gambiae* and *Aedes aegyptii* were dominated by highly processed fucosylated

N-glycan $Man_2GlcNAc_2Fuc$, while high mannose N-glycans were much less abundant (Kurz et al., 2015). That is in contrast to beetle larvae and more similar to beetle adults.

Several studies on *Drosophila* adult N-glycan profiles showed that 20-40% of the N-glycans are of the high mannose type, 60-80% of the paucimannose type and 1-5% are of the complex type (Fabini et al., 2001; Rendic et al., 2007; Sarkar et al., 2006). These investigations also showed a much higher abundance of fucosylation (40-50%) compared to values found in the study of larval N-glycans. These values are similar to those found for *Tribolium* adults and although there is no study directly comparing larval and adult N-glycans in *Drosophila* it appears likely that an increase of fucosylation and a decrease in content of high mannose N-glycans are associated with development from larva to adult in both of these species. Changes in the total N-glycan profile between post-embryonic life stages were shown in the nematode *Caenorhabiditis elegans*. In this organism the life stages differed mainly in the number and abundance of complex and phosphocholine modified N-glycans (Cipollo et al., 2005) suggesting that glycosylation dynamics during development may be a evolutionary widespread phenomenon.

The most striking difference between *Drosophila* and *Tribolium* was the content of difucosylated glycans. In the fruit fly difucosylated glycans represented below 1% of the total N-glycan profile. In contrast, levels of these glycans in beetles were markedly higher, that is up to 16%. Intriguingly, difucosylated glycans in *Drosophila* are found mainly in the central nervous system or male reproductive organs (Rendić et al., 2006), thus much higher levels of these glycans in beetles suggest their broader distribution and potentially divergent functions.

The most elaborated structure in the *Tribolium* glycome was $GlcNAc_2Man_3GlcNAc_2Fuc$ and no sialylated glycans were detected in any of the life stages. This might be due to the activity of fdl (β-N-acetylglucosaminidase) which redirects the pathway towards synthesis of paucimannose glycans and reduces the content of complex glycans (Leonard et al., 2006). Another reason might be low expression level of the sialyltransferase gene and other genes involved in production of complex glycans. This hypothesis will be tested in Chapter 5.

The method we finally applied was optimal for quantitative analysis of N-glycans but it does not allow studying the entire diversity of N-glycan structures which might be present in *T. castaneum*. New developments of the technology revealed that insect glycomes might comprise of nearly 90 distinct N-glycan structures. The majority of those is present in trace amounts and only about 20-30% of all glycans is present at significant levels. Recently, Kurz et al. 2015 were able to extensively

characterize mosquito and fruit fly N-glycans owing to the use of huge sample amounts (2 grams) and a combination of analytic techniques such as ion-exchange chromatography, PA-labeling, normal-phase chromatography, MALDI-MS, exoglycosidase treatments, permethylation and nanospray ionisation-MS. This elaborate approach enabled the detection of low-abundant glycans but did not allow for precise quantitative analysis.

It is still an open question what is the biochemical basis of the observed differences in N-glycosylation profile. A first possibility could be that glycoproteins expressed during metamorphosis and in adults could have their specific N-glycosylation patterns different from those expressed in larval stages. In this scenario the global N-glycan composition change would be determined mostly by protein structural features and would not require increased expression of N-glycan processing genes. Such an influence of protein structure on the final form of the N-glycan has been demonstrated for single insect proteins (Kanie et al., 2009, Hang et al., 2015). The second option is that the increased expression of the N-glycan processing genes drives the N-glycosylation shift of the proteins expressed throughout all life stages and allows them to mediate metamorphosis specific functions. Previous analyses of adult *T. castaneum* glycoproteins revealed that Fasciclins, Laminins, Catalases and V-type ATPases are N-glycosylated (Vandenborre et al., 2011a). Interestingly, RNAi of these proteins in beetle larvae or pupae prevented metamorphosis or caused defects in appendage development, which would support the second hypothesis (Mummery-Widmer et al., 2009). The biochemical basis of the observed shift in N-glycan composition and its importance for the physiology of the *Tribolium castaneum* metamorphosis will be the main subjects of the following Chapter 5.

The marked discrepancies between different insect species and orders indicate that generalization on the N-glycan structures and functions even among insects might be misleading. Separate studies on the N-glycosylation in particular insect pests appears to be crucial in order to fully exploit the potential of glycobiology in crop protection.

Chapter 5

Functional analysis of genes involved in N-glycosylation in *Tribolium castaneum*

Results shown in this chapter are part of manuscript in preparation:

Walski, T., Van Damme, E. J. M., Smargiasso, N., Christiaens, O., De Pauw, E., & Smagghe, G. Differential N-glycan processing is required for successful metamorphosis in the pest beetle *Tribolium castaneum.*

5.1. Abstract

In holometabolous insects the transition from immature larvae to adult requires a complete body reorganization and relies on N-glycosylated proteins. N-glycosylation is an important posttranslational modification that influences the biological activity of proteins. We previously found that the global N-glycan composition is shifted towards more processed forms in the adult stage compared to larvae but the role of this process in the metamorphosis has not been studied yet. Here we used the red flour beetle, *Tribolium castaneum*, to perform a first comprehensive study of the involvement of the entire protein N-glycosylation pathway in metamorphosis. We found that the enrichment of trimmed and modified glycans that coincides with transition from larvae to adults is driven by developmentally regulated expression of genes encoding N-glycan processing enzymes. Additionally, RNAi of the genes involved in the N-glycan attachment resulted in larval mortality while interruption of the N-glycan processing disturbed pupation, adult eclosion and appendage formation. Interestingly, silencing of a single gene coding for α-mannosidase Ia, was sufficient to affect the entire N-glycan processing pathway. Our findings revealed that next to hormonal control insect metamorphosis depends on changes in protein N-glycosylation. Consequently, disruption of this process could be an effective approach for insect control.

5.2. Introduction

N-glycosylation or the attachment of an oligosaccharide to asparagine (N) is one of the most important post-translational modifications (PTM) of proteins. This process begins in the endoplasmic reticulum (ER) with the transfer of a glycan precursor from a lipid carrier to a N-X-T/S/C consensus sequence (X is any amino acid except proline) in a newly translated protein (Stanley et al., 2009). Following the oligosaccharide attachment which is catalyzed by the oligosaccharyltransferase (OST), the nascent N-glycoprotein is transported through the ER to the Golgi apparatus. In those organelles the N-glycan moiety is processed by multiple glycosyl hydrolases and transferases (Fig. 5.1.). The early stage of N-glycan processing involves the sequential action of α-glucosidases that remove three glucose residues capping the immature N-glycan to produce the $Man_9GlcNAc_2$ N-glycan. This stage is conserved among eukaryotes and is crucial for the control of protein folding, quality and ER-associated degradation (Helenius and Aebi, 2004). N-linked glycans can be further processed by class I and II α-mannosidases, N-acetylglucosaminyltransferases, N-acetylglucosaminidases and fucosyltransferases to form an array of over 20 different high mannose and paucimannose N-glycans. In vertebrates glycans can be extended with additional galactose, GalNAc and sialic acid residues to form diverse complex N-glycans, but in insects and other invertebrates these glycans are usually found at low levels (Gutternigg et al., 2004; Katoh et al., 2013; Kurz et al., 2015).

N-glycosylation is crucial for protein folding, transport and secretion (Helenius and Aebi, 2004) but may also affect protein activity (Sekine et al., 2013). Studies in *Drosophila melanogaster* have shown that N-glycosylated proteins are involved in receptor functions, transport or cell adhesion and play important roles in immunity and in the central nervous system (Koles et al., 2007; Mortimer et al., 2012; Sarkar et al., 2010). Notably, multiple proteins implicated in insect metamorphosis have recently been shown to be N-glycosylated (Gui et al., 2006; Vandenborre et al., 2011a; Zhang et al., 2014a; Zielinska et al., 2012). However, no research has addressed the intriguing question of a potential role for protein N-glycosylation in the development from juvenile to adult life forms.

The life cycle of holometabolous insects such as beetles includes metamorphosis or transition between morphologically distinct larval, pupal and mature adult stages. The induction of this process is controlled by the levels of juvenile and ecdysteroid hormones but the understanding of the mechanisms behind insect metamorphosis is far from complete (Jindra et al., 2013; Truman and Riddiford, 2002). Especially little is known with regard to the involvement of protein post-translational modifications such as N-glycosylation in the execution of the metamorphosis. *Tribolium casataneum*,

the red flour beetle appears to be an outstanding model to study these aspects thanks to a completely sequenced genome and systemically working RNA interference (RNAi). Through injection of mixture of dsRNAs it is possible to silence up to three genes simultaneously, which allows to uncover phenotypes masked in single knock-downs by functionally redundant genes. Additionally, differences in *Tribolium* development including the formation of wing covers (elytra), which are not present in the fruit fly, enable investigation of novel gene functions (Linz and Tomoyasu, 2015). Importantly, *T. castaneum* is a pest, thus deeper insight into the biochemistry of its metamorphosis may bring relevant ideas for novel insect control strategies replacing the currently used synthetic insecticides that may pose a risk to human health or environment (Tunaz and Uygun, 2004; Wilson, 2004).

In this study we address the question of the involvement of protein N-glycosylation in post-embryonic development and metamorphosis in *Tribolium castaneum*. For that purpose we studied the expression of genes coding for all steps of the N-glycosylation pathway from glycan attachment to the final maturation steps producing paucimannose and complex oligosaccharides. Additionally, we looked into the role(s) of these genes in metamorphosis using RNAi-mediated gene knockdown. Here we show, for the first time, that the observed changes in N-glycome composition were driven by developmentally regulated expression of genes coding for N-glycan processing enzymes. Furthermore, the arrest of N-glycan attachment blocked larval growth while disruption of N-glycan processing revealed novel N-glycosylation roles in pupa and adult development, particularly in elytra formation. Based on our data, we postulate that enhanced N-linked glycan processing is crucial for successful metamorphosis in insects.

5.3. Materials and methods

5.3.1. Phylogenetic analyses

Tribolium orthologs (from Tcas 4.0 genome assembly) putatively involved in *N*-glycosylation were identified using *Tribolium* BLAST tool (http://bioinf.uni-greifswald.de/blast/tcas/blast.php) with *Drosophila melanogaster* sequences used as queries. Gene functions and specificities were inferred through phylogenetic relationships with known fruit fly and human sequences using MEGA 5.2 and Maximum Likelihood method based on the Poisson correction model. The bootstrap consensus trees were inferred from 1000 replicates (Tamura et al., 2011).

5.3.2. Beetle cultures

For most experiments the red flour beetle, *T. castaneum* GA-1 strain was used, except for the assessment of RNAi effects on wing discs, in which case the enhancer trap line *pu11* was used. The *pu11* line expresses enhanced yellow fluorescent protein in eyes, neurons as well as in hindwing and elytron discs enabling to track their development. All beetles were cultured at 30°C and 60% humidity in darkness. Whole wheat flour containing 5% brewer's yeast was used as a diet.

5.3.3. Gene expression analysis

For gene expression approx. 20 mg of insects at 2nd, 4th and 6th (last) larval instar, prepupal, early pupal (0-48h), late pupal (72-120 h), pharate adult and mature adult stages were homogenized in Trizol and stored at -80°C. RNA was isolated using the Qiagen RNeasy mini kit with on column DNase digestion. cDNA was synthesized with SuperScript™ II Reverse Transcriptase (Invitrogen) using 1 µg of total RNA. qRT-PCR was performed using GoTaq® qPCR Master Mix (Promega) and Bio-Rad CFX Connect robot, cycles included 15s denaturation at 95°C and 30s annealing/extension at 60°C. Melt curve analysis was included at the end of the run. *TcRpS6* and *TcRpL32* encoding ribosomal proteins were used as reference genes. Final concentration of each primer was 300 nM. Expression at early larval stage (2nd instar) was used as a baseline expression for further comparisons. The whole experiment was done in three independent biological replications. Quantification of the relative transcript abundance using ΔΔCq method, as well as statistical analyses (ANOVA) were performed in qbase+ software (Biogazelle, Zwijnaarde, Belgium). T-tests were done using SPSS 22. Build-in correction for multiple testing was applied in qbase+. Primers for qPCR were designed using Primer blast tool: http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi (Ye et al., 2012). Detailed information on primer sequences and amplification efficiency can be found in Supplementary Table S5.2.

5.3.4. Larval RNAi

Gene-specific dsRNAs (without 19-23 bp 'off-targets') and primers were designed using E-RNAi tool: http://www.dkfz.de/signaling/e-rnai3// (Horn and Boutros, 2010). Templates were prepared by qPCR using cDNA from 4th instar larvae and specific primers containing T7 promotor sequences at 5' ends according to standard methods (Arakane et al., 2005a; Clark-Hachtel et al., 2013). dsRNA was produced using MEGAscript® RNAi Kit (Life Technologies). Some of dsRNAs were obtained from

Eupheria Biotech (Dresden, Germany). At least two genes were silenced for each step of the N-glycosylation pathway to further exclude the possibility of off-target RNAi effects. A detailed list of primers and dsRNAs can be found in Supplementary Table S5.1. 4th instar larvae weighing 1.2±0.2 mg were anesthetized for 4 min using diethyl ether and injected with 150-250 nL of dsRNA at 1 µg/mL by a capillary needle attached to a FemtoJet® injector (Eppendorf). In the case of double and triple RNAi experiments larvae were injected with equal mixtures of two or three dsRNA solutions at total dsRNA concentration of 1 µg/mL. Approx. 60 larvae were individually injected for each gene. For each gene or set of injections on a given day an equal number of larvae were injected with dsGFP to serve as a control group, except for the experiment with *pu11* line in which dsRNA for *TcST6Gal* was used. Silencing of this gene caused no detectable effects. The knockdown efficiency was assessed six days after injection using qRT-PCR as described for gene expression analysis except that three randomly selected individual larvae were used for RNA isolation.

5.3.5. Analysis of RNAi effects

Following the dsRNA injection larvae were allowed to recover for several hours, weighed on a microbalance and placed individually in test tubes containing whole wheat flour diet. Prior to weighing any dead larvae or those with visible cuticle tanning were considered as mechanically damaged due to injection (usually <10%) and discarded. After 6 days of incubation living larvae were weighed again.

Relative larval growth was calculated for each individual larva as the ratio of net larval weight increase to larval weight at day 0. Larvae were then returned to the incubator and examined every other day for mortality, phenotype abnormalities as well as pupation and adult eclosion timing.

Images were taken using a Leica SD6 stereomicroscope. Extended depth of field pictures were prepared in FiJi (Schindelin et al., 2012) to present whole beetles in full focus. Briefly, 4-6 pictures taken at different focus levels were aligned using 'Linear stack alignment with SIFT' plugin (Lowe, 2004) and finally combined in 'Extended depth of field' plugin (Aguet et al., 2008). The length of pupal elytra was measured, on unprocessed images, as the distance from the joint between T1 tibia and tarsus to the tip of the elytra, to avoid errors caused by elytra curvature. The gap between elytra was measured at 0.5 mm from the elytra tip (see the measurement scheme on Fig. 5.7 A). To image adult wings, elytra and legs beetles were fixed overnight in 95% ethanol and dissected. For walking speed measurements three- to seven-day-old adults were selected to avoid differences caused by age. Individual beetles were placed in the middle of a square cardboard arena using a brush and allowed to

adapt for 30s. Then beetles were placed back in the middle of arena and filmed for at least 30 seconds using a digital camera. The recorded videos were converted to image sequences at one second intervals which were used to measure beetle speed in FiJi software. Briefly, images were combined into stacks, converted to 8-bit format and threshold was adjusted to isolate pixels representing the beetle. Beetle position coordinates were recorded using the 'Analyze Particles' function (with 'centroid' option selected). The average distance between coordinates on adjacent images was calculated as beetle walking distance per second. Statistical analyses, ANOVA, t-tests or nonparametric median tests in case of non-normal data distribution, were performed using SPSS 22.

5.3.6. Confocal microscopy

Confocal images of *pu11* prepupae were acquired with a confocal microscope A1R (Nikon), a 405 nm laser and 450/50 emission filter for cuticle autofluorescence (laser power: 4.0, PMT: 100) and a 488 nm laser and 525/50 nm emission filter for EYFP (laser power: 6.0, PMT: 100). The acquisition settings: 6.21 µm pixel size, 1/8 scan speed, 47.3 µm pinhole and Plan Fluor 4x (NA = 0.13) objective were used. All pictures were adjusted identically for brightness and contrast in FiJi software. Ten z stacks were obtained at 50 µm steps and combined to obtain maximum projection images.

5.3.7. N-glycan analysis in Tcα-Man-Ia RNAi beetles

Approx. 60 mg of 1-2 week old adults eclosed after larval RNAi of *Tcα-Man-Ia* were starved for 24 hrs to exclude the impact of dietary glycoproteins and frozen at -80°C until use. Whole insects were ground in liquid nitrogen to a fine powder and extracted using 4% SDS, 0.1 M DTT, 0.1 M Tris-Cl pH 7.6. Tryptic peptides were prepared as described by Zielinska et al. (2012). In brief, protein extracts were purified by four rounds of washes with 8M urea in 0.1M Tris/HCl pH 8.6 (UT) on Amicon Ultra-0.5 mL centrifugal filters (30 kDa cut off). Subsequently, thiol groups were carboxymethylated by incubation of the samples with 50 mM iodoacetamide in UT for 20 mins in dark. Next proteins were washed twice with UT and twice with 40 mM ammonium bicarbonate. Porcine trypsin (Thermo Scientific) was added at a 1:100 w/w ratio to the protein extracts on filters (5 µg trypsin per 500 µg peptides per filter) and digestion was performed on filters at 37°C for 16 hrs. Tryptic peptides were collected by centrifugation and cleaned up using Sep-Pak C18 cartridges (Waters) and 1-propanol/5% acetic acid system. N-glycans were released from approx. 2 mg of tryptic peptides using either 0.4 mU PNGase A from almonds (ProGlycAn, Vienna, Austria) in 0.1M Phosphate-citrate buffer pH 5 for 16 hrs. Released

glycans were separated from peptides on Sep-Pak C18 cartridges (Waters) using 1-propanol/5% acetic acid system. Samples were desalted using a Glycoclean H-cartridge (Prozyme) according to the manufacturer's protocol and then evaporated to dryness. Glycans were resuspended in labelling solution ($NaBH_3CN$ 750 mM, 2-AB 175 mM in DMSO/acetic acid at 10:3 ratio) and incubated at 65°C for two hours. After further purification on Glycoclean S-cartridge (Prozyme) according to the manufacturer's protocol, glycans were resuspended in 50% acetonitrile in water for mass spectrometry analysis. Samples were prepared in four biological replicates. MALDI-TOF analyses of RNAi samples were performed on an UltraFlextreme (Bruker) mass spectrometer (500 laser shots per spectrum). The matrix was 2,5-DHB prepared at 20 mg/mL in 50% acetonitrile, 0.1% formic acid. Three mass spectra were recorded for each biological replicate. Laser intensity was optimized for every sample depending on the signal obtained. For each spectrum, the intensities of glycan peaks were normalized on the total glycan signal allowing relative proportions to be determined. Detected peaks were annotated using GlycoWorkbench 2 (Ceroni et al., 2008) based on N-glycans described in *Drosophila*. Ambiguous peaks were annotated based on MS/MS fragmentation spectra. Differences between relative glycan content were analyzed using one sided t-test in SPSS 22.

5.4. Results

5.4.1. *T. castaneum* and *D. melanogaster* genomes encode similar array of N-glycosylation genes

Blast tools were used to identify putative genes involved in N-glycosylation in *Tribolium* with *D. melanogaster* sequences as queries and subsequently phylogenetic trees were constructed using fly, beetle and human proteins to confirm the true homology of the identified sequences. These analyses revealed that the genome of *T. castaneum* encodes a similar array of genes (Fig. 5.1 and Supplementary Figs. S5.1 and S5.2) involved in the N-glycan attachment and processing as previously described for the model insect *D. melanogaster.*

Drosophila gene	*Tribolium* ortholog	In this paper	Function
l(2)k12914	TC016366	*TcDad1**	Subunits of OST complex attaching
CG1518	TC010433	*TcStt3A**	*N*-glycan precursor to a polypeptide
OstStt3	TC009183	*TcStt3B**	
GCS1	TC011354	*TcGCS1*	ER α-glucosidases trimming terminal
GCS2α	TC032148	*TcGCS2α*	glucose residues from N-glycan
α-Man-Ia	TC011089	*Tcα-Man-Ia*	Type I α-mannosidases processing
α-Man-Ib	TC002991	*Tcα-Man-Ib*	$Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ N-glycan
Mgat1	TC009001	*TcMgat1*	GlcNAc transferase
α-Man-IIa	TC009186	*Tcα-Man-IIa*	Type II α-mannosidases responsible
α-Man-IIb	TC014283	*Tcα-Man-IIb*	for production of paucimannose glycans†
fdl	TC009779	*Tcfdl*	N-acetylhexosaminidase‡
FucT6	TC008521	*TcFucT6*	Core α1,6-fucosyltransferase
FucTA	TC014343	*TcFucTA*	Core α1,3-fucosyltransferases
FucTB	TC005825	*TcFucTA/C*§	
FucTC	TC007385	*TcFucTB*	
	TC008651	*TcFucTC*	
	TC008652		
Mgat2	TC001867	*TcMgat2*	GlcNAc transferases involved in production
CG9384	TC003870	*TcMgat4*¶	of complex glycans
CG17173			
β4GalNAcTA	TC006388	*Tcβ4GalNAcTA*	GalNAc/Gal transferases putatively involved in
β4GalNAcTB	TC006987	*Tcβ4GalNAcTB*	complex N-glycan synthesis#
ST6Gal	TC014265	*TcST6Gal*	Sialic acid transferase
Csas	TC015995	*TcCsas*	Synthesis of donor substrate for ST6Gal

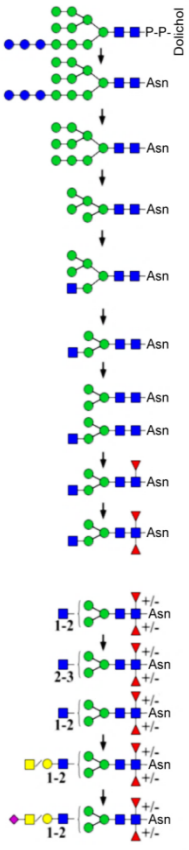


Fig. 5.1. Overview of *T. castaneum* genes putatively involved in N-glycosylation. ■ or GlcNAc – N-acetylglucosamine, ● or Man – mannose, ● or Glc – glucose, ▲ or Fuc – fucose, □ or GalNAc – N-acetylgalactosamine, ○ or Gal – galactose, ◆ – sialic acid, Asn – asparagine in a polypeptide chain, P-P-dolichol – lipid carrier of N-glycan precursor. * – Names based on homology with human OST subunits, † – Drosophila α-Man-IIb may function downstream to α-Man-IIa to produce paucimannose glycans (Rosenbaum

et al., 2014), but can also trim single α1,2 mannose residues from $Man_{9-8}GlcNAc_2$ and process $Man_5GlcNAc_2$ into $Man_3GlcNAc_2$ (Nemcovicova et al., 2013), ‡ – Enzyme encoded by fdl can also remove terminal N-acetylhexosamine residues from biantennary LacdiNAc or core fucosylated glycans (Dragosits et al., 2015), § – TC005825 has similar evolutionary relationship with Drosophila FucTA and FucTC (see Supplementary Fig. 5.2i), ¶ – Named based on human ortholog MGAT4, # – homolog from moth Trichoplusia ni next to GalNAc may transfer Gal as well (Vadaie and Jarvis, 2004). Insect β4GalNAcT's may also be involved in the synthesis of glycolipids.

Matches between *D. melanogaster* and *T. castaneum* included genes coding for two isoforms of Stt3 – catalytic subunit of OST, two alpha glucosidases (GCS1 and GCS2 involved in glucose trimming), a single α1,2 mannosylglycoprotein 4-β-N-acetylglucosaminyltransferase (Mgat1) and two class II Golgi α-mannosidases (α-Man-IIa and α-Man-IIb). Similar to *Drosophila*, the *Tribolium* genome encodes two putative GalNAc transferases and a single sialic acid transferase. Yet, there were some differences in the genetic organization of N-glycosylation between the red flour beetle and the fruit fly. For instance there are two class I α-mannosidases (α-Man-Ia and α-Man-Ib) in the *Tribolium* genome while there are three such genes in *D. melanogaster* (α-Man-Ia, b and c). Additionally, only single genes coding for each of the human α1,3 and α1,6 mannosylglycoprotein 4-β-N-acetylglucosaminyltransferases (Mgat2 and Mgat4) were found in *T. castaneum*. In contrast, there are three such genes in *D. melanogaster*.

Both *D. melanogaster* and *T. castaneum* have an array of five fucose transferases although as indicated by phylogenetic analysis there was no clear one to one homology between the genes. For instance in case of TC005825 analysis revealed similar evolutionary distance to *Drosophila* FucTA and FucTC, while there was no clear *Tribolium* homolog of *Drosophila* FucTD.

5.4.2. Expression of N-glycosylation genes is developmentally regulated in *T. castaneum*

To gain a first insight into the importance of N-glycosylation for *Tribolium* post-embryonic development, we analyzed the expression patterns of 22 genes putatively involved in the process of protein carbohydrate modification. For that we used qRT-PCR to estimate changes in the transcript abundances at eight life stages between 2nd instar larvae and mature adults (Fig. 5.2 and Table 5.1). This approach revealed that the expression of genes coding for proteins involved in the N-glycosylation pathway is developmentally regulated. There were statistically significant differences in mRNA expression between life stages for all of the tested genes (ANOVA, $P<0.05$), except for *TcStt3B*

(P=0.547). Interestingly, nineteen of the genes showed the highest expression levels during the pupal stage (twelve in early and seven in the late pupae). In contrast, there was only one gene, α-1,3-fucosyltransferase *TcFuctA,* which showed the highest expression during the larval stage (4th instar).

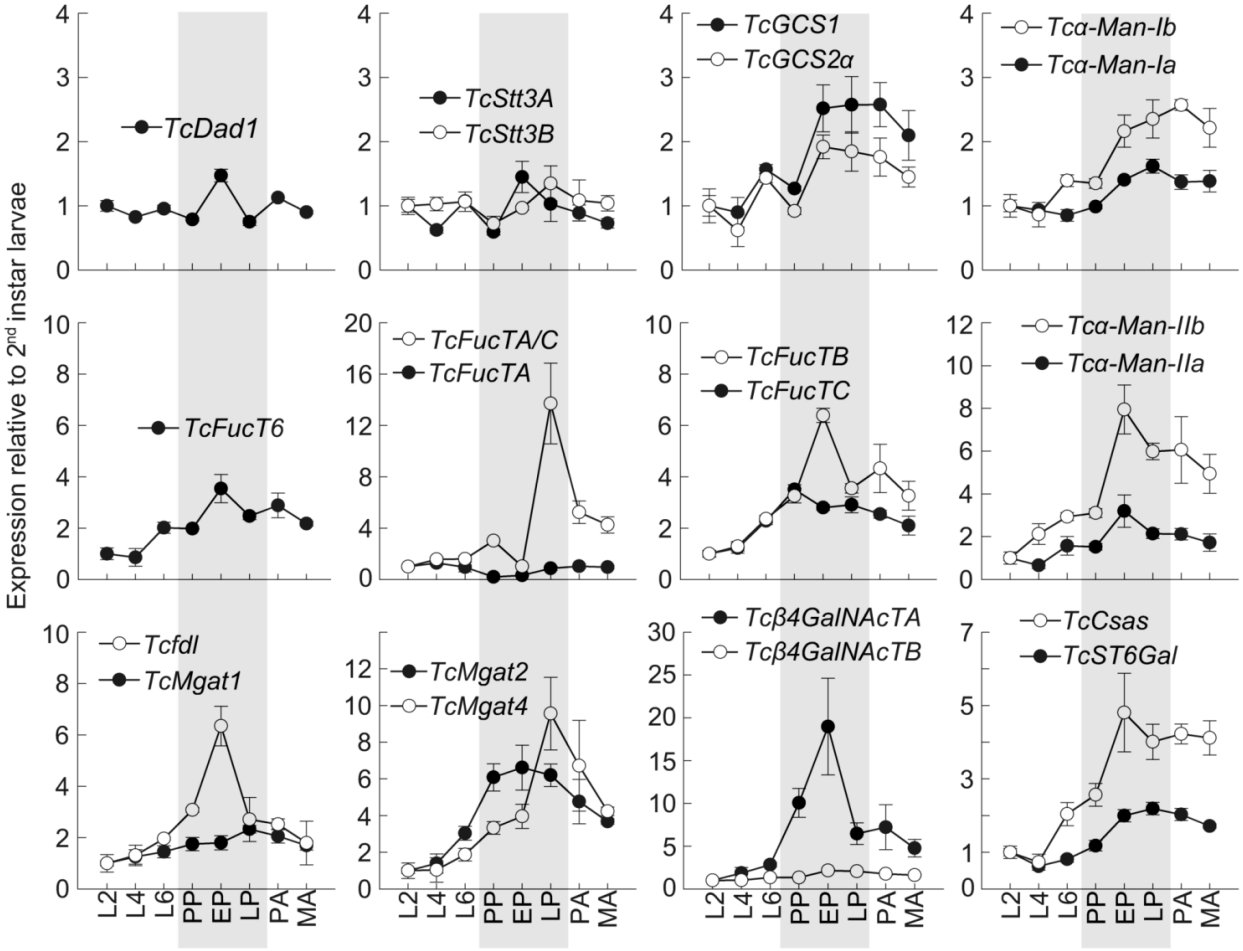


Fig. 5.2. Expression of genes involved in N-glycosylation pathway changes during post-embryonic development. L2, L4, L6 – 2nd, 4th, 6th instar larvae; PP, EP, LP – pre-, early, late pupae; PA, MA – pharate, mature adults. Relative gene expression was quantified with qRT-PCR using TcRpL32 and TcRpS6 as reference genes and normalized to L2 expression level. Error bars are SD (n=3). Note that the highest expression of most of the genes was observed during pupal periods (marked in grey). See Table 5.1 for statistics.

Additionally, mRNA levels of most of the genes involved in N-glycan processing in the ER and Golgi were higher in mature adults compared to 4th instar larvae (midpoint of larval development). Notably, class I and II α-mannosidases, key players in the trimming of N-glycans, were 1.5 to 2.5 fold upregulated, while the expression of genes potentially involved in the synthesis of complex N-glycans was 2-6 fold higher.

Table 5.1. Developmental changes in expression of genes putatively involved in N-glycan attachment and processing in *T. castaneum*

		Stage with the highest expression vs 4th instar larvae			Mature adults vs 4th instar larvae	
Gene	ANOVA P	Stage	Expression ratio	t-test P	Expression ratio	t-test P
TcDad1	<0.001	Early pupae	1.87	<0.001	1.08	0.262
TcStt3A	0.011	Early pupae	2.35	0.029	1.15	0.123
TcStt3B	0.547	Late pupae	1.32	0.998	1.01	0.472
TcGCS1	0.001	Late pupae, pharate adults	2.86	0.018	2.25	0.032
TcGCS2α	0.013	Early pupae	3.03	0.018	2.30	0.029
Tcα-Man-Ia	<0.001	Late pupae	1.74	0.002	1.46	0.031
Tcα-Man-Ib	<0.001	Pharate adults	2.97	0.001	2.53	0.012
TcMgat1	0.025	Late pupae	1.86	0.111	1.35	0.128
Tcα-Man-IIa	<0.001	Early pupae	3.88	0.004	2.33	0.008
Tcα-Man-IIb	<0.001	Early pupae	3.59	0.002	2.23	0.022
Tcfdl	0.009	Early pupae	4.86	<0.001	1.37	0.316
TcFucT6	0.003	Early pupae	4.14	<0.001	2.55	0.011
TcFucTA	0.001	4th inst. larvae	1.00	-	0.74	0.101
TcFucTA/C	<0.001	Late pupae	8.11	<0.001	2.55	0.006
TcFucTB	<0.001	Early pupae	4.56	<0.001	2.35	0.012
TcFucTC	<0.001	Prepupae	2.68	<0.001	1.63	0.059
TcMgat2	<0.001	Early pupae	4.77	0.004	2.65	0.007
TcMgat4	0.004	Late pupae	9.21	0.003	4.08	0.006
TcβGalNAcTA	<0.000	Early pupae	10.24	0.003	2.57	0.037
TcβGalNAcTB	<0.000	Early pupae	2.15	0.001	1.60	0.016
TcST6Gal	<0.000	Late pupae	3.55	<0.001	2.77	0.001
TcCsas	<0.000	Early pupae	6.60	0.001	5.65	0.002

ANOVA P – p-value obtained using ANOVA in qbase+ software to reveal life stage dependent changes in expression. T-test P – p-value obtained in independent sample t-test to compare expression between 4th instar larvae and life stage with highest expression or between 4th instar larvae and mature adults.

The levels of upregulation of N-glycan processing genes in adults were moderate but consistent across the entire N-glycan processing pathway. In contrast, genes involved in the attachment of the N-glycan precursor to a polypeptide (*TcDad1*, *TcStt3A*, and *TcStt3B*) were expressed

at similar levels in larvae and adults. This observation is consistent with previously found shift in the N-glycan composition towards more processed forms associated with the transition from larvae to adults.

5.4.3. Silencing of genes involved in N-glycan attachment and early processing blocks development

The elevated expression of genes encoding enzymes responsible for N-glycan trimming and modification during later developmental stages as well as enrichment of more processed N-glycans in adults suggested that N-glycan processing is implicated in metamorphosis. To verify this hypothesis we knocked-down an array of genes involved in N-glycosylation and studied the consequences for insect development. Injection of specific dsRNAs into *Tribolium* larvae induces RNAi-mediated gene silencing that persists throughout metamorphosis and in the mature adults. In our hands injection of gene-specific dsRNA into 4th instar larvae generally resulted in a 80-95% reduction of transcript abundance (Supplementary Fig. S5.2).

Silencing of *TcDad1*, encoding an essential subunit of the OST complex catalyzing the attachment of the N-glycan precursor to a polypeptide, completely inhibited larval growth and caused 96.4% mortality during the larval stage (Table 5.2, Fig. 5.3). The OST contains either the Stt3A or Stt3B isoform of the catalytic subunit determining activity of the complex (Sato et al., 2012; Shrimal et al., 2013). When we silenced either of these subunits we observed only weak phenotypes: minor reduction of larval growth or adult movement speed. It has been shown that the mammalian Stt3A and B isoforms have partially redundant activities (Ruiz-Canada et al., 2009), so we asked if this can also be the case in *T. castaneum*. Co-silencing of both *Tribolium Stt3* isoforms resulted in cessation of growth and high larval lethality similar to that caused by RNAi of *TcDad1* (Fig. 5.3 A, C), coding for an essential subunit of OST. This supports functional compensation between *TcStt3A* and *TcStt3B* and indicates that N-glycan attachment is crucial for larval growth and survival.

Table 5.2. Summary of phenotypes induced by larval RNAi.

Gene silenced	Mortality (%)			Phenotype description	
	larva	pupa	adult		
TcDad1	96.4	1.8	0	Larval growth blocked	Mid-larval mortality
TcStt3A	6.0	0	0	Growth reduced by ~20%	
TcStt3B	6.9	1.7	8.6	Adult speed reduced	
TcStt3A+TcStt3B	95.6	4.4	0	Larval growth blocked	Mid-larval mortality
TcGCS1	89.7	5.2	1.7	Growth reduced by ~40%	Prepupal mortality
TcGCS2α	90.9	3.6	0	Growth reduced by ~20%	Prepupal mortality
Tcα-Man-Ia	8.3	10.0	0	Pupal & adult elytra malformed	Adult speed reduced
Tcα-Man-Ib	5.8	0	1.9	Pupal elytra apart	Adult speed reduced
Tcα-Man-Ia+Tcα-Man-Ib	16.1	73.2	0	Legs, wings and elytra malformed	Adult eclosion blocked
TcMgat1	3.7	0	0	Pupal elytra apart & shorter	Adult speed reduced
Tcα-Man-IIa	1.8	0	0	Pupal elytra apart & shorter	Adult speed reduced
Tcα-Man-IIb	5.5	0	0	Pupal elytra apart & shorter	
Tcα-Man-IIa+Tcα-Man-IIb	1.9	0	0	Pupal elytra apart & shorter	Adult speed reduced
Tcfdl	1.5	0.0	4.5	Pupal elytra apart & shorter	Adult speed reduced
TcMgat2	3.1	0	0	none	
TcMgat4	7.1	0	0	none	
TcMgat1+TcMgat4	4.0	0	0	Pupal elytra apart	Adult speed reduced
TcMgat1+TcMgat2+ +TcMgat4	0	0	0	Pupal elytra apart & shorter	Adult speed reduced
TcFucT6	0	0	0	none	
TcFucTA/C	0	0	0	none	
TcFucTA	5.3	0	0	none	
Tcβ4GalNAcTA	0	0	0	Growth reduced by ~20%	
Tcβ4GalNAcTB	4.5	0	4.5	none	
Tcβ4GalNAcTA+ +Tcβ4GalNAcTB	4.2	0	0	Growth reduced by ~20	
TcST6Gal	0	3.3	0	none	
TcCsas	0	0	0	none	

The first step of N-glycan maturation of glycoproteins involves the action of two α-glucosidases, GCS1 and GCS2, which act in the ER to sequentially remove three glucose residues from the immature *N*-linked glycan to form the $Man_9GlcNAc_2$ structure (Stanley et al., 2009). Silencing of *TcGCS1* and *TcGCS2α* resulted in a reduced larval growth by 19% (P=0.006) and 41% (P<0.001), respectively, and prevented pupation (Fig. 5.3. B-D). In both cases, approximately 90% of all beetles

died during the last larval or prepupal stage. Notably, in most of the prepupae that died due to silencing of those genes, there was a characteristic dorsal split in the old cuticle which normally precedes pupal emergence, suggesting that pupation was initiated but could not be completed.

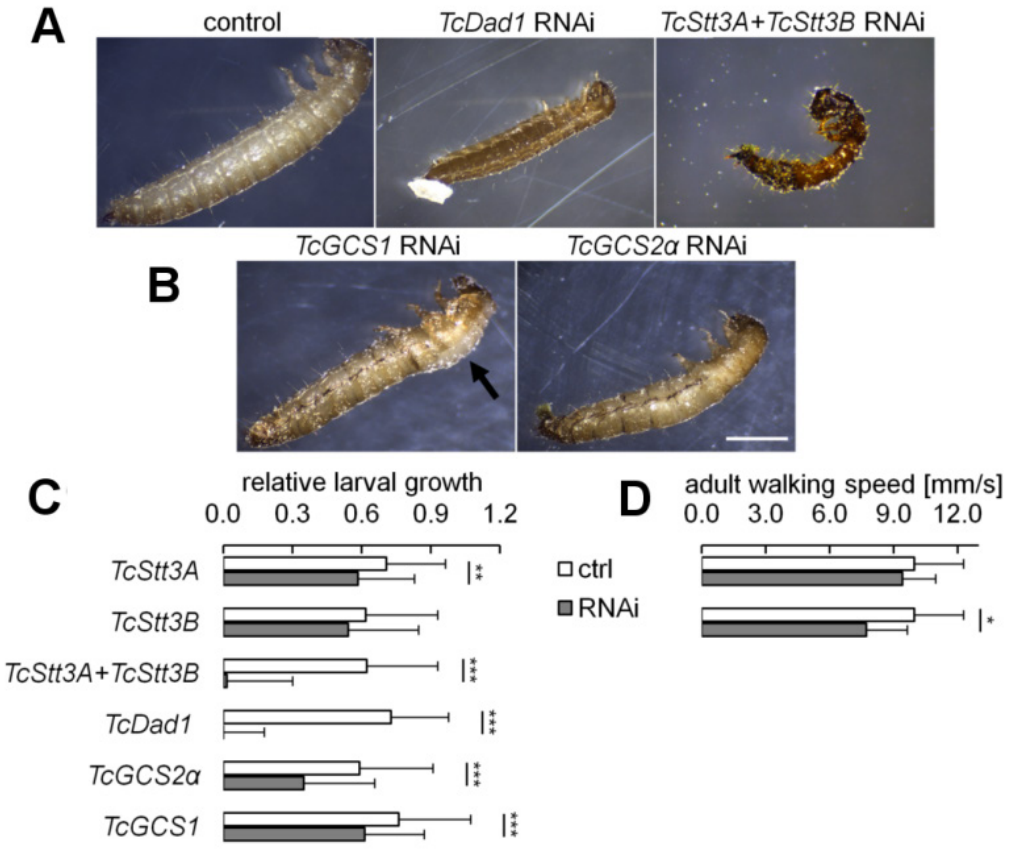


Fig. 5.3. Silencing of genes involved in N-glycan attachment and early trimming disrupts larval development and prevents pupation. 4th instar larvae were injected with appropriate dsRNAs to induce gene silencing or with dsGFP in case of control. (A) RNAi of TcDad1 and double RNAi of TcStt3A and TcStt3B isoforms resulted in early larval mortality. (B) RNAi of TcGCS1 and TcGCS2α resulted in mortality during last larval and prepupal stages. Black arrow indicates dorsal split in the cuticle suggesting that pupation was initiated before the larvae died. (C) RNAi effects on larval growth (n=51, 61, 43, 56, 58, 61 – top to down). (D) RNAi of TcStt3B but not of TcStt3A induced minor reduction of adult walking speed, n=13. Error bars are SD, ***P<0.001, **P<0.01, *P<0.05. Scale bar is 1 mm.

5.4.4. Disruption of trimming of high mannose N-glycans affects pupal and adult development

Following the action of the α-glucosidases the $Man_9GlcNAc_2$ can be trimmed to $Man_5GlcNAc_2$ by class I α-mannosidases. The *Tribolium* genome encodes two enzymes potentially involved in these

reactions: α-Man-Ib thought to specifically cleave a single mannose residue from the middle antenna of a glycan and α-Man-Ia cleaving additional three mannoses (Nemcovicova et al., 2013).

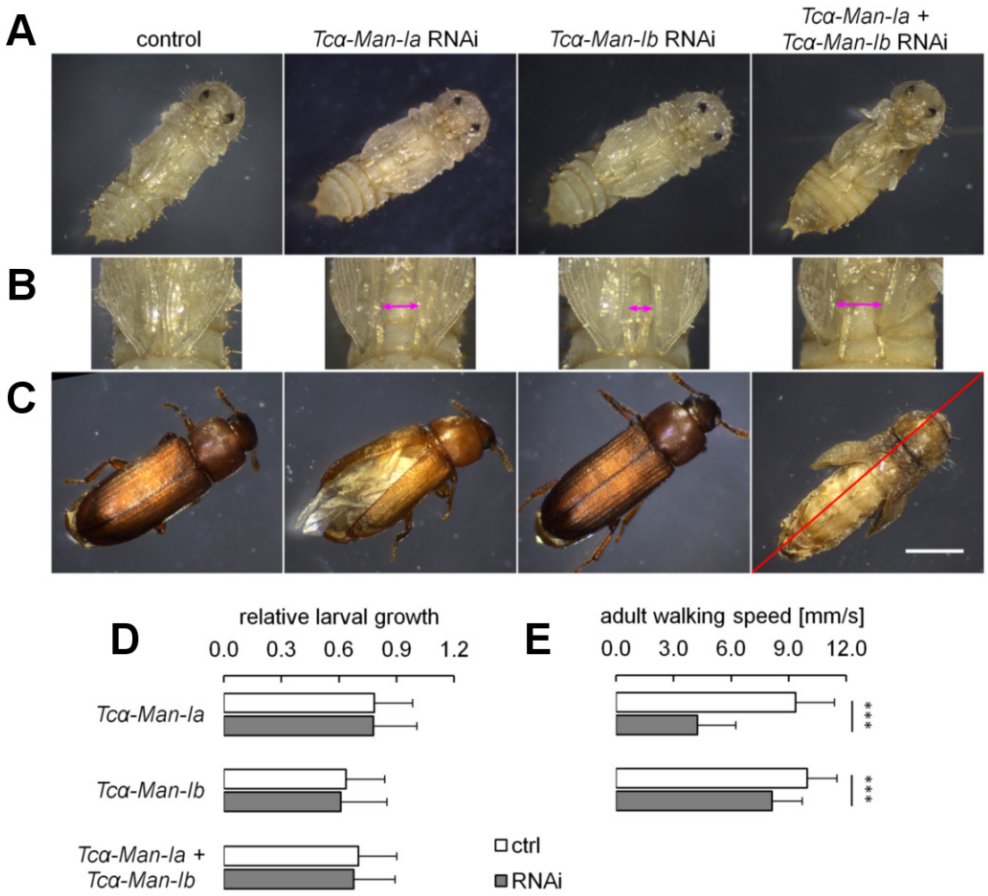


Fig. 5.4. Silencing of class I α-mannosidases affects pupa and adult development. 4th instar larvae were injected with appropriate dsRNAs to induce gene silencing or with dsGFP in case of control. (A) Single knockdowns by RNAi of class I α-mannosidases induced minor changes in position and shape of pupal elytra, while double knockdown of Tcα-Man-Ia and Tcα-Man-Ib caused severe pupal malformations. (B) Zoomed view on pupal elytra. (C) Silencing of Tcα-Man-Ia disrupted adult elytra development, but double RNAi of Tcα-Man-Ia and Tcα-Man-Ib completely prevented adult eclosion. Red diagonal line indicate dead beetle. (D) and RNAi of class I α-mannosidases did not affect larval growth (n=61, 53, 53). (E) RNAi of Tcα-Man-Ia and of Tcα-Man-Ib decreased adult mobility (n=15, 22). No living adults eclosed after double RNAi of Tcα-Man-Ia and Tcα-Man-Ib. Scale bar is 1 mm in (A) and (C) and 0.5 mm in (B). Error bars are SD, ***P<0.001 (t-test).

In contrast to silencing of the genes implicated in the initial steps of the N-glycosylation pathway, knock-down of *Tcα-Man-Ia* and *Tcα-Man-Ib* did not affect survival, larval growth or time of pupation but rather affected the morphology of the pupae (Figs. 5.4 A). In control pupae, the elytra (wing covers) almost completely cover the abdomen and the third pair of legs. In contrast, in insects treated with dsRNA for class I α-mannosidases the elytra were mispositioned (Figs. 5.4 B and 5.7). The gap between the elytra was 0.31±0.04 and 0.15±0.03 mm in the case of *Tcα-Man-Ia* and *Tcα-Man-Ib*

RNAi, respectively, compared to 0.06±0.03 mm in the control beetles (both P<0.001). Silencing of the α-Man-Ia gene also yielded shorter pupal elytra: 1.31±0.07 mm compared to 1.42±0.06 mm in the control group (P<0.001) and induced effects on the adult appendages. The adult elytra were wrinkled and not closed, revealing the underlying flight wings which appeared smaller and crumbled (Figs. 5.4 C and 5.8). In both cases RNAi reduced the adult walking speed; by over 50% for *Tcα-Man-Ia* (P<0.001) and approx. 20% for *Tcα-Man-Ib* (P=0.003).

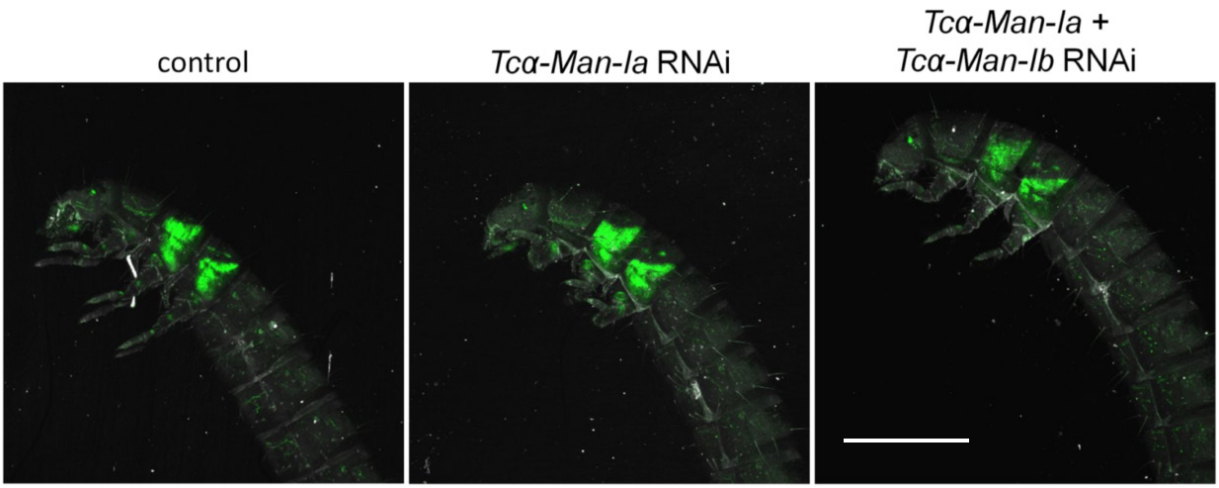


Fig. 5.5. RNAi of class I α-mannosidases had no clear effect on development of wing and elytra wing discs in pu11 strain. This strain expresses EYFP in wing and elytron primordia, eyes and neurons. Maximum projection confocal images are shown: green - EYFP, white – cuticle autofluorescence. Scale bar is 1 mm.

Remarkably, double knockdown of Tcα-Man-Ia and Tcα-Man-Ib resulted in more pronounced pupa malformations and in an arrest of wing and leg development implying some functional overlap between the genes (Figs. 5.4, 5.7, 5.8). All double RNAi insects that reached the pupal stage (~73%) died before the adult eclosion was completed. This observation implies that trimming of high mannose N-glycans is necessary for metamorphosis. To verify whether the observed wing and elytra malformations could be due to the effect of RNAi on development of the wing imaginal discs, we repeated the knockdown experiments in the pu11 nub enhancer trap line, which allows following of proliferation of the wing discs by observation of yellow fluorescent protein (EYFP) expression. However, silencing of the Tcα-Man-Ia gene alone or in combination with Tcα-Man-Ib caused no obvious effects on the formation of the wing imaginal discs (Fig. 5.5.). This suggests that α-mannosidases are implicated in later phases of wing development.

5.4.5. Paucimannose N-glycans are involved in elytra development and adult movement

Silencing of Mgat1, α-Man-IIa, α-Man-IIb and fdl genes, which are involved in the production of paucimannose glycans did not affect larval growth, mortality, or timing of pupation and adult eclosion. Similarly to RNAi of *Tcα-Man-Ia* and *Tcα-Man-Ib* we observed minor effects on pupal elytra length and position (Figs. 5.6-5.8). Additionally, reduced expression of *TcMgat1*, *Tcα-Man-IIa* and *Tcfdl* impaired adult movement speed by approximately 40%, 20% and 20% respectively (P<0.001, <0.001 and 0.033, respectively).

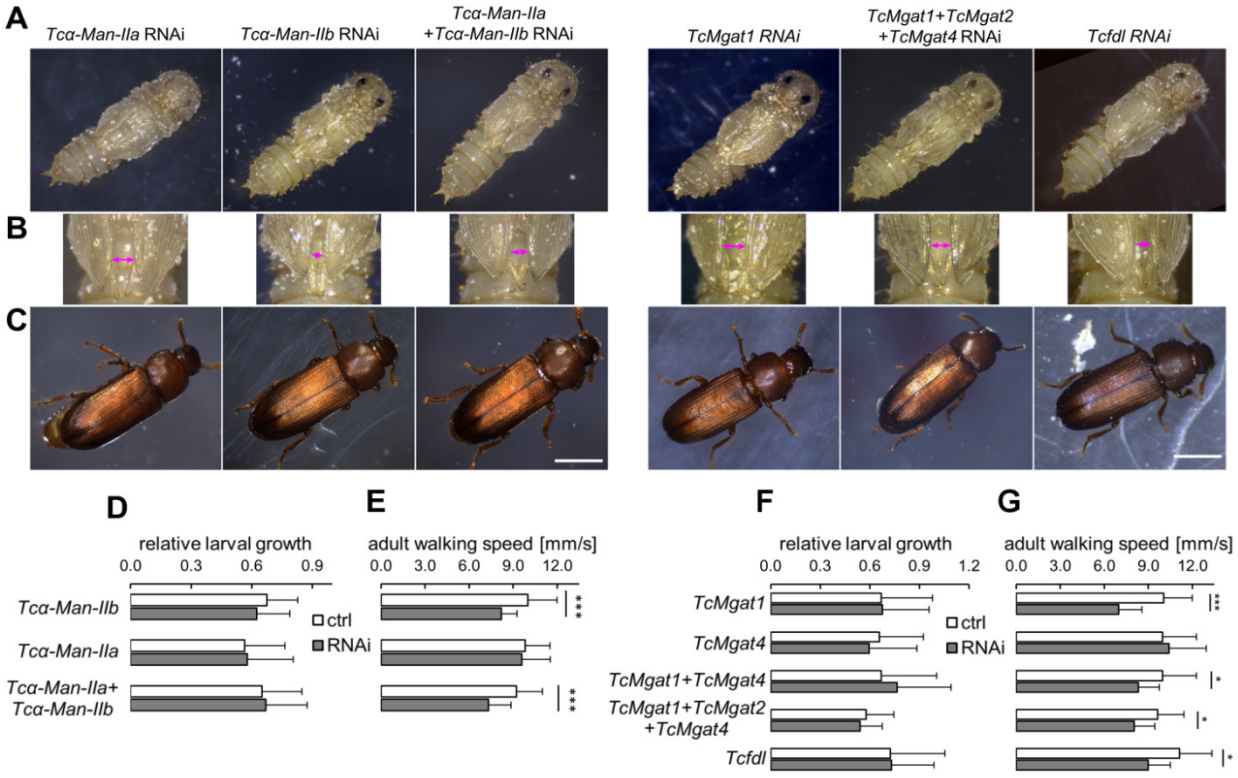


Fig. 5.6. RNAi of genes involved in paucimannose N-glycans production cause minor developmental defects. 4th instar larvae were injected with appropriate dsRNAs to induce gene silencing or with dsGFP in case of control. (A) and (B) Knockdowns caused minor disruption of pupal elytra (A) and (B) but not there was no effect on (C) adult morphology. (D) RNAi of class II α-mannosidases did not affect larval growth (n=60, 52, 61). (E) RNAi of Tcα-Man-IIa and double RNAi of Tcα-Man-IIa and Tcα-Man-IIb decreased adult mobility (n=24, 29, 29). (F) RNAi of Tcfdl or TcMgats genes did not affect larval growth (n=57, 57, 58, 58, 69). (G) Silencing of TcMgat1 and Tcfdl reduced adult mobility (n=20, 12, 12, 12, 12). Double RNAi of TcMgat1 and TcMgat4 as well as triple RNAi of TcMgat1, TcMgat2 and TcMgat4 did not cause any increase in phenotype severity. Scale bar is 1 mm in (A) and (C) and 0.5 mm in (B). Error bars are SD, asterisks indicate statistically significant differences: *** P<0.001, ** P<0.01, * P<0.05 (t-test).

Yet, silencing of these genes caused no clear malformation of flight wings or legs (Fig. 5.8). Sequential action of Mgat1, α-Man-II and fdl may also be bypassed by the activity α-Man-IIb, which in

turn would prevent more serious phenotypes. Thus to test this possibility *Tcα-Man-IIa* and *Tcα-Man-IIb* were simultaneously silenced. However, this did not result in any enhanced phenotypes compared to the single knockdowns, which may advocate the presence of yet additional enzyme(s) involved in the process.

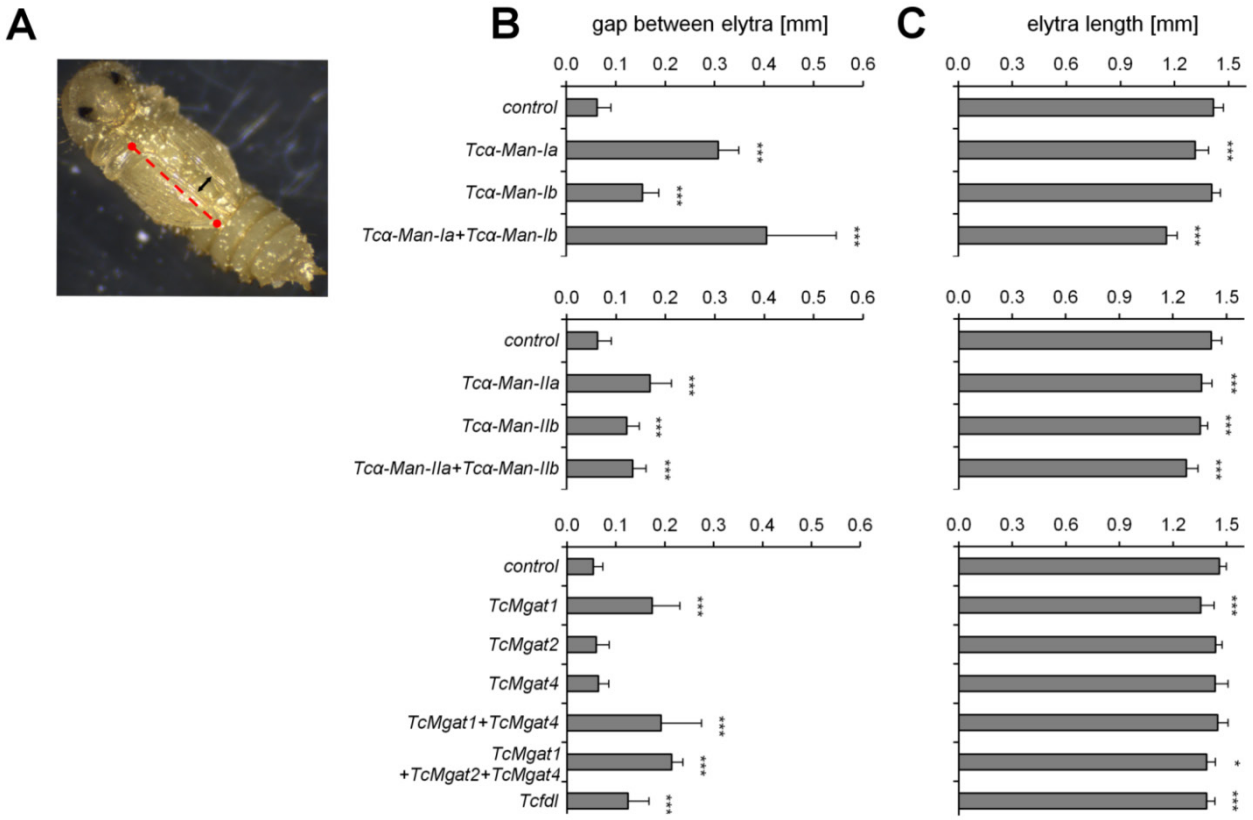


Fig. 5.7. RNAi of genes involved in N-glycan processing affect pupal elytra morphology. (A) Scheme of elytra length (red dashed line) and gap (black double-headed arrow) measurement. To avoid errors caused by elytra curvature, the length was measured as distance from the joint between T1 leg tibia and tarsus to the tip of the elytron. The gap between elytra was measured at 0.5 mm from the elytra tip. (B) Knockdown effects on elytra position. Elytra gap is enlarged in case of RNAi of each of the genes involved in N-glycan processing towards paucimannose glycans (all four α-mannosidases, TcMgat1 and Tcfdl) and not after RNAi of TcMgat2 and 4 which are involved in complex glycan synthesis. (C) Effects of N-glycan processing genes RNAi on pupal wing length (n=11-17). Error bars are SD, asterisks indicate statistically significant differences compared to control: *** P<0.001, ** P<0.01, * P<0.05 (t-test).

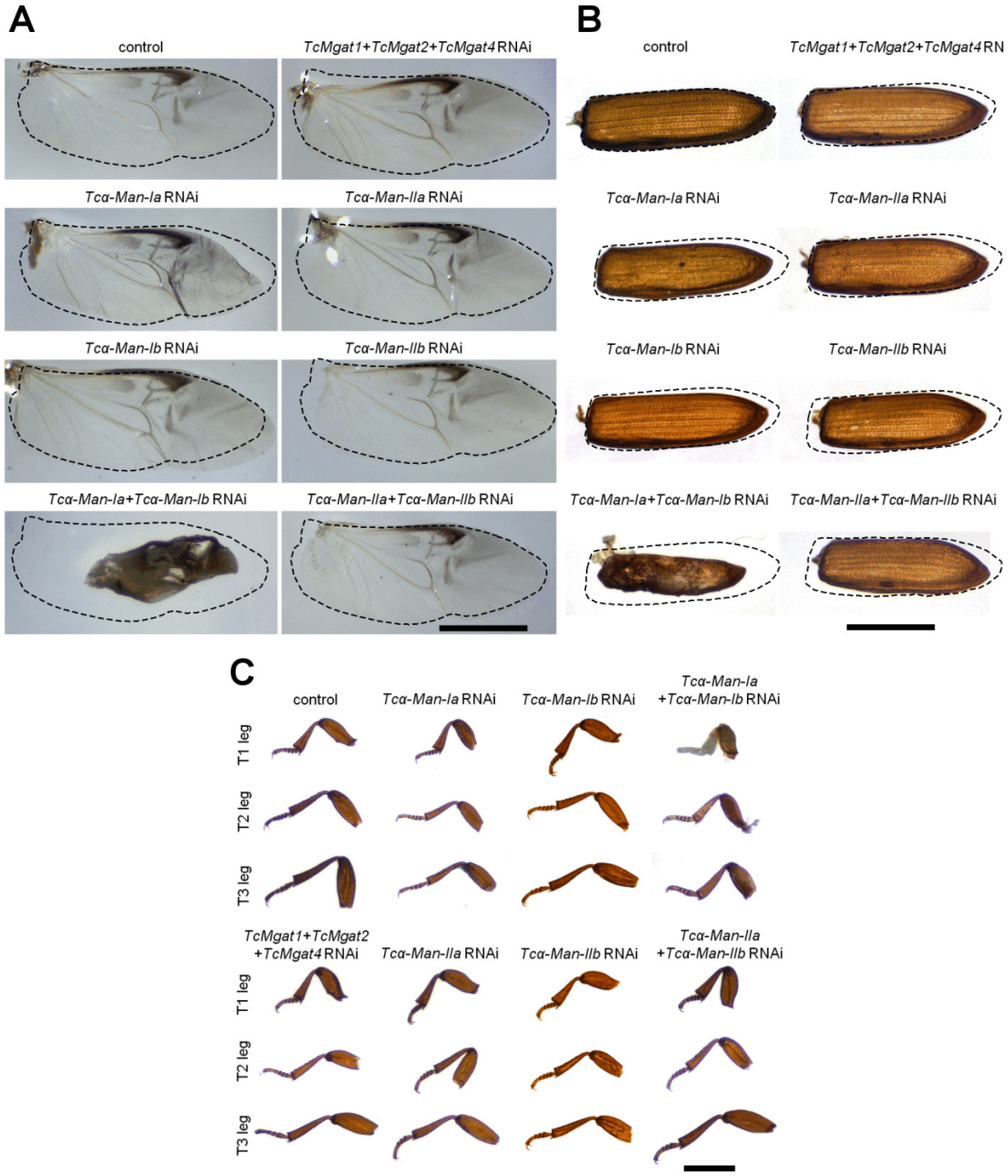


Fig. 5.8. RNAi of class I α-mannosidases affect adult appendage development. (A) RNAi effects on adult wings, (B) adult elytra and (C) adult legs. Knockdown of Tcα-Man-Ia caused minor malformations: wrinkled and smaller wings and elytra. Double knockdown of Tcα-Man-Ia and Tcα-Man-Ib completely blocked wing and elytra development and interfered with proper leg sclerotization (especially visible on T1 legs). Dashed line indicate shape of wing and elytron of control beetles. Scale bars in (A) and (B) are 1 mm. Scale bar in (C) is 0.5 mm.

5.4.6. RNAi of genes involved in complex N-glycan synthesis and fucosylation causes minor effects

Alternatively to the production of paucimannose oligosaccharides, N-glycans can be extended to form complex structures. However, in line with minute amounts of complex N-glycans found in the MS, we did not observe any appreciable phenotypes caused by the knockdown of genes putatively responsible for their synthesis, except for a minor (~20%) decrease in larval growth observed after injection of dsRNA for *Tcβ4GalNAcTA* (Fig. 5.9).

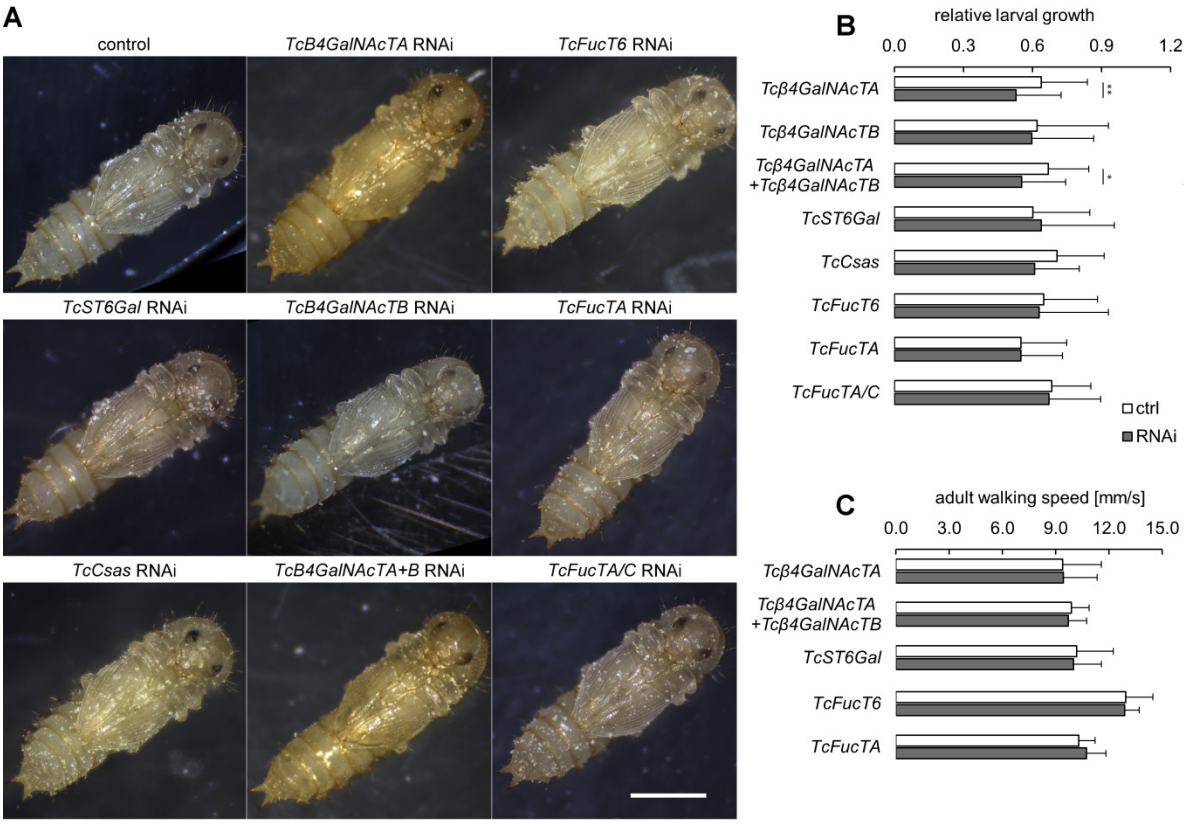


Fig. 5.9. Silencing of genes potentially involved in complex glycan synthesis and sialylation caused minor or no effects. (B) Only RNAi of Tcβ4GalNAcTA caused minor reduction of larval growth (n=28-63). (C) There was no effect on adult movement speed (n= 10-25). Error bars are SD, asterisks indicate P<0.001 (***), P<0.01 (**), P<0.05 (*). Scale bar is 1 mm.

In an attempt to block the GlcNAc addition and prevent complex glycan synthesis completely, we injected larvae with a mixture of dsRNAs for *TcMgat1*, *TcMgat2* and *TcMgat4.* This resulted in pupal elytra phenotypes and adult movement speed reduction similar to the effects observed when *TcMgat1* was silenced alone (Figs. 5.6-5.8).

Finally, we silenced the *Tribolium* ortholog of the *Drosophila* α1,6-fucosyltransferase (*TcFucT6*) and two putative α1,3-fucosyltransferases (*TcFucTA* and *TcFucTA/C*). Knockdown of these genes did not cause any negative effects. Although in the case of the *FucT6* gene the absence of a phenotype could probably be due to the low knockdown efficiency (37±9%), the RNAi of the genes *TcFucTA* and *TcFucTA/C* was successful (80±3% and 85±2% reduction, respectively). The attempt to silence all three genes together was not successful as the knockdown efficiency was low for each of the genes (Supplementary Fig. S5.4) and also resulted in wild type pupae and adults. The roles of the other two putative fucosyltransferase genes *TcFucTB* and *TcFucTC* were not tested as the dsRNAs for these genes could not be produced.

5.4.7. RNAi of α-Man-Ia causes disruption of the entire N-glycome

To look more closely at the biochemical basis for the disruption of appendages and adult development caused by RNAi of *Tcα-Man-Ia* the effects of this knockdown on the N-glycan profile were studied using mass spectrometry. The analysis of N-glycans isolated from adults that eclosed after knockdown of *Tcα-Man-Ia* revealed an evident accumulation of high mannose N-glycans and a clear reduction but not total absence of paucimannose N-glycans (Fig. 5.10, Table 5.3). $Man_9GlcNAc_2$ was still the most prevalent glycan and its relative abundance was increased by 60% compared to control adults (P=0.010). Additionally, each of the other high mannose substrates ($Man_{6\text{-}8}GlcNAc_2$) of the class I α-mannosidase were enriched by 2-4 fold. In total high mannose N-glycans amounted to 77.4 ± 0.8% of all N-glycans in *Tcα-Man-Ia* RNAi beetles compared to 37.8 ± 8.3% in the control (P<0.001). Surprisingly, the amount of $Man_5GlcNAc_2$, the end product of α-Man-Ia, was not reduced but enriched by ~20% (P=0.013). Interestingly, the glycan that is formed by the addition of a terminal GlcNAc, $GlcNAcMan_5GlcNAc_2$, was below the detection limit in *Tcα-Man-Ia* knockdown adults. $GlcNAcMan_5GlcNAc_2$ is a precursor for complex and core fucosylated oligosaccharides, thus consequently, the prevalence of each of these glycans was reduced by 3 to 20 fold. In total, monofucosylated, difucosylated and complex N-glycans were decreased by 4.7, 6.6 and 5.2 fold, respectively (P=0.001, 0.002 and 0.011, respectively). In contrast, the content of non-fucosylated paucimannose N-glycans in *Tcα-Man-Ia* RNAi adults was not significantly different from that in the control adults (P=0.069, Table 5.3.)

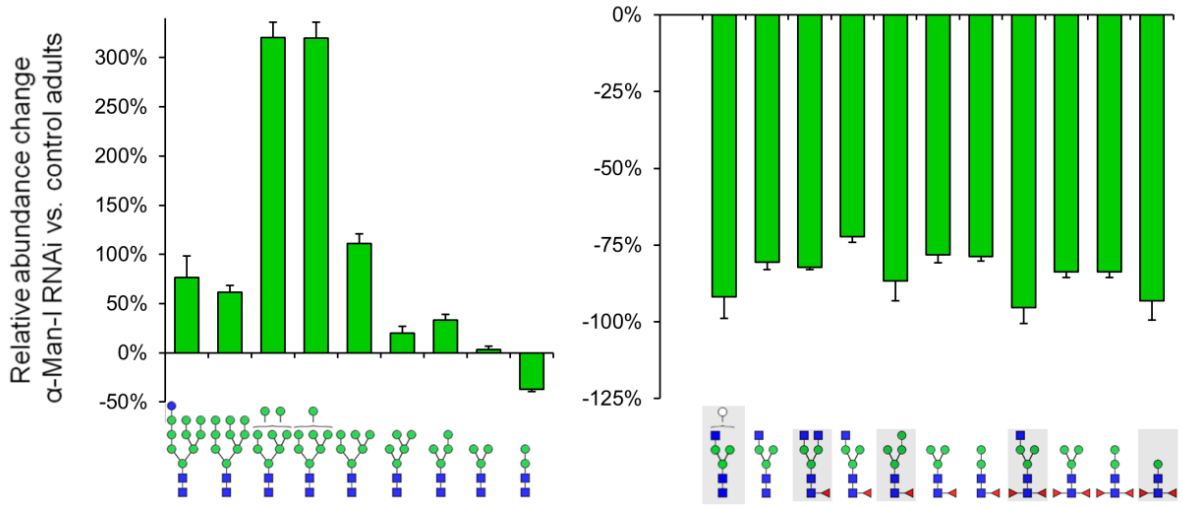


Fig. 5.10. RNAi of Tcα-Man-Ia RNAi induces changes in entire adult N-glycome. Proteins were extracted from whole 4th instar larvae or mature adults. N-glycans were released using PNGaseA, labeled with 2-AB and analyzed using MALDI-MS. Upper panel shows relative changes in adult N-glycan abundance in adults eclosed after silencing of Tcα-Man-Ia compared to wild type adults. Left: mannose series N-glycans, right: insect complex, hybrid and fucosylated N-glycans. Error bars are SD (n=4). Glycans in grey boxes indicate N-glycans found below 0.1% in RNAi adults. See Fig. 5.1. for N-glycan symbol description.

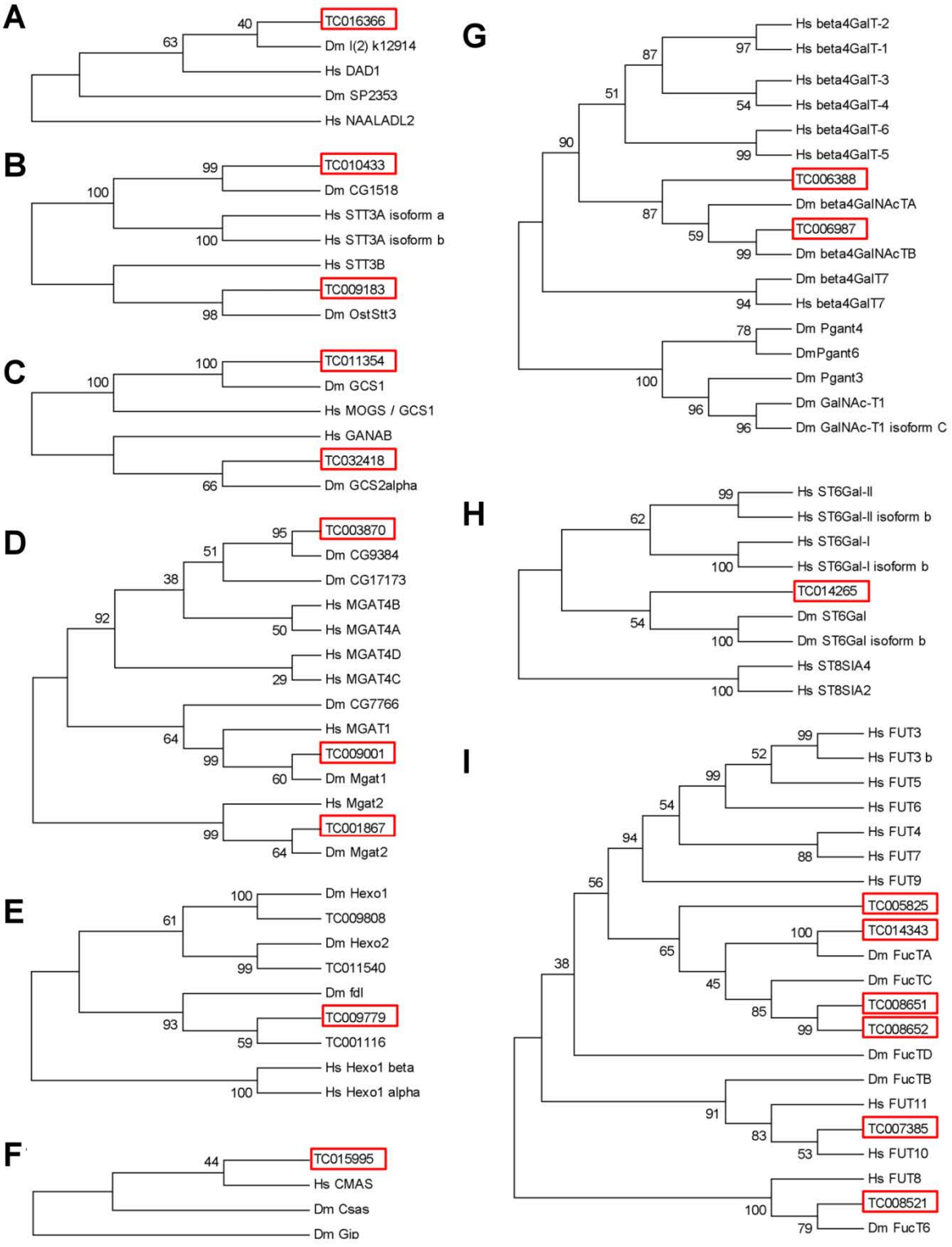
However, the levels of particular N-glycans among this group were differentially affected. In that the $Man_4GlcNAc_2$ was enriched by ~35% (P=0.001), the $Man_3GlcNAc_2$ was unchanged (P=0.294) and the $Man_2GlcNAc_2$ was downregulated by ~40% (P=0.007) in *Tcα-Man-Ia* RNAi adults. These pronounced changes in the N-glycan profile indicate clearly that the gene coding for Golgi class I α-mannosidase is a key element of the N-glycan processing pathway.

Table 5.3. Abundance of different N-glycan groups in adults eclosed after silencing of Tcα-Man-Ia.

	% total profile (±SD) Adults	α-Man-Ia RNAi Adults	p-value A vs RNAi
High mannose	37.8 ± 8.3	77.4 ± 0.8	<0.001
Paucimannose:			
non-fucosylated	15.3 ± 1.7	13.5 ± 0.3	0.069
monofucosylated	33.1 ± 6.4	7.0 ± 0.4	0.001
difucosylated	13.8 ± 3.3	2.1 ± 0.3	0.002
Complex	3.1 ± 1.2	0.6 ± 0.1	0.011

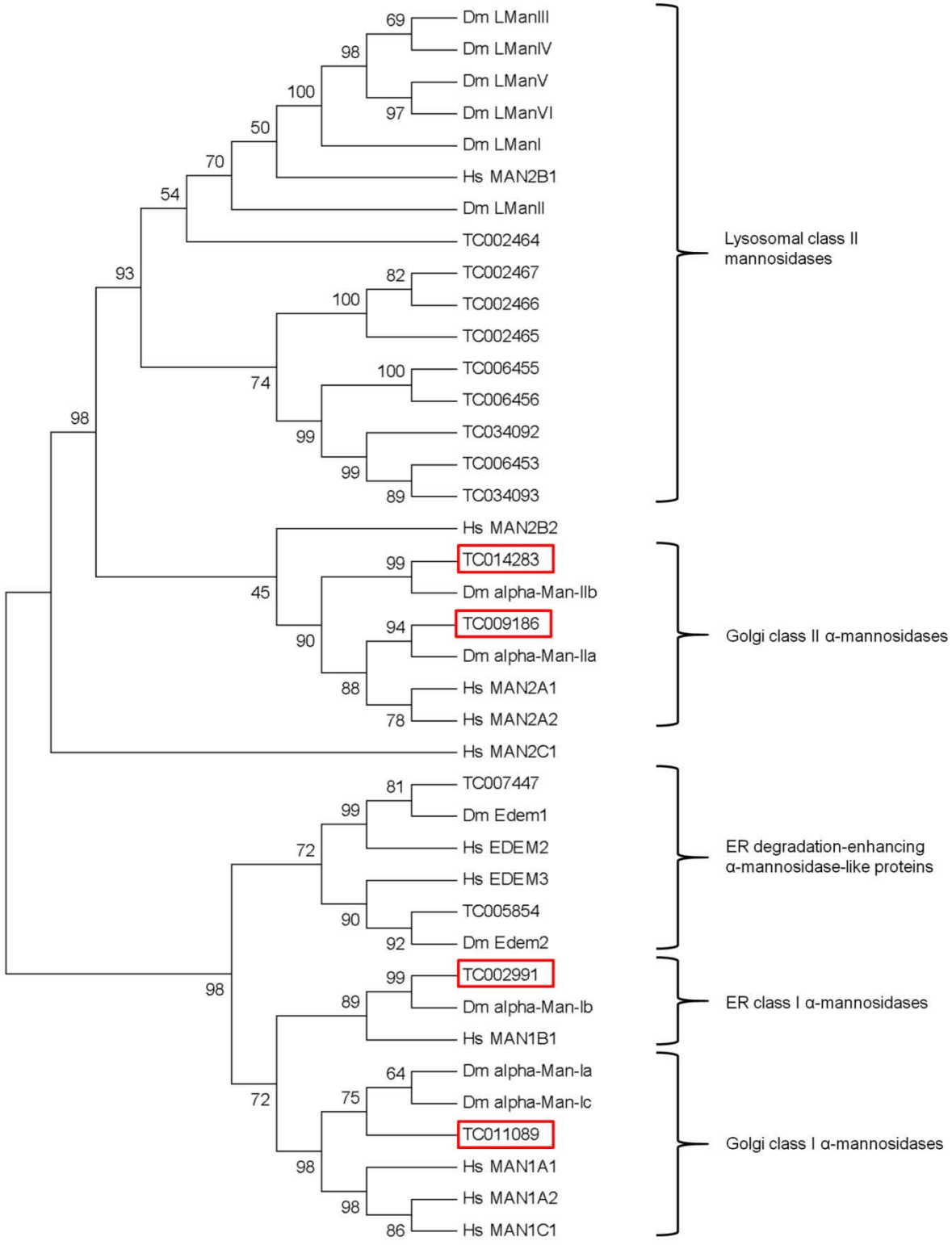
p-values were obtained using independent samples t-test, n=4.

5.4.8. Supplementary figures



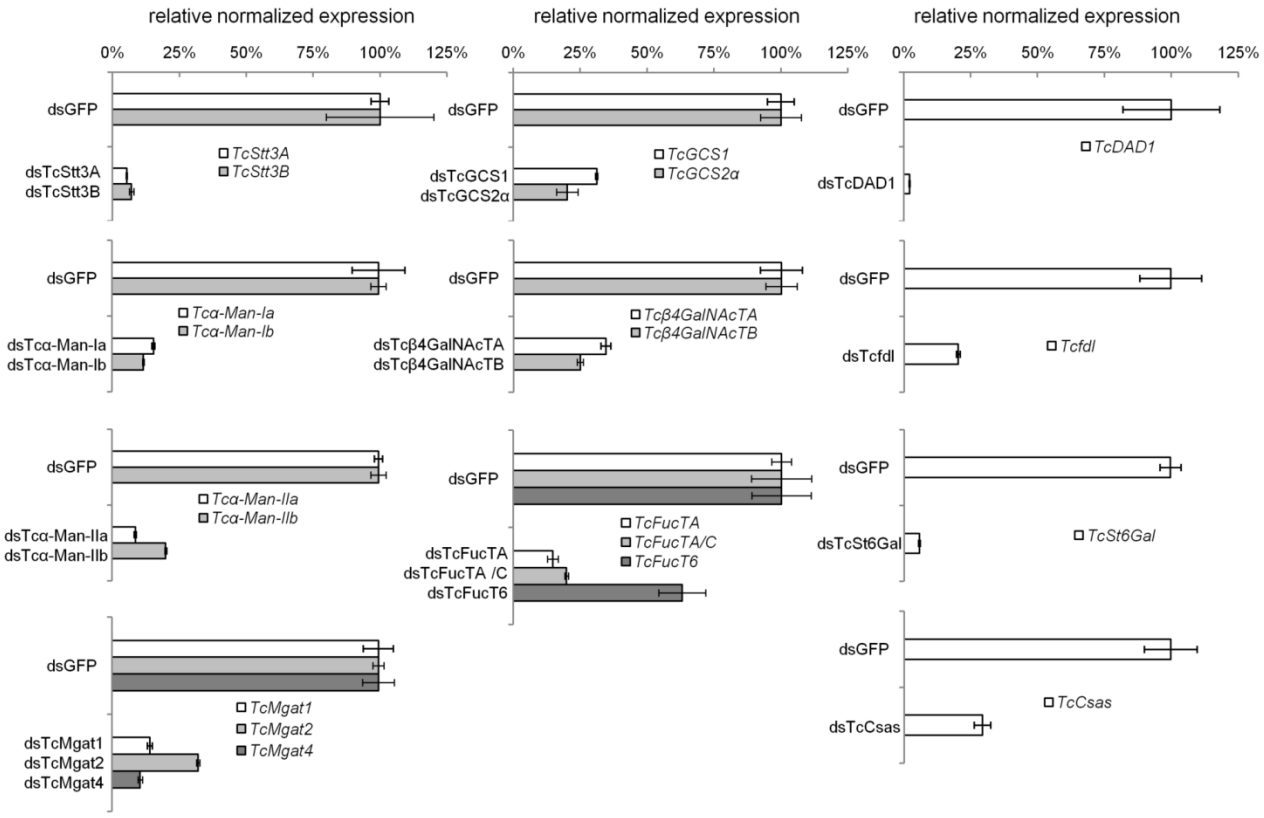
Supplementary Fig. S5.1. Phylogeny of proteins putatively involved in N-glycan attachment and processing in *Tribolium castaneum*. The evolutionary history was inferred by using the Maximum Likelihood method based

on the Poisson correction model. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Hs - homo sapiens, Dm – Drosophila melanogaster, TC0##### are putative *Tribolium castaneum* orthologs obtained from http://bioinf.uni-greifswald.de/gb2/gbrowse/tcas4. *Tribolium* orthologs studied in this chapter are in brackets. (a) DAD1 orthologs. RefSeq accessions: Hs DAD1 - NP 001335.1; Dm lethal (2) k12914 - NP 609222.1; Dm SP2353 - NP 611082.2; Hs NAALDL2 - XP 011510919.1. The latter two sequences are remotely related and are used as outgroups to improve the quality of tree. (b) Orthologs of STT3 subunits. RefSeq accessions: Hs STT3A - NP 689926.1, NP 001265433.1; Hs STT3B - NP 849193.1; Dm CG1518 - NP 608425.1; Dm OstStt3 - NP 524494.1. (c) Orthologs of α-glucosidases. RefSeq accessions: Hs MOGS/GCS1 - NP_001139630.1; Hs GANAB - NP_001265121.1; Dm GCS1 - NP_001245621.1; Dm GCS2α - NP_652145.1. (d) Orthologs of GlcNAc transferases, RefSeq accessions: Hs MGAT1 - NP 002397.2; Hs MGAT2 - NP 002399.1; Hs MGAT4A - NP 036346.1; Hs MGAT4B - NP 055090.1; Hs MGAT4C - NP 037376.2; Hs MGAT4D - NP 001264282.1; Dm Mgat1 - NP 525117.2; Dm Mgat2 - NP 001263096.1; Dm CG9384 - NP 648720.3; Dm CG17173 - NP 648721.1; Dm CG7766 - NP 001138180.1. (e) Orthologs of N-acetylglucosaminidases, RefSeq accessions: Hs Hexo1α - NP 000511.1; Hs Hexo1β - NP 000512.1; Dm Hexo1 - NP 728975.1; Dm Hexo2 - NP 525081.1; Dm fdl - NP 001286351.1. (f) Orthologs of CMP-sialic acid synthase, RefSeq accessions: Hs CMAS - NP 061156.1; Dm Csas - ACH92492.1; Dm Gip - NP 511106.1 (distantly related sequence used as an outgroup for tree construction). (g) Orthologs of Gal/GalNAc transferases RefSeq accessions: Hs beta4GalT-2 isoform b - NP 003771.1; Hs beta4GalT-2 - NP 085076.2; Hs beta4GalT-1 - NP 001488.2; Hs beta4GalT-3 - NP 003770.1; Hs beta4GalT-4 - NP 003769.1; Hs beta4GalT-6 - NP 004766.2; Hs beta4GalT-5 - NP 004767.1; Hs beta4GalT7 - NP 651319.2; Dm beta4GalNAcTA - NP 610946.1; Dm beta4GalNAcTB - NP 651657.1; Dm beta4GalT7 - NP 009186.1; Dm Pgant4 - NP 001137779.1; Dm Pgant6 - NP 647749.2; Dm GalNAc-T1 - NP 611043.1; Dm GalNAc-T1 isoform C - NP 001286461.1. (h) Orthologs of sialic acid transferases, RefSeq accessions: Hs ST6Gal-II - NP 115917.1; Hs ST6Gal-II isoform b - NP 001135824.1; Hs ST6Gal-I - NP 003023.1; Hs ST6Gal-I isoform b - NP 775324.1; Hs ST8SIA4 - NP 005659.1; Hs ST8SIA2 - NP 006002.1; DmSt6Gal - NP 726474.1; DmSt6Gal isoform b - NP 523853.1. (i) Orthologs of Fucosyltransferases, RefSeq accessions: Hs FUT3 - NP 000140.1; Hs FUT5 - NP 002025.2; Hs FUT6 - NP 000141.1; Hs FUT4 - NP 002024.1; Hs FUT7 - NP 004470.1; Hs FUT9 - NP 006572.2; Hs FUT11 - NP 775811.2; Hs FUT10 - ref|NP 116053.3; Hs FUT8 - NP 835368.1; Dm FucTA - NP 648754.2; Dm FucTC - NP 001036320.3; Dm FucTD - NP 612107.1; Dm FucTB - |NP 609288.4; Dm FucT6 - NP 572740.1.

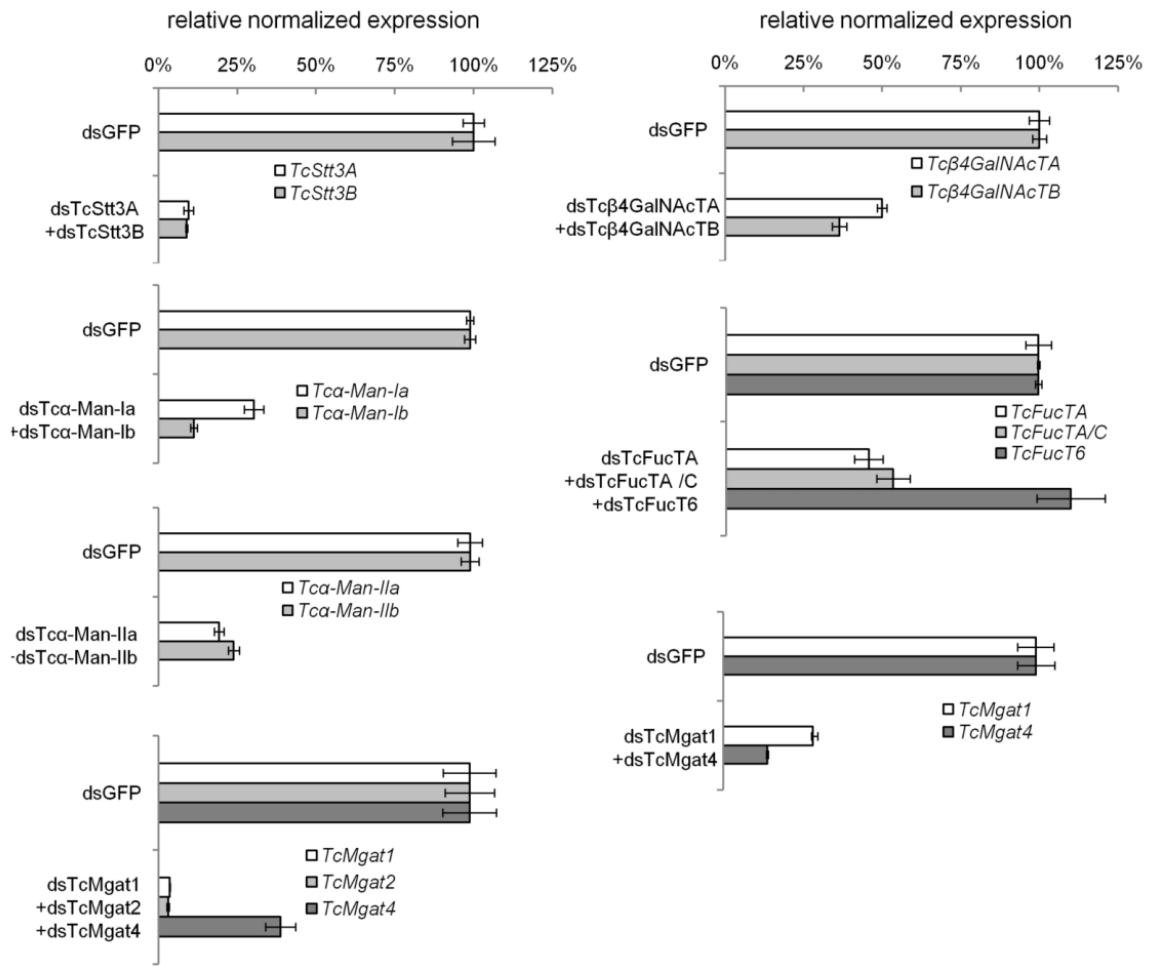


Supplementary Fig. S5.2. Phylogeny of putative *Tribolium* α-mannosidases. The evolutionary history was inferred by using the Maximum likelihood method based on the Poisson correction model. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are

shown next to the branches. Hs - homo sapiens, Dm – Drosophila melanogaster, TC0##### are putative *Tribolium castaneum* orthologs obtained from http://bioinf.uni-greifswald.de/gb2/gbrowse/tcas4. *Tribolium* orthologs studied in this chapter are in brackets. RefSeq accessions: Hs MAN2B1 - NP 000519.2; Hs MAN2B2 - AAH94773.1; Hs MAN2A1 - NP 002363.2; Hs MAN2A2 - NP 006113.2; Hs MAN2C1 - NP 006706.2; Hs MAN1B1 - NP 057303.2; Hs MAN1A1 - NP 065112.1; Hs MAN1A2 - NP 006690.1; Hs MAN1C1 - NP 005898.2; Hs EDEM2 - NP 060687.2; Hs EDEM3 - XP 005245556.1; Dm LManIII NP 609250.2; Dm LManIV - NP 609251.2; Dm LManV - NP 609252.1; Dm LManVI - NP 609253.1; Dm LManI - NP 609407.1; Dm LManII - NP 609408.1; Dm α-Man-IIb - NP 650494.2; Dm α-Man-IIa - NP 524291.2; Dm Edem1 - NP 001259152.1; Dm Edem2 - NP 609611.1; Dm α-Man-Ia - NP 727408.2; Dm α-Man-Ib - NP 651667.1; Dm α-Man-Ic - NP_733331.1



Supplementary Fig. S5.3. Efficiency of RNAi mediated single gene knockdowns. 4th instar larvae were injected with gene specific dsRNAs to induce gene silencing or with dsGFP in case of control. Total RNA was isolated 6 days post injection. Relative gene expression was quantified with qRT-PCR using TcRpL32 and TcRpS6 as reference genes and normalized to control. Data are averages ± SD (n=3).



Supplementary Fig. S5.4. Efficiency of RNAi mediated knockdowns of multiple genes. 4th instar larvae were injected with a mixture of two or three indicated dsRNAs to induce gene silencing or with dsGFP in case of control. Total RNA was isolated 6 days post injection. Relative gene expression was quantified with qRT-PCR using TcRpL32 and TcRpS6 as reference genes and normalized to control. Data are averages ± SD (n=3).

Supplementary Table S5.1. dsRNAs utilized in RNAi experiments and primers used to obtain them.

Target gene	*Tribolium* accession number		Primer sequence	dsRNA length
TcDad1	TC016366	F	taatacgactcactatagggTGCCACAGGTGACTTCAGTT	367
		R	taatacgactcactatagggATGTAGGACCACATGGGCG	
TcGCS1	TC011354	F	taatacgactcactatagggCAACAACCAATCGTGAGCTG	374
		R	taatacgactcactatagggGACCAATCGGTATTCGGGAT	
Tcα-Man-Ia	TC011089	F	taatacgactcactatagggCTTGGGGCAAGAACGAACT	381
		R	taatacgactcactatagggCCAGTCTGTGTCTGAAACGC	
Tcα-Man-Ib	TC014283	F	taatacgactcactatagggCTAGTCTCGACCGAATCCGA	385
		R	taatacgactcactatagggTGTCAATCGAGTCAACAATGC	
Tcα-Man-IIa	TC009186	F	taatacgactcactatagggTGTCCATGGAAAGTGCCC	365
		R	taatacgactcactatagggAAAAATCGCCCGATAACGA	
Tcα-Man-IIb	TC002991	F	taatacgactcactatagggGGCCATAGGCCTCACAATAA	423
		R	taatacgactcactatagggTTCATTATCTGGCGGGAGAC	
TcFucTA/C	TC005825	F	taatacgactcactatagggAATCGAAATTTTCAGCGTCG	435
		R	taatacgactcactatagggCACAATTCGAGCCCATGAT	
TcFucTA	TC014343	F	taatacgactcactatagggATGCCTCCGCGACTCTCT	378
		R	taatacgactcactatagggGGCAAGAATCACTTTCTCCG	
Tcβ4GalNAcTA	TC006388	F	taatacgactcactatagggACGCTCCATCTCAAGAAAGC	500
		R	taatacgactcactatagggTCTTCCGGCAACAAATCAAT	
TcST6Gal	TC014265	F	taatacgactcactatagggACGCTCATGATTTGGTTTTG	374
		R	taatacgactcactatagggCAAAAACCCCGACGATAGTG	
TcCsas	TC015995	F	taatacgactcactatagggTCTTTTTCATGCAAATCCAGC	395
		R	taatacgactcactatagggACGTACACTGCACCAAAGCA	
			Eupheria catalog number	
TcStt3A	TC010433		iB_01689	479
TcStt3B	TC009183		iB_01492	497
TcGCS2α	TC032148		iB_00458	501
TcMgat1	TC009001		iB_01454	489
Tcfdl	TC009779		iB_04695	490
TcMgat2	TC001867		iB_03261	482
TcMgat4	TC003870		iB_00631	483
TcFucT6	TC008521		iB_04509	481
Tcβ4GalNAcTB	TC006987		iB_06724	391

Eupheria catalog number refers to dsRNAs obtained from Eupheria Biotech (Dresden, Germany). F, R – forward or reseverse primer.

Supplementary Table S5.2. Primers used for qRT-PCR studies of gene expression.

Target gene		primer sequence	Amplicon length	Efficiency (%)
TcDad1	F	CCACAGGTGACTTCAGTTATCG	147	92.5
	R	AAAAGTGCCCACAAGACAGC		
TcStt3A	F	TGTGCTACTACCGCTTTGGG	148	94.7
	R	ATACGCACGAGCCAGTGTTC		
TcStt3B	F	CCCGCTTCATGTGTTTGTCC	141	101.1
	R	TCGCTGGTACGAATGGGTTG		
TcGCS1	F	ATCGGCCCTCAGTAACATGC	132	95.5
	R	AAACTTCGAGACGGGACTGC		
TcGCS2α	F	GGGAAGGGGAACAAGAGGTG	103	97.6
	R	TCAACACCCACTGCTTCAGG		
Tcα-Man-Ia	F	AATGCAAGCTGTCCTCCAAC	124	97.6
	R	CAGACCACCCGAAAAACAAC		
Tcα-Man-Ib	F	ACGTGCTATTTGCCTGGAAC	83	98.9
	R	TTCGGGTGCTAGAAATGTGG		
TcMgat1	F	ACCAGGAGTTGAAAGAGGGC	147	94.1
	R	TACCCTTGTACGCAGTTCGG		
Tcα-Man-IIa	F	CAAAGCGAAGAAAGGAGTGG	104	97.2
	R	ACGATCGGTCCTTGAATCAC		
Tcα-Man-IIb	F	GGAGTTCCCCTGTGATACGC	124	95.2
	R	ACTCACAGCACAGTCGTAGC		
Tcfdl	F	CCCAGAGTTTTCCTTTGGTG	123	91.6
	R	CTCTGATTCGGGCATATTCG		
TcFucT6	F	ATTCGTTGCGTGGAGACCAG	117	103.5
	R	TGGGCTCTTCTTTCGCACTC		
TcFucTA/C	F	ACGTCACGGAGAAGCTTTGG	83	94.2
	R	CCTGTAACTCGCCACGGAAG		
TcFucTA	F	CACCCTCCAGTTTCCAGACC	136	92.0
	R	CGACCAGATCGCTGTCTCTG		
TcFucTB	F	ACAACGAACAACTGCTCCAC	80	91.6
	R	GATGTAGTCGTCGCAAATGG		
TcFucTC	F	CGACAAACCAACCTTTCTGG	71	90.6
	R	GGCTTTGGAACATTGGTGTC		
TcMgat2	F	CCCATACTCGATCCAAACACATCC	112	97.7
	R	GAGGGCATTGTTGCATTTCTGG		
TcMgat4	F	GGTGCACTACAAACCTTCGC	147	94.3
	R	ATTCCTGAGACGACTTCCGC		
Tcβ4GalNAcTA	F	GCAAGATACACGATGCTGACG	92	91.0
	R	TCCTTGTCGAAGCGTTTGGG		
Tcβ4GalNAcTB	F	CGGTTGTGTGAGCAAACCAAG	103	97.1
	R	CGATGAAATCGCCACAACTCC		
TcST6Gal	F	CTCACCGAGCAGACTTAGGAG	124	94.0
	R	GCGTTTAGTGACCCTGGTTG		

TcCsas	F	CAAGGGAAAGGAGTGTGTCTTC	119	92.5
	R	CTTGTCTTCGTGGTCGATTG		
TcRps6	F	GAATATCGGGCGGGAACGAC	150	95.7
	R	TGCACCCTCTAACTGACTTGC		
TcRpL32	F	ACCAGTCTGACCGTTATGGC	114	94.2
	R	CGTAGCCAATGTTGGGCATC		

F, R – forward or reseverse primer.

5.5. Discussion

The investigation of mRNA levels of genes involved in the N-glycosylation process in *T. castaneum* indicated that the expression of most of these genes changes between the life stages and thus appears to be developmentally regulated. Most importantly, genes involved in N-glycan processing were upregulated in adults compared to larvae, but those coding for proteins responsible for N-glycan attachment were expressed at similar levels in adult and larval stages. mRNA levels do not always correspond to protein abundance, however usually upregulation at mRNA level results in higher protein expression (Koussounadis et al., 2015). Therefore increased expression of N-glycan processing genes seems to be responsible for the previously observed enrichment of trimmed and fucosylated N-glycans in the adult beetles. This observation points towards the conclusion that N-glycosylation is involved in metamorphosis in the red flour beetle. There are no studies focused on changes in expression of N-glycosylation related genes during the development of other insects although large-scale transcriptome analyses in *D. melanogaster* also pointed towards an upregulation of some of genes encoding N-glycan processing enzymes in later developmental stages (Gelbart and Emmert, 2013; Graveley et al., 2011).

The correlation between the gene expression changes and the N-glycan abundance was not clear for all of the steps of the N-glycan processing pathway. For instance, all genes involved in complex N-glycan production were upregulated in adults although mass spectrometry did not show an increase in complex N-glycan content in adults compared to larvae (Chapter 4). Additionally, sialylated N-glycans could not be detected even though *TcCsas* and *TcST6Gal,* which encode enzymes involved in sialic acid activation and attachment, were expressed throughout the development. The analysis of gene expression changes was performed using relative rather than absolute quantitation approach, yet it could be roughly estimated that, even at their peak expression, the transcripts of *TcCsas* and

TcST6Gal were 20-100-fold less abundant than those of genes involved in the earlier steps of the pathway. This observation is in agreement with the low level and restriction of expression to subset of cells in the central nervous system of the sialyltransferase ortholog in *D. melanogaster* (Repnikova et al., 2010).

In line with minute amounts of complex N-glycans and the generally low mRNA expression levels of the genes involved in their production we did not observe any appreciable phenotypes caused by disruption of this phase of the pathway through RNAi. This lack of phenotypes in *Tribolium* metamorphosis, in contrast with serious neurophysiological defects and abnormal movement caused by mutations in ST6Gal and β4GalNAcTA genes in *Drosophila* (Haines and Stewart, 2007; Repnikova et al., 2010; Yamamoto-Hino et al., 2015), points out that the complex glycans might be primarily involved in insect embryonic development. On the other hand the highest expression for most of the genes was observed in pupae, thus it would be interesting to analyze the N-glycan composition in this life stage, which might be even more shifted towards highly processed oligosaccharides and potentially contain higher levels of complex N-glycans.

The RNAi approach revealed multiple development and metamorphosis related functions for other genes involved in N-glycosylation (Fig. 5.11). The N-glycan attachment to a protein catalyzed by OST (oligosaccharyltransferase) can be considered as the initial step in the N-glycosylation pathway. Single knockdowns of *Tribolium* genes coding for the Stt3A and Stt3B isoforms of the catalytic subunit of OST caused only minor defects. However, double silencing of both isoforms resulted in a complete blockage of the larval growth and prevented development into pupae. Very similar phenotypes were observed after silencing of the *TcDad1*, an essential subunit of the OST. This observation indicates that the attachment of N-glycans is crucial for larval growth and proper development. Additionally, this experiment showed that isoforms of Stt3 have overlapping roles and each isoform can compensate for the lack of the other. Similar observations were made for Stt3A and B subunits in mammals and in *Arabidopsis thaliana* (Ruiz-Canada et al., 2009). Yet, this is in contrast to *Caenorhabiditis elegans* which has a single Stt3 isoform (Kelleher et al., 2003) and it also differs from *D. melanogaster* which has two Stt3 isoforms but mutation in one of them, the ortholog of Stt3B, is enough to cause lethality (Spradling et al., 1999).

The processing of the protein linked N-glycan is initiated by α-glucosidases, which sequentially remove three glucoses capping the native oligosaccharide. These deglucosylation reactions are crucial for N-glycoprotein folding in the calnexin/calreticulin (CNX) cycle. Silencing of each of the *Tribolium* α-

glucosidases, GCS1 and GCS2, affected larval growth and blocked the development at the late larval/prepupal stage. Notably, morphological characteristics of the dead prepupae suggested that pupation was initiated but could not be completed. A comparable phenotype was observed for RNAi of TC012278 (Dönitz et al., 2014), a putative *Tribolium* ortholog of calreticulin, one of the key players in N-glycoprotein folding (Helenius and Aebi, 2004) which supports the importance of N-glycoprotein maturation in the ER for successful metamorphosis. Interestingly, knockdown of the GCS1 ortholog in *C. elegans* affected worm growth, movement and viability, which was attributed to an alteration of the entire N-glycome, compromised protein quality control and chronic ER stress (Katoh et al., 2013). The only studies of α-glucosidases in *Drosophila* involved transgenic RNAi restricted to the notum (dorsal part of the thoracic segment). This did not prevent the pupation but resulted in some lethality before the end of the pupal stage (Mummery-Widmer et al., 2009) suggesting timing differences in the requirement for the first steps of N-glycan maturation. *GCS1* and *GCS2α* are required for pupation in *Tribolium* while in *Drosophila* they appear to be essential for pupa-adult transition.

Silencing of class I α-mannosidases involved in trimming of high mannose glycans did not affect larval growth or metamorphosis but disrupted the formation of wings and wing covers and affected adult locomotor behavior. This suggested that some glycoproteins require processing of the attached high mannose N-glycan to properly function in development of pupal and adult appendages and potentially in the nervous system. In *D. melanogaster* mutations of the *α-Man-Ia* ortholog are also not lethal, but affect the immunity, nervous system, wings and eyes (Kerscher et al., 1995; Mortimer et al., 2012; Rosenbaum et al., 2014). These relatively weak phenotypes were explained by the presence of an alternative N-glycan processing pathway inferred from the observation that N-glycan processing is not blocked completely in α-Man-Ia mutant flies (Roberts et al., 1998). It was suggested that an unusual class II α-mannosidase (α-Man-IIb or α-Man-III) could be involved in this alternative trimming route (Kawar et al., 2001; Nemcovicova et al., 2013).

The N-glycan profile of *Tribolium* adults after Man-Ia RNAi supports this notion. In the absence of Man-Ia the N-glycan processing was clearly less efficient, as indicated by accumulation of $Man_{9-6}GlcNAc_2$ glycans. Yet, it was not blocked completely. In the RNAi adults the complex and paucimannose N-glycans were present but at 3-20 fold lower levels compared to the control. This lack of complete blockage of N-glycan processing is in line with the hypothetical partially compensatory activity of *Tcα-Man-IIb*.

Overexpression of a mammalian ortholog of this gene, α-Man-IIx, in CHO cells lead to a reduction of the complex N-glycan content caused by an accumulation of $Man_3GlcNAc_2$ and $Man_4GlcNAc_2$ (Oh-eda et al., 2001) that are not efficiently processed by the subsequent enzyme Mgat1 (Altmann et al., 1993). In invertebrates Mgat1 activity is required for a subsequent fucose attachment and the synthesis of complex glycans (Paschinger et al., 2005; Schachter, 2010; Zhang et al., 2003). In *Tcα-Man-Ia* RNAi beetles $GlcNAcMan_5GlcNAc_2$ was undetectable while $Man_4GlcNAc_2$ was enriched, so indeed *Tribolium* Man-IIb can be responsible for the alternative N-glycan processing and the reduction of paucimannose and complex N-glycans by a similar mechanism to that observed in CHO cells.

Additionally, the profile of the N-glycans after *Tcα-Man-Ia* silencing indicated that even though there is a significant redundancy between N-glycan processing enzymes, knockdown of a single gene can disrupt the entire pathway including the fucosylation reactions occurring far downstream.

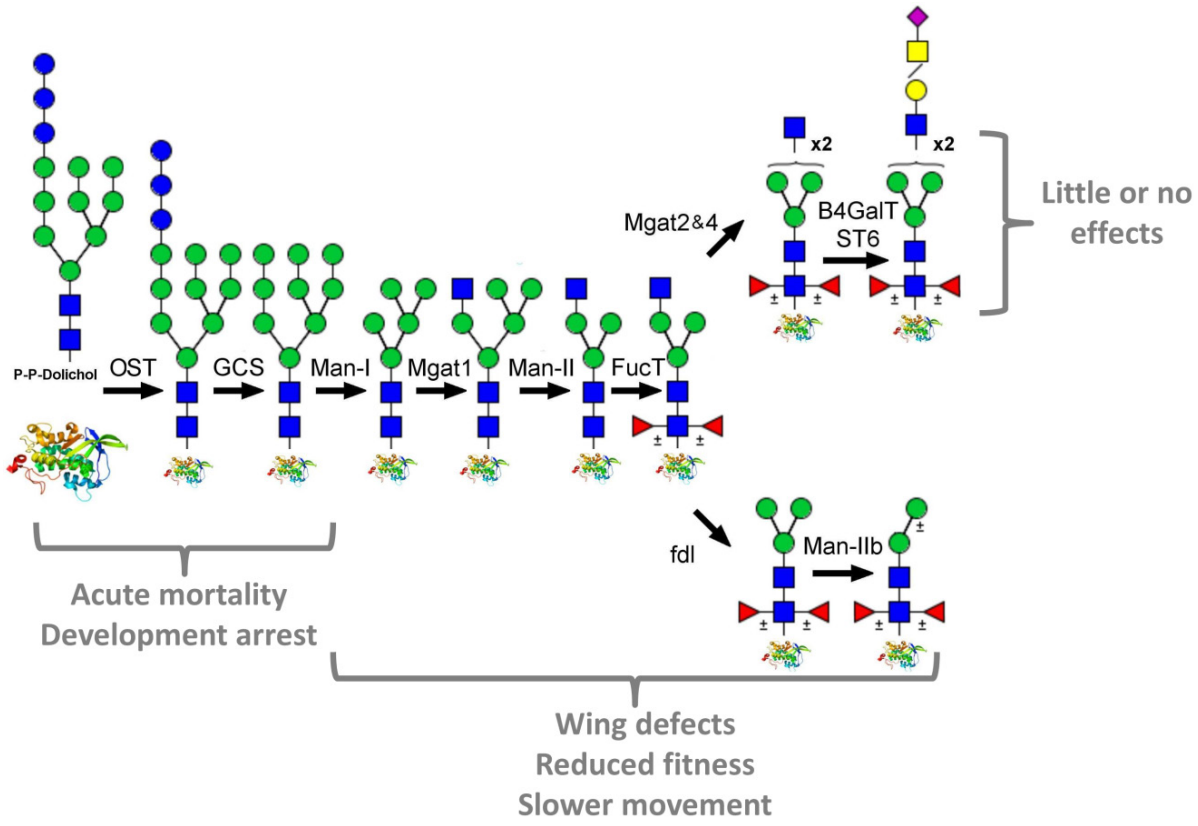


Fig. 5.11. Schematic representation of phenotypes caused by disruption of genes involved in N-glycosylation pathway. Note that the RNAi earlier the genes that occur early in the pathway result in more severe the phenotypes. ■ or GlcNAc – N-acetylglucosamine, ● or Man – mannose, ● or Glc – glucose, ▲ or Fuc – fucose, □ or GalNAc – N-acetylgalactosamine, ○ or Gal – galactose, ◆ – sialic acid, Asn – asparagine in a polypeptide chain, P-P-dolichol – lipid carrier of N-glycan precursor.

It is thought that Man-Ib is mainly involved in the folding of N-glycosylated proteins in the ER (or ERQC) while Man-Ia is responsible for the trimming of high mannose N-glycans attached to proteins in the Golgi apparatus (Benyair et al., 2015a; Kawar et al., 2000). Yet, the lack of mortality in case of single knockdowns and severe phenotypes (developmental arrest and mortality) after double knockdown of both class I mannosidase genes indicated that the proteins they encode might have overlapping functions. Even more importantly, it seems clear that $Man_9GlcNAc_2$ N-glycans need to be further trimmed for the metamorphosis to be completed and *Tcα-Man-IIb* does not efficiently contribute to this process in the absence of both *Tcα-Man-Ia* and *Tcα-Man-Ib*.

RNAi of α-Man-IIa in *D. melanogaster* was lethal (Yamamoto-Hino et al., 2015) while genes coding for Mgat1, α-Man-IIa, α-Man-IIb and fdl were shown not to be required for fruit fly survival but were implicated in nervous system development, locomotor behavior, longevity and immunity (Leonard et al., 2006; Mortimer et al., 2012). In our hands RNAi of these genes in the red flour beetles induced no mortality but affected development of pupal wing covers and adult walking speed. The reduction of mobility of RNAi beetles indicated evolutionary conserved roles of these genes in the nervous system, while wing cover phenotypes indicate novel roles for N-glycosylation in the development of elytra. The sequential action of Mgat1, α-Man-II and fdl may also be bypassed by the activity α-Man-IIb, which could explain the lack of lethality in beetles after single knockdowns. However, co-silencing *Tcα-Man-IIa* and *Tcα-Man-IIb* did not evoke any enhanced phenotypes compared to single knockdowns. This suggests that synthesis of paucimannose N-glycans is not necessary for the development and survival or on the other hand may advocate the presence of yet additional enzyme(s) involved in the process.

In the previous chapter we observed that adults contained more N-glycans carrying core one or two fucose residues. In the dipterans *Drosophila melanogaster* and *Anopheles gambiae* and in lepidopteran Sf-9 cells (Kurz et al., 2015; Paschinger et al., 2005; Staudacher and Marz, 1998) it was shown that difucosylated N-glycans are produced in a strict order. First, FucT6 attaches the N-glycans with a α1,6 fucose followed by the addition of a second fucose in α1,3 position by FucTA. Consistently with the increased fucosylation, *TcFucT6* expression was upregulated in adults compared to larvae, but on the contrary *TcFucTA* expression was not. The latter gene was the only one with the highest relative expression during the larval stage. Thus it is likely that core fucosylation is in part performed by other *Tribolium* fucosyltransferases, particularly by *TcFucTA/C* which was upregulated in adults. Interestingly,

this protein has no close ortholog among *Drosophila* proteins and appears to have differentiated from a common ancestor sequence of FucTA and FucTC. Intriguingly, silencing of *Tribolium* FucTA and FucTA/C genes did not cause any detectable defects, which points towards additional putative fucosyltransferase genes, such as *TcFucTB* and *TcFucTC* potentially contributing to core fucosylation. However, *Drosophila* orthologs of these genes have not been proven to encode functional proteins yet. The best way to verify the importance of these proteins for glycan fucosylation would be to analyze the N-glycans produced by beetles after RNAi of a given gene and combinations thereof.

In conclusion, we demonstrated that N-glycan attachment to proteins is crucial for growth and survival of the red flour beetle, *Tribolium castaneum.* Additionally, we observed for the first time that the global N-glycosylation profile changes towards more modified paucimannose glycans during transition from larval to adult stages. This increased N-glycan processing is required for successful pupation and adult eclosion and appendage development. Altogether our data provide a new link between protein N-glycosylation and execution of metamorphosis, and indicate that genes involved in this process may be promising targets for RNAi-mediated pest control.

Chapter 6

General discussion and perspectives for future research

N-glycosylation is one of the most important posttranslational modifications of proteins. It is assumed that even more than half of all the proteins might be modified with an oligosaccharide chain (Apweiler et al., 1999). The basic principles underlying sugar attachment and modifications are common for eukaryotic organisms, yet the devil is in the details. Animals produce well above 20 (possibly closer to 100) different glycan structures and a significant share of them appears to be order-specific. For instance N-glycans carrying sialic acids are plentiful in vertebrates and very low abundant in insects. In contrast, the invertebrates are capable of synthesizing difucosylated N-glycans which is not the case for the vertebrates (Varki and Lowe, 2009). N-glycosylated proteins are involved in a multitude of processes including embryonic development, immunity, and nervous system function and in metamorphosis (Sarkar et al., 2006).

Since glycosylation appears crucial for the functionality of proteins it is possible that interference with this process might be a successful strategy to kill pest insects. However, the current knowledge on N-glycosylation in insects comes mostly from *Drosophila* or insect cell lines studied in the context of recombinant protein production. Yet, even in those models the understanding of N-glycosylation is far from complete. Scarce data regarding N-glycosylation in other insects indicate that N-glycan patterns can be highly variable between different insect orders. Thus the data obtained for *Drosophila* cannot be simply extrapolated to distantly related pest insects such as moths, aphids or beetles. Consequently, prospective disruption of glycosylation for pest control purposes requires detailed research on agriculturally relevant pest insect models.

This thesis addressed these issues at two levels. First, plant and fungal lectins, or glycan binding proteins, were tested for their insecticidal activity against a selection of insect cell lines and against *Tribolium castaneum*, the red flour beetle, a recalcitrant pest of dry food storage facilties. Second, we performed detailed investigation of the functions of N-glycosylation in *T. castaneum* by studying changes in N-glycan composition, expression of genes involved in the N-glycosylation pathway. Additionally, we analyzed the consequences of glycosylation disruption through RNA interference and its applicability for pest control.

This chapter situates the obtained results in a broader context, proposes future research directions and discusses application perspectives in insect management.

6.1. Lectin interactions with insect cells

Using an array of fluorescently labeled lectins, we showed in Chapter 2, that glycan epitopes are distributed asymmetrically in insect midgut epithelial cells. As demonstrated by the prominent binding of RSA and SSA lectins GalNAc/Gal containing oligosaccharides are enriched in the microvilli present at the pole of the of the epithelial cells that is exposed to the gut lumen. SNA-I binding suggested also that sialylated glycans might also be present in this zone of the cell membrane. On the other hand lectins that recognize high mannose N-glycans, GNA and HHA, bound predominantly to the basal pole of the epithelial cells. The high abundance of GalNAc/Gal glycans exposed to the lumen of the midgut justifies the use of lectins capable of recognizing this epitope as potential insecticidal toxins disrupting gut cell function. This is in line with the observation of toxicity of these lectins to cultured midgut cell line as shown in Chapter 2, and in previous reports (Hamshou et al., 2013).

These experiments indicated that lectins labeled with fluorescent tags and confocal microscopy can be effectively utilized to study the microheterogeneity and spatial distribution of sugar motifs at the single cell level, which is not possible yet with other thechnologies as mass-spectrometry-based (MALDI) imaging technologies. In MALDI-imaging methodology specimen (or tissue section) is covered with matrix compound which absorbs laser energy and promotes the ionization of the analyzed compounds. The mass spectrometer analyzes the ion composition in a specimen “spot by spot” and then records the localization creating a two-dimensional map of the analyte content. Analysis of N-glycans using this technology requires tissue sectioning, proteolytic digestion, glycosidase treatment and preferably also derivatization of glycans to enhance their ionization efficiency. Such an extensive processing of tissue might result in diffusion of glycans and distort the actual resolution. So far, the highest resolution reported of N-glycan analysis was 50 µm (Powers et al., 2014). This pixel size is roughly equal to the size of an insect midgut columnar cell, thus this technology requires significant resolution improvement to be useful for precise localization of N-glycans in different regions of the cell membrane.

Binding patterns of the used lectins make them potentially useful in labeling of different regions of the midgut cell membrane which are not clearly distinguishable otherwise. Microvilli can be visible in traditional transmitted light microscopy, but this approach does not allow optical sectioning and 3D reconstruction of the cell structures (Robinson, 2001). Thus fluorescently-labeled RSA and SSA may be particularly effective for protein localization studies in combination with antibody detection in the cell membrane of the primary midgut cells.

6.2. Mechanisms of lectin toxicity to insects cells

In experiments using various cultured insect cell lines we observed that lectins not only inhibit cell proliferation but also cause cell death. The morphological changes and DNA fragmentation caused by the exposure of cells to lectins were not consistent with a single particular mode of cell death. The data indicated a mixture of autophagic, necrotic and apoptotic features. Detailed investigation of the mode of action of lectins against insect cells was not the main focus of this thesis, but it would be of great interest to investigate this topic further. Apoptosis could be confirmed by measurement of the activity of caspases-9 and -3 which are involved in induction and execution of apoptosis (Wu et al., 2011) and can be further distinguished from necrosis by staining with Annexin V (Krysko et al., 2008). Upregulation of the Atg8 protein could be used as a marker for autophagy (Park et al., 2015). Cell death can be either rapidly executed upon specific induction or could be delayed and occur as a result of an unsuccessful stress response and damage repair after prolonged exposure to harmful stimuli (Maiuri et al., 2007). Determination of the cell death mode induced by lectins and timing of these events could be another criterion for selection of the most promising lectins, since the proteins that would rapidly induce apoptosis or necrosis in insect culture cells would most likely cause acute toxicity to insects in vivo.

Another captivating issue that arose from this thesis was the contribution of the carbohydrate binding specificity to lectin insecticidal potency and mode of action. Two of the lectins toxic to *T. castaneum* cells, RSA and SNA-II are specifically recognizing glycans containing GalNAc/Gal groups, thus they should bind to the same receptors on the cell surface. Furthermore, since there are no additional functional domains in the structure of these proteins except for the carbohydrate binding domain, it could be expected that these lectins would act in a similar way. However, as tested via *in vitro* experiments SNA-II was 60 times more toxic than RSA, which suggests that next to the binding specificity additional factors might play a role in toxicity. These additional factors could be the affinity to target glycans (or strength of interaction), promiscuity - ability to interact with other than the preferred oligosaccharides or some yet uncharacterized structural features of lectin domains. Binding promiscuity might result in a higher number of receptors at the cell surface and, in turn, enhanced lectin internalization and toxicity (Sandvig et al., 2010). On the other hand some non-specifically bound glycans might be present on “non-productive” receptors. Binding with such receptors would not result in lectin internalization, but would trap the lectin at the cell surface potentially decreasing the overall toxicity (McClain et al., 2000). Conversely, internalization may be not necessary for lectin toxicity. It

was proposed for RSA and ricin that cell toxicity might by induced solely by lectin binding to the cell surface, interference with functions of protein receptors, induction of signaling pathways and siruption of membrane biology (Audi et al., 2005; Hamshou et al., 2013; Hamshou et al., 2012).

Another hint which possibly explains the differences between RSA and SNA-II comes from the work of Shang et al. (2015) in which it was shown that SNA-II can inhibit protein translation even though it does not contain a classical ribosome inactivating (RIP) domain. Such an activity has not been reported yet for RSA.

The importance of binding specificity is also intriguing in the case of the third lectin SNA-I. This lectin recognizes sialylated glycans which are synthesized in insects in minute amounts in contrast to the GalNAc/Gal motif (Kurz et al., 2015). Still, SNA-I was nearly 8-fold more toxic than RSA. This can lead to two contradictory conclusions. First, highly specific lectins require binding with the glycan receptors and can induce considerable toxicity even if the binding receptors are present at minimal amounts. In support of this theory, it was shown that expression of an SNA-I variant with a mutated glycan-binding site in tobacco resulted in reduced protection against the aphid, *Myzus nicotianae* (Shahidi-Noghabi et al., 2008). This provided a strong indication that the ability to bind glycans is important for its toxicity. SNA-I, next to four lectin domains, contain also four A-chains which have strong ribosome inactivating activity (Shang et al., 2015). This raises the second possibility that the lectin could cause the toxicity owing to its RIP activity upon internalization via micropinocytosis even without association with membrane receptors (Jones, 2007).

The contribution of glycan-specificity for lectin toxicity could be resolved by disruption of the synthesis of glycans recognized by the lectins. If the binding to specific glycan receptors is crucial for lectin action, a reduction of such receptors would result in decreased lectin toxicity. This could be achieved through generation of knockout lines using CRISPR-CAS system (Wang et al., 2013) or by gene knockdown through RNAi by simply soaking TcA cells with the appropriate dsRNA (Silver et al., 2014). The *Tribolium* genome encodes over 10 putative GalNAc and galactose transferases involved in the production of glycans recognized by RSA and SNA-II, so it would be impractical to knock out all of these genes and impossible to silence all of them simultaneously by RNAi. Yet, there are only two genes coding for the UDP-galactose 4'-epimerase. This enzyme produces UDP-Gal and UDP-GalNAc which are substrates for all of the GalNAc and Gal transferases (Sanders et al., 2010). Therefore, a reduction of the expression of just two genes should result in a drastic decrease in abundance of all glycans containing GalNAc or galactose.

There is only a single gene coding for sialyltransferase in the *Tribolium* genome (*Tribolium* Genome Sequencing Consortium, 2008). Therefore, reduction of the sialylated glycan content through RNAi or knockout should be relatively straightforward.

Alternatively, the importance of lectin specificity could be tested by mutagenesis of amino acids involved in the glycan binding. If the glycan binding capability would be shut down completely, it would most likely preclude the glycan-based interactions and in turn would allow to infer on the contribution of non-receptor-mediated internalization of lectins. In a complementary approach, it could be theoretically possible to modify the glycan binding pocket of the lectin to accommodate a broader range of glycans. This could show whether binding of non-specific glycans increases or decreases the toxic activity.

6.3. Lectin activity against the red flour beetles

Contrary to the high toxicity observed in cell based assays, the three tested lectins were not highly active against *T. castaneum* larvae. Instead of the expected acute toxicity, SNA-II and RSA caused growth retardation and only minor mortality after chronic exposure of larvae to high doses of lectins. Moreover, SNA-I delivered in the diet caused no negative effects whatsoever. These observations are in line with previous reports that lectins are usually most toxic to hemipteran (e.g. aphids, planthoppers) insects and have much lower insecticidal activity against other insect orders (Bandyopadhyay et al., 2001; Fitches et al., 1997; Gatehouse et al., 1999; Hamshou et al., 2013; Shahidi-Noghabi et al., 2010b; Upadhyay et al., 2010). This low susceptibility of flour beetles against lectins might be a result of their adaptation to the food source. Currently, flour beetles are synanthropes (organisms that are associated with human-made habitats) mainly found in dried products storage facilities, but they are thought to be originally feeding on damaged grains (Trematerra et al., 2000). Numerous plant seeds including wheat and beans contain considerable amounts of lectins (Van Damme et al., 1998b). Therefore, to use such food source flour beetles must had developed resistance to toxic lectins. Altogether, the three tested lectins, RSA, SNA-I and SNA-II appear, so far, to have a higher application potential as resistance factors against aphids rather than against pest beetles.

6.4. Possible means to enhance lectin toxicity

An investigation of the fate of SNA-I, SNA-II and RSA in the *T. castaneum* midgut revealed multiple factors responsible for low toxicity caused by orally-delivered lectins (Fig 6.1). A first element was the reduction of the amount of lectins by partial digestion (or complete digestion in the case of SNA-I) by gut proteolytic enzymes. The undigested lectin was further constrained by the peritrophic matrix (PM) that lines the midgut epithelium. This protective structure appears to restrict passage of molecules larger than several nanometers. Additionally, proteins embedded in the PM are heavily glycosylated (Hegedus et al., 2009) and thus might be bound by lectins, which could have further reduced the amount of lectin that could interact with midgut epithelium cells. A low amount of lectin interacting with the midgut epithelium resulted in only minor cytotoxicity. Furthermore, insect midguts have remarkable regeneration ability, thus limited numbers of killed epithelial cells could be rapidly replenished by differentiation of the midgut stem cells (Hakim et al., 2010). Most likely an even less significant fraction of lectin could pass through the midgut to the hemolymph, therefore systemic toxicity was not induced by feeding. The lectin molecules that resisted digestion and did not pass through the PM were excreted with the feces.

Taking into account the mechanisms that restrict the lectin toxicity several methods can be proposed to enhance the lectin effectiveness (Fig 6.2). Lectin stability could be improved by the use of protease inhibitors. Such compounds of synthetic or biological origin have already been suggested as standalone pesticides interfering with insect digestion (Lawrence and Koundal, 2002; Oppert et al., 2011), but a combination with lectins might result in synergistic improvement of insecticidal effects.

The PM appears to be the major obstacle, thus improvement of lectin passing through this structure could be the best approach to increase lectin toxicity. Lectin permeation could be enhanced by disruption of the PM. The polysaccharide chitin is a major component of this protective layer, thus use of chitin degrading enzymes, e.g. bacterial chitinases (Ozgen et al., 2013) or enhancin produced by nuclear polyhedrosis virus (Toprak et al., 2012) might break down the structure and disable the barrier function of the PM. Moreover, it has been shown that RNAi mediated knockdown of *TcCHS2* (coding for chitin synthase) disrupted chitin production in the midgut of *T. castaneum* larvae resulting in an increase of PM permeability, inability to digest food and death of starvation (Arakane et al., 2005b). Similar, effects were achieved by silencing of two PM structural proteins that regulate its permeability (Agrawal et al., 2014) and by oral administration of diflubenzuron, a chitin synthesis inhibitor (Kelkenberg et al., 2015). Gene silencing can be achieved in several beetle species by the incorporation

of dsRNA into the diet (Baum et al., 2007), thus it seems possible to create transgenic plants with pyramided genes encoding lectins and any combination of protein inhibitors, chitinases and dsRNA to silence insect genes involved in the synthesis of PM components.

The PM is a porous structure and the larger the molecule the less efficient its passage through this barrier. The three tested lectins, SNA-I, SNA-II and RSA can assemble into octa-, tetra- and dimeric structures. In the SNA-I molecule subunits are kept together by intermolecular disulphide bridges. Thus, mutation of the cysteine residues that are involved in the formation of disulphide bridges would generate a lectin with significantly lower molecule dimensions and most likely better ability to pass the PM. In contrast RSA monomers are not interconnected by disulphide bridges but are held together by multiple hydrogen bonds formed by N-terminal aminoacids (Skamnaki et al., 2013). Thus for this lectins monomerization could be achieved by mutation (or removal) of the lectin N-terminus. Moreover, it seems justified to select toxins with a small molecular dimensions (<3 nm) similar to those present in spider venoms (King and Hardy, 2013). However, such venoms have rather low toxicity to midgut cells and are much more effective when they are delivered into the hemolymph. Fitches et al. (2010) successfully improved the transport of the toxin from the gut to the circulatory system and achieved a much higher toxicity by fusing the toxin with a small monomer of another lectin GNA known to accumulate in the hemolymph. Possibly, the fusion of the lectins tested in this thesis with GNA monomers could also result in enhanced toxicity. Yet, it would still require the use of some agent to disrupt the PM to counteract the increase in the molecular size of the fusion protein.

6.5. Direct delivery of lectins to the insect hemolymph

Contrary to the low oral toxicity, SNA-I and SNA-II effectively killed *T. castaneum* larvae after injection into the hemolymph. The doses of the lectins that were effective upon injection were up to 10,000-fold lower than those administered through the diet. This observation underlined the importance of lectin delivery to the insect circulatory system for high toxicity.

Obviously, manual injection of toxins into insects is irrelevant in terms of pest control, but theoretically lectins could be be delivered into insect bodies through other mechanisms than feeding. Surfaces of numerous plants, including crops such as wheat, soya, cotton, potatoes and beans bear epidermal appendages called trichomes (Wagner, 1991).

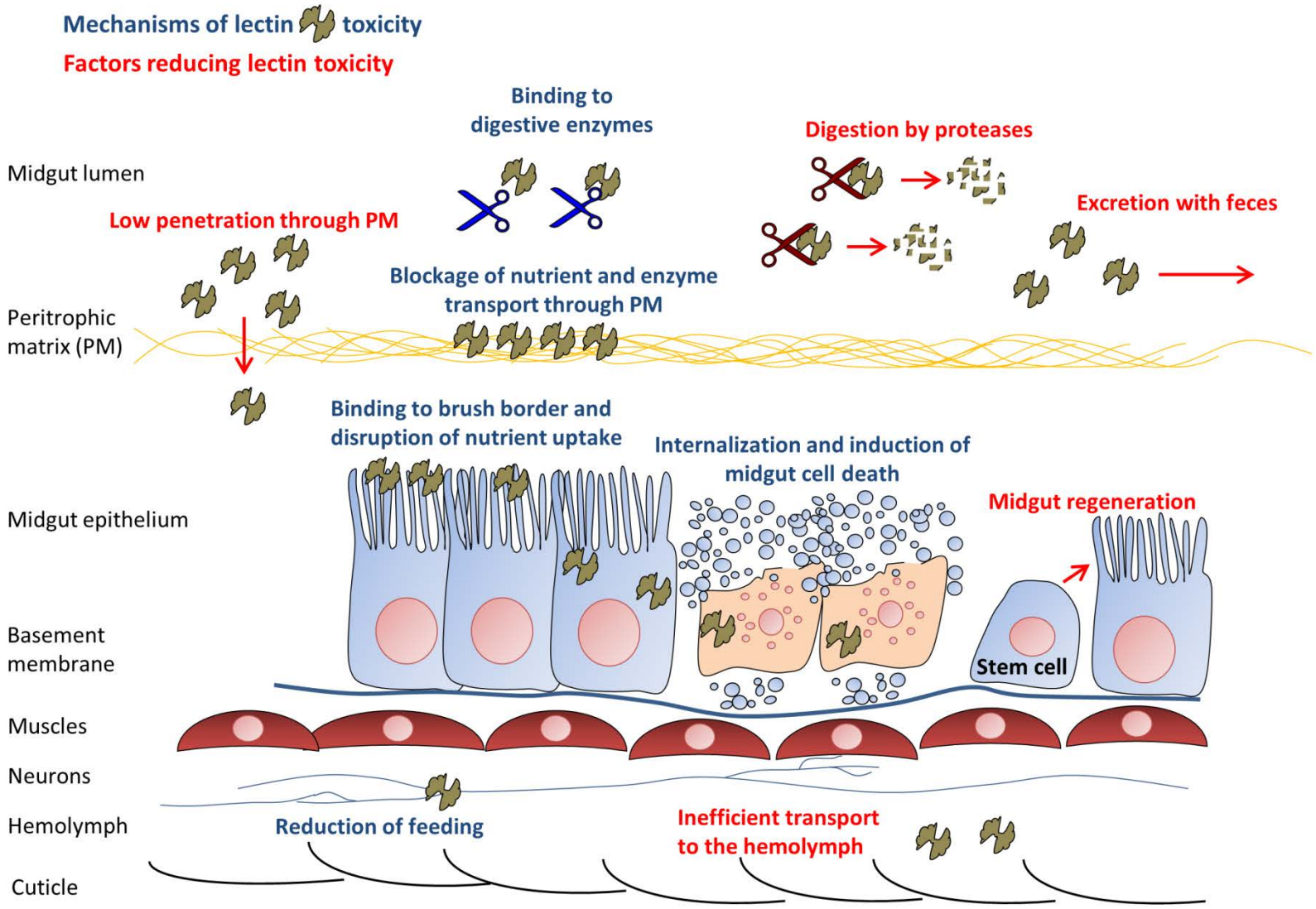


Fig. 6.1. Mechanisms responsible for lectin toxicity **and** factors restricting insecticidal effects.

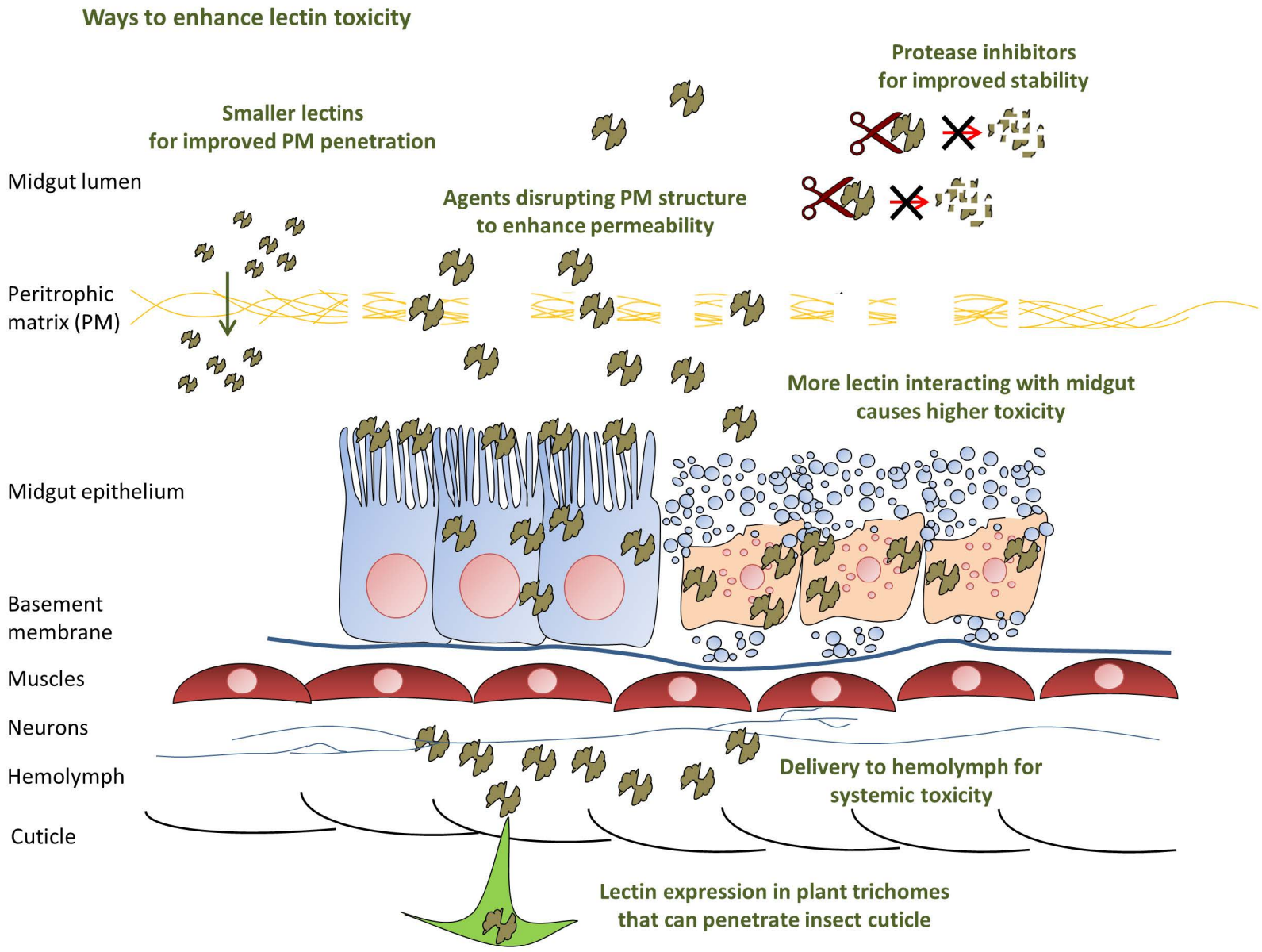


Fig. 6.2. Proposed means to increase insecticidal efficacy of lectins.

These hair or needle like structures may offer protection against pests by restricting their movement, feeding and oviposition (Hoxie et al., 1975). Yet, trichomes produced by some plants may penetrate the insect cuticle (Krings et al., 2003; Szyndler et al., 2013). Therefore, it seems plausible that production of lectins or other insecticidal agents in the trichomes could result in lectin delivery through the cuticle directly into the insect hemolymph. Interestingly, several promoters for the specific expression of proteins in the trichomes have already been described and could be used for that purpose (Gutiérrez-Alcalá et al., 2005; Wang et al., 2002).

As an additional benefit, next to enhanced protection against pests, expression of the toxic protein in the trichomes would result in selective killing of the insects that damage the leaves but would not affect pollinators, such as bees, which get into contact only with flowers. It has to be noted, however, that trichomes may also harm beneficial insects such as predatory beetles or wasps (Riddick and Simmons, 2014). Clearly, there are no silver bullets, so all the benefits and risks should be evaluated for every novel crop protection approach.

6.6. Toxin injection as a preliminary test for insecticidal activity

Feeding tests for oral toxicity often require milligram amounts of the purified proteins. Such quantity of protein might be difficult to obtain, especially at the early stages of screening. Therefore, it is a common practice to perform tests on cultured insect cell lines as this approach allows preliminary assessment of toxicity using 200-500 µg of a protein (Hamshou et al., 2013; Shahidi-Noghabi et al., 2010a; Smagghe, 2007). From our work with *Tribolium* it seems clear that lectin injection into larvae might be a complementary approach for the testing of protein toxicity. Although the manual injection might not be compatible for high-throughput screening, the major benefit of this technology is that it requires minimal amounts of the tested compound. As little as 20 µg of lectin is enough to test several concentrations on a large groups of larvae and in a few biological replications. Another advantage of this approach is that injection into the hemolymph puts the lectin into contact with virtually all the insect cells which allows determining the lectin potential to induce systemic effects or neurotoxicity. This is in contrast to cell based assays which usually involve a single cell type and appears to be particularly important in light of the results, obtained in Chapters 2 and 3, indicating that lectin effectiveness differ between cell types. This kind of assay has already been successfully applied for the initial testing of several insecticidal proteins such as ButalT, SFI1 (arachnid venoms) or chitinase (Fitches et al., 2008; Fitches et al., 2010; Pham Trung et al., 2006).

It has to be noted however that assays on insect cell lines and hemolymph injections will only give preliminary indication of potential cytotoxicity when interaction between the compound of interest and target cells is unrestricted. On the contrary, the predictive value of these assays is rather limited for the compounds delivered through the diet because of the gut defensive mechanisms and factors mentioned in Section 6.4. (Fig. 6.1.).

6.7. General considerations on the use of lectins in pest control

The introduction of genetically modified (GM) crops expressing the insecticidal Bt (or Cry) toxins from *Bacillus thuringensis* has been a major breakthrough for pest control. This approach resulted in increased crop yields and at the same time reduced the usage of conventional insecticides for the benefit of the farmer health and the environment (Christou et al., 2006). Yet, the rapid emergence of insect resistance against Bt toxins threatened further use of GM crops. Likewise, resistance gene pyramiding, or the introduction of plants expressing multiple entomotoxic proteins did not overcome the problem of the development of insect resistance. Resistance gene pyramiding strategy is based on the concept that insects will be less likely to become unsusceptible to multiple toxins compared to single ones. One of the reasons for the reduced efficiency of this approach is the similarity in mode of action and amino acid sequences of the expressed insecticidal proteins. Clearly, to utilize the full benefits of this strategy and effectively counteract rapid resistance development a combination of diverse insecticidal factors should be used (Brévault et al., 2013; Carrière et al., 2015). Lectins, protease inhibitors, arachnid venoms, viral proteins or dsRNAs are interesting candidates for additional elements of pyramids that could enhance insecticidal efficiency of those currently used (Baum et al., 2007; Christou et al., 2006). Another original approach was proposed by Mehlo et al. (2005). They engineered a fusion protein composed of a Cry toxin and a lectin domain of the ricin B chain which increased the number of binding targets for the fusion protein and resulted in higher toxicity. Using a combination of the two approaches, future insect resistant crops might produce multiple fusion proteins targeting diverse receptors and acting through multiple modes of action.

One major concern about using lectins as insecticidal agents is their potential impact on non-target organisms. Unlike Cry toxins, which are usually specific to a single insect order, lectins are often toxic to multiple insects including caterpillars, aphids and beetles (Macedo et al., 2015; Vandenborre et al., 2011b). Such a lack of specificity can be beneficial for protection of crops infested by diverse pests (e.g. cotton) but raises the possibility of the harmful impact on beneficial insects. Some data indicate

that lectins might be toxic to human cells. For instance, SNA-I and SNA-II were toxic to HeLa cells while they were virtually non-toxic to human fibroblasts (Shang et al., 2015). Nevertheless, because of the variety of lectin structures, binding specificities, modes of action and differential activities towards different organisms and cell lines, each individual case would require dedicated assessment of the potential impact on human health. In the case of plants cultivated for purposes other than food and feed like energy crops, cotton or tobacco the human health issues are less relevant, but yet the possible impact on non-target organisms including pollinators and natural enemies should be evaluated (Koch et al., 2015).

6.8. Glycosylation dynamics in the post embryonic development

The enrichment of more extensively trimmed and modified N-glycans in *T. castaneum* adults compared to larvae (described in Chapter 4) indicated that N-glycosylation plays a role in the metamorphosis of this beetle. Life stage specific dynamics of N-glycosylation might be widespread in invertebrates. Such a phenomenon has been shown in the nematode *Caenorhabditis elegans* (Cipollo et al., 2005). In this organism the life stages differed in the abundance of nematode-specific complex glycans modified with phosphocholine chains, but differences in the amounts of high and pauci-mannose N-glycans were not reported. Among insects, it has been found that the N-glycan composition changes during embryonic development of the fruit fly (Aoki et al., 2007). The nature of the change was similar to that observed between larvae and adults of the flour beetles, in that late embryos contained less high mannose N-glycans but were enriched in pauci-mannose and fucosylated N-glycans, in comparison to early embryos. Although the level of complex glycans was also increased during fly embryo development, this was not observed during beetle metamorphosis.

It is tempting to generalize that metamorphosis of insects is associated with differential N-glycan processing. However, except for this work, such changes between post-embryonic life stages have not been shown yet for any insect other than *Tribolium*. Beetles, flies, butterflies and many other insects are holometabolous, that is their life cycle consists of distinct larval, pupal and adult stages. In contrast adults of hemimetabolous insects e.g. aphids, grasshoppers or dragonflies develop from morphologically similar nymphs. This mode of development is also called incomplete metamorphosis (Truman and Riddiford, 1999). Thus another intriguing question arises: whether enhanced N-glycan processing occurs also in insects undergoing incomplete metamorphosis?

The observed shift in the N-glycan composition was driven by the increased expression of N-glycan processing genes in the red flour beetle adult tissues. Interestingly, the expression of multiple genes involved in glycan processing in pupa was even higher compared to adults. This suggested that the N-glycans synthesized in this life stage might be even more dominated by heavily processed paucimannose and potentially include higher amounts of complex N-glycans. Gene expression studies indicated also some differences between early, middle and late larval stages. Therefore, analysis of the N-glycan dynamics in larval and pupal stages seems a natural continuation of this work that may shed even more light on the importance of glycosylation for the physiology of the insect post-embryonic development.

The current strategies for N-glycan analysis require relatively large amounts of sample which brings technical difficulty especially when tissue microdissection is required. Novel mass spectrometry techniques such as MALDI imaging could be used to determine the spatial distribution of different N-glycans in single dissected guts or brains (Powers et al., 2014). This approach would be particularly useful to determine the localization of the difucosylated N-glycans. These structures were among the most abundant glycans in beetles. This is in stark contrast to fruit fly in which difucosylation is rare and limited to the nervous system (Rendić et al., 2010). Therefore, MALDI imaging could be used to verify if the high content of difucosylated N-glycans in *Tribolium* is a result of extraordinary enrichment in the neurons or broader tissue distribution.

6.9. Roles of N-glycosylation in the post embryonic development

The majority of work related to functions of N-glycans in insects involves the analysis of *Drosophila* loss of function mutants. In this strategy, most of the phenotypes arise from impaired embryonic development (Attrill et al., 2015). Induction of gene knockdowns specifically in later developmental stages is also possible but usually requires elevated temperature for induction (Nagarkar-Jaiswal et al., 2015). However, high temperature also induces a heat shock response which makes it difficult to dissect the roles of N-glycosylation in normal conditions. In contrast, our approach, that is larval RNAi, allowed precise timing of depletion of a target gene mRNA and lead to the discovery of the importance of N-glycosylation in post embryonic development and metamorphosis. Using the RNAi strategy we revealed that different steps of the N-glycosylation pathway are required for particular stages of post embryonic development (Fig. 6.3.).

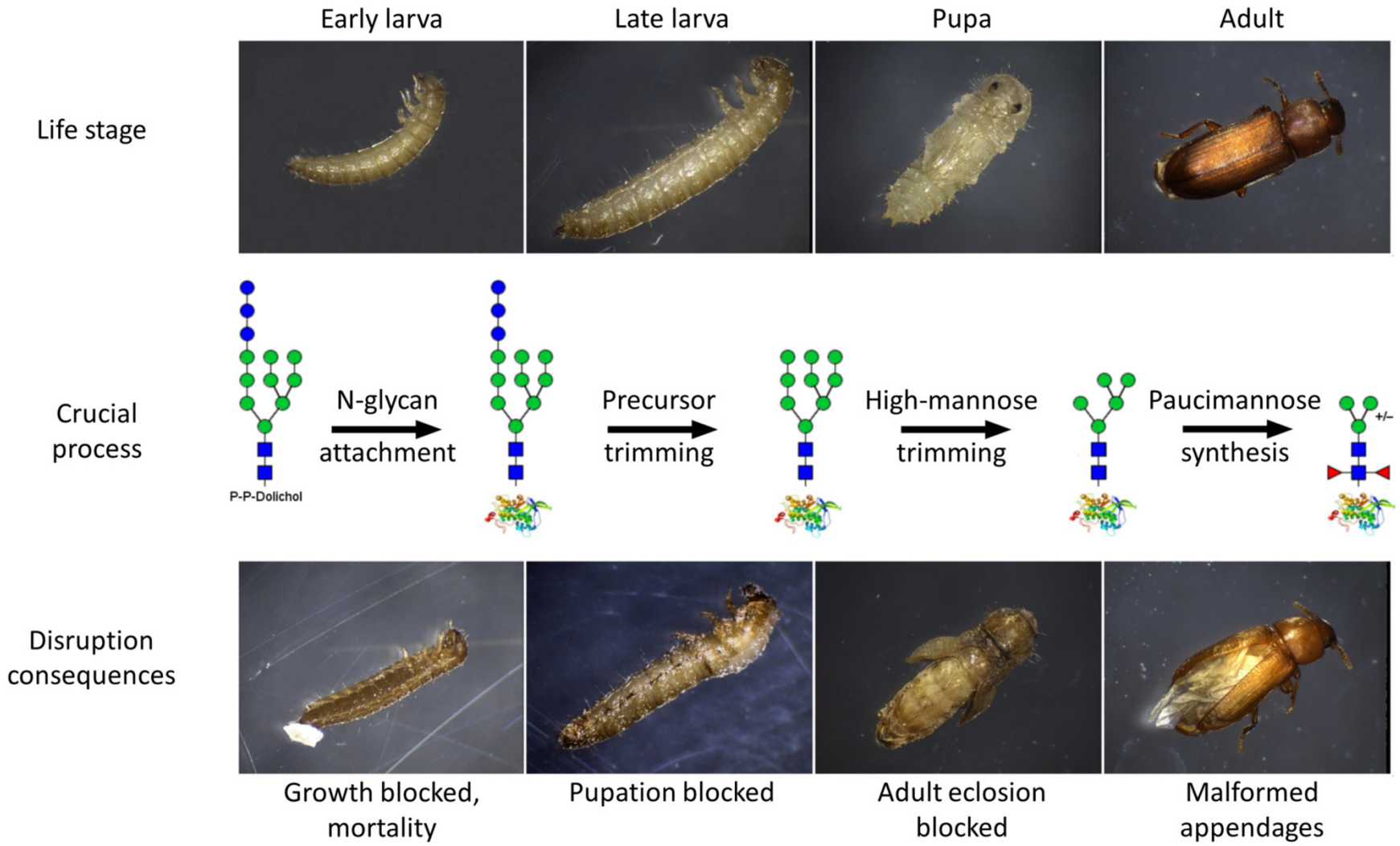


Fig. 6.3. Generalized model for the involvement of particular parts of N-glycosylation pathway in post-embryonic development and metamorphosis.

The attachment of an N-glycan precursor to the protein, which could be considered as the very first step in the pathway, turned out to be crucial for larval survival and growth. It has been shown in other models that blockage of the N-glycosylation results in the accumulation of misfolded proteins in the ER, the formation of toxic aggregates, autophagy and finally cell death (Høyer-Hansen and Jäättelä, 2007; Kelleher and Gilmore, 2006). The next step in the process, the cleavage of the glucoses that cap the early N-glycan, plays a role for the entry into metamorphosis, as disruption of this process prevented larval transition into pupa. Subsequent trimming of high mannose N-glycans was required for adult eclosion, as well as for formation of wings and legs.

The final stages of the processing leading to formation of paucimannose N-glycans appeared to be involved in the functioning of the nervous and/or muscular system as judged from impaired mobility. The generally mild phenotypes caused by the disruption of genes involved in the production of paucimannose glycans suggests that enzymatic organization of this part of the pathway may be more complicated than previously thought (Nemcovicova et al., 2013). Paucimannose N-glycan synthesis relies mostly on two Golgi class II α-mannosidases (α-Man-IIa and α-Man-IIb) but silencing of both of these genes did not result in the expected severe phenotypes, which suggested that some other enzymes might compensate for the lack of these two. Possible candidates could be among the putative *Tribolium* lysosomal class II α-mannosidases. Lysosomal mannosidases are though to be involved in the degradation of glycoproteins rather than in N-glycan modification. The assignment of *D. melanogaster* proteins to this group was based solely on sequence similarity with a single murine lysosomal mannosidase Man2b1 (Rosenbaum et al., 2014). We used the same approach in our phylogenetic analyses but we found that the amino acid sequences of *Tribolium* mannosidases are only remotely similar to Man2b1 (37-44% sequence identity). Moreover, the *T. castaneum* genome encodes nine putative lysosomal class II mannosidases, while the genome of *D. melanogaster* encodes six such genes. Thus, it seems plausible that some of those additional class II mannosidases might be involved in paucimannose N-glycan production.

One way of verifying the roles of these proteins would be by studying their subcellular localization. Recently a plasmid for transient transformation of *Tribolium* cells has been developed (Silver et al., 2014). It could be used for co-expression of Man-Ib or Man-Ia (known to be mainly localized to ER or Golgi, respectively) together with putative lysosomal mannosidases fused to fluorescent tags. If any of those proteins would localize to the Golgi or ER instead of lysosomes, it would be a strong indication for the involvement in N-glycan processing rather than degradation.

The generation of mutants or transcript depletion of all putative lysosomal mannosidases might prove to be impossible, but the involvement of these genes in glycan processing could be studied using swainsonine treatment (Shah et al., 2003). This plant alkaloid is a selective inhibitor of class II α-mannosidases. Therefore, the consequences of complete ablation of paucimannose N-glycan production could be achieved through injection of this compound into larvae.

We did not observe any particular phenotypes after silencing of the genes involved in N-glycan fucosylation. This is particularly bothering, when one takes into account that fucosylated N-glycans contributed to approximately half of all the adult N-glycans. There are at least five putative fucosyltransferases, therefore their complementary enzymatic activities may mask the phenotypes of single knockdowns. Unfortunately, it is feasible to silence only up to three genes using RNAi in *Tribolium* (Linz and Tomoyasu, 2015). Hence a different approach would be required to decode the function(s) of fucose attachment. It has been shown in *Drosophila* that mutation in the *nac* gene causes depletion of GDP-fucose which is a substrate for fucosyltransferases and, in turn, reduces fucosylation of N-glycans (Geisler et al., 2012). However, GDP-fucose serves also as a substrate for O-glycan fucosylation crucial for Notch and EGFR signaling in wing and neuronal development (Sasamura et al., 2003). Thus this strategy would not allow determining the roles of N-glycan fucosylation. In future this could be possible with the use of N-glycan fucosyltransferase inhibitors. However, currently available compounds are based on GDP-fucose analogs and thus lack desired specificity (Tu et al., 2013).

6.10. N-glycosylated proteins and metamorphosis

We described N-glycan dynamics during *T. castaneum* metamorphosis and characterized the genetic basis of this phenomenon. Yet, there is still one important piece of the puzzle missing. A complete understanding of the link between metamorphosis and N-glycan processing necessitate detailed characterization of N-glycosylated proteins in larval, pupal and adult stages of the red flour beetle. Using a combination of lectin-based glycopeptide enrichment with bottom-up mass glycopeptidomics approach, it is possible to identify glycoproteins, but also importantly to elucidate which glycans decorate particular proteins (Quinton et al., 2011; Song et al., 2014). This will allow answering three fascinating questions: (i) are there N-glycosylated proteins which are life-stage specific? (ii) Do the N-glycosylated proteins expressed in multiple life stages bear the same or different N-glycans? And ultimately (iii) what are the functions of these N-glycosylated proteins?

So far only approximately 100 N-glycosylated proteins have been identified in *Tribolium* adults. which means that several thousand still remain to be discovered. Those already identified include proteins involved in development-related signaling, such as juvenile hormone esterase and insulin receptor (Vandenborre et al., 2011a). Blocked N-glycosylation of these proteins could prevent their transport to the cell membrane and impair their function in regulation of growth and organ development (Shingleton et al., 2005). Another group of interesting N-glycosylated proteins in *Tribolium* included proteases and chitinases, which may be involved in the degradation of old larval tissues, as well as chitin synthases, laminins and integrins which, in contrast, are involved in the restructuration of the tissue and the development of new pupal and adult organs (Arakane et al., 2005b; Domínguez-Giménez et al., 2007). Multiple of those proteins require to be secreted to the body cavity and/or circulatory system and it has been shown in *Trichoplpusia ni* cells that the excreted protein usually contain paucimannose or complex N-glycans while membrane proteins carry high mannose N-glycans (Hsu et al., 1997). Thus it seems likely, that enhanced N-glycan processing is necessary for extracellular localization of the proteins involved in tissue remodeling during the metamorphosis. Moreover, *Tribolium* fasciclin and tropomyosin were found to be N-glycosylated. These proteins are involved in axon guidance and muscle activity (Beck et al., 2012; Tansey et al., 1991). Thus the observed locomotor phenotypes in RNAi-mediated disruption of N-glycan processing in beetles (Chapter 5) could be the result of an impairment of the activity of these proteins. Verification of these hypotheses requires a study focused on the localization and activity of those proteins after silencing of N-glycan processing pathway components.

6.11. Disruption of N-glycosylation as a pest control strategy

In numerous insects RNAi mediated gene silencing is effective when dsRNA is delivered with the diet, which lead to the idea that RNAi could be used not only for functional genomics but also for pest control. Gene silencing through RNAi requires a high level of similarity between a target mRNA sequence and the dsRNA probe. Therefore it allows designing selective reagents active against the particular pest but safe to beneficial species (Huvenne and Smagghe, 2010). In this strategy dsRNA is the molecule conferring the resistance, so there is no need for energy consuming process of protein translation, contrary to crops expressing Cry toxins or lectins, which may result in a better yield. Proof-of-concept studies have shown that this approach can be succesful against the Colorado potato beetle, the western corn rootworm or the cotton bollworm (Baum et al., 2007). Recently, a growing number of studies showed that targeting an essential insect gene(s) by dsRNA produced in a transgenic crop results in

effective protection against pests (Zhang et al., 2015). Selection of the genes to be targeted with RNAi is crucial for this strategy of crop protection. The optimal target should be ubiquitously expressed and its knockdown should result in high mortality (Price and Gatehouse, 2008). Genes involved in the early part of the N-glycosylation pathway: *TcDad1, TcGcs1, TcGcs2α* meet these criteria and appear suitable for use in RNAi-mediated control of pest beetles.

6.12. Conclusive remarks

This PhD thesis accomplished the three major goals: (i) the study of insecticidal activity of lectins and their suitability for control of pest beetles, (ii) the elucidation of the importance of N-glycosylation in postembryonic development of beetles, (iii) the evaluation of the disruption of the N-glycosylation through RNAi as a pest control strategy.

Lectins have high insecticidal activity against insect cells but when fed to the red flour beetle their efficiency was greatly impaired. Characterization of the factors restricting the insecticidal properties of lectins could be generalized to virtually all insecticidal proteins and can be used for a more rational selection of novel insecticidal toxins and enhancement of activity of the ones that are currently used.

By studying glycan composition, gene expression analysis and functional genomics we determined that N-glycosylation is highly dynamic during post embryonic development. We found that N-glycosylation is involved in larval growth, progression of the life stages and development of adult appendages. Finally, it can be concluded that disruption of the early stages of the N-glycosylation pathway appears to be promissing strategy for future control of insect pests.

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Summary / Samenvatting

The majority of all the proteins undergoes glycosylation. This post-translational modification of proteins is involved in many biological processes and an erroneous glycosylation is often lethal. Following this logic interference with insect glycosylation is likely to be an effective way to control insect pests. Unfortunately, most of the knowledge on insect glycobiology comes from the research on *Drosophila* which lacks relevance for the agricultural context. This work focused on the discovery of the physiological importance of N-glycosylation in the red flour beetle, *Tribolium castaneum,* which is a pest and a model insect. Additionally, this PhD thesis presents verification of use of glycan-binding proteins (lectins) and disruption of N-glycosylation as control strategies against pest beetles.

Experimental data shown in **Chapter 2** confirmed clearly that lectins can be toxic to insect cells but their insecticidal effects depend on the type of insect cells used. Lectin toxicity was much higher against the midgut cell line compared to a cell line derived from embryos and this supported the notion that lectin feeding may be a relevant approach for insect control. Moreover, the use of fluorescently labeled lectins and confocal microscopy revealed that GalNAc/Gal and sialic acid containing glycans are enriched at the apical surface of the midgut cells, while high-mannose type glycans are more prevalent at the basal pole of the cells. This result puts forward the lectins specific for GalNAc/Gal and sialic as promising candidates for the tests in vivo.

In **Chapter 3** a variety of in vitro, feeding and injection assays was used to verify the toxicity of the selected lectins against the red flour beetle. Experiments with the *Tribolium* cell line confirmed high toxicity of the tested lectins. Surprisingly, feeding assays indicated a much lower toxicity against larvae than expected. The toxicity of the lectins was associated with their resistance the proteolysis and ability to pass through the peritrophic matrix – a protective mesh like structure lining the gut. The efficiency of passing through the peritrophic matrix correlated with lectin size but was not dependent on lectin charge. Interestingly, injection of the lectins directly into the larval hemolymph caused high toxicity at doses up to four orders of magnitude lower compared to those in the feeding assays. This showed high a toxic potential of the tested lectins and indicated that lectins need to accumulate in the hemolymph in order to induce high insecticidal effect.

Chapter 4 focused on a detailed analysis of N-glycans found on beetle proteins, which was a first prerequisite for functional studies of N-glycosylation. For this purpose we employed several techniques including high pressure liquid chromatography and mass spectrometry to analyze both monosaccharide compositions and intact N-glycans in larvae and adults. In both life stages a similar range of over 20 different glycans was found, mainly of high mannose and paucimannose type, and only a minor fraction of complex structures. Interestingly, the composition of the adult N-glycome was shifted towards more trimmed forms. $Man_{9-6}GlcNAc_2$ structures were reduced in adults while glycans containing one to five mannoses were enriched compared to the 4th instar larvae. These observations suggested that changes in the global profile of N-glycans may be involved in the transition from larval to adult stages. Moreover, marked differences in N-glycome composition between the red flour beetle and the fruit fly indicated limited validity of *D. melanogaster* as a model for pest insects and supported the need of glycomic studies on particular pest species.

Chapter 5 describes functional studies of N-glycosylation in the red flour beetle. We showed that mRNA levels of the genes involved in the N-glycosylation process are developmentally regulated and that a shift of the N-glycan composition (described in **Chapter 4**) is driven by change in the expression of the genes encoding N-glycosyltransferases and hydrolases during pupal and adult stages. Additionally, the analysis of phenotypes caused by RNAi mediated gene knockdown revealed that N-glycan attachment to proteins is crucial for larval growth and survival, while the first steps of N-glycan trimming are required for a transition of larvae to pupae. Moreover, silencing of genes involved in later parts of the pathway demonstrated novel roles of N-glycosylation in adult development and the formation of wings, wing covers and legs. All together these data showed that the postembryonic development and the metamorphosis of *Tribolium castaneum* rely on proper N-glycan processing and that RNAi mediated disruption of this pathway can be an effective strategy for insect pest control.

De meerderheid van alle eiwitten ondergaat glycosylatie. Deze post-translationele eiwitmodificatie is van groot belang voor heel wat biologische processen en een foutieve glycosylatie kan soms letaal zijn. De verstoring van dit proces bij plaaginsecten kan om die reden een effectieve strategie zijn in de plaagbestrijding. Echter, onze kennis van de glycobiologie bij insecten is tot op heden nog steeds beperkt tot het modelorganisme *Drosophila*, dat nauwelijks relevant is in een gewasbeschermingscontext. Dit onderzoek richt zich op het fysiologisch belang van N-glycosylatie in de kastanjebruine rijstmeelkever *Tribolium castaneum*, dat zowel een plaaginsect als een modelorganisme is. Deze thesis toont bovendien ook het potentieel van suiker-bindende eiwitten (lectines) en de verstoring van N-glycosylatie voor plaagbestrijding.

De experimentele data in **Hoofdstuk 2** toonden aan dat lectines een toxisch effect kunnen hebben op insectencellen. Hun insecticidale effecten waren afhankelijk van het celtype dat onderzocht werd. Zo was de toxiciteit van het lectine veel hoger in cellen afkomstig van de middendarm dan bij cellen die afkomstig waren van embryo's, wat het potentieel voor een toepassing in de gewasbescherming, via een orale toediening, bevestigde. Lokalisatiestudies, gebruik makende van fluorescent-gelabelde lectines en confocale microscopie, toonden aan dat GalNAc/Gal- en siaalzuur-bindende suikers voornamelijk intercatie vertoonden met het apicale oppervlak van de middendarmcellen, terwijl high-mannose type glycanen eerder met de basale polen van de cellen interageerden. Hieruit was duidelijk dat lectines die specificiteit vertonen voor GalNAc/Gal en siaalzuur veelbelovende kandidaten waren voor de in vivo experimenten.

In **Hoofdstuk 3** werd de toxiciteit van de geselecteerde lectines bij de kastanjebruine rijstmeelkever onderzocht door middel van in vitro, voedings- en injectie-experimenten. Bij de experimenten met een Tribolium cellijn werd de sterke toxiciteit van de geteste lectines bevestigd. Toediening van het lectine aan larven via de voeding leidde echter tot een veel lagere toxiciteit dan verwacht. De toxiciteit van de lectines kon worden gelinkt aan hun gevoeligheid voor proteolyse en de mate waarin de lectines door de peritrofe matrix, een netvormige beschermende structuur die het darmoppervlak bedekt, konden migreren. Dit laatste kon worden gecorreleerd aan de grootte van het lectine maar bleek onafhankelijk te zijn van de lading van het eiwit. Rechtstreekse injectie van het lectine in het hemolymfe van de larven veroorzaakte wel een hoge toxiciteit, zelfs wanneer dosissen tot vier maal kleiner dan deze bij de voedingsexperimenten werden toegediend. Deze resultaten bevestigden de

hoge toxiciteit van de geteste lectines en toonden aan dat de lectines in het hemolymfe moeten terechtkomen en accumuleren om een sterk insecticidaal effect te veroorzaken.

Hoofdstuk 4 bevat een gedetailleerde analyse van de N-glycanen die aangetroffen worden op eiwitten bij kevers, wat een eerste vereiste is vooraleer verder functionele studies van N-glycosylatie kunnen worden uitgevoerd. Hiervoor werden verschillende technieken aangewend, zoals hogedrukvloeistofchromatografie en massaspectrometrie, om zowel de monosaccharide samenstelling en de intacte N-glycanen te analyseren in larven en adulten. In zowel de juveniele als de adulte stadia werd een vergelijkbare verscheidenheid van meer dan 20 verschillende glycanen teruggevonden. Hierbij kwamen vooral high mannose- en paucimannose-type glycanen voor, en slechts een kleine fractie van meer complexe structuren. Een interessante bevinding was dat bij het adulte N-glycoom een hoger aandeel getrimde vormen werd aangetroffen. $Man_{9-6}GlcNAc_2$ structuren kwamen bij adulten in mindere mate voor, terwijl glycanen die één tot vijf mannose residu's bevatten, vaker voorkwamen in vergelijking met kevers in het 4^{de} larvale stadium. Deze bevindingen suggereren dat veranderingen in het globale N-glycaanprofiel een rol kunnen spelen in de overgang van het larvale naar het adulte stadium. Bovendien werden ook verschillen in de N-glycoom samenstelling tussen de kastanjebruine rijstmeelkever en de fruitvlieg opgemerkt, wat de status van *D. melanogaster* als modelorganisme bij insecten in vraag stelt. Deze bevindingen geven de noodzaak aan voor glycaananalysen bij specifieke plaaginsecten.

Hoofdstuk 5 beschrijft de functionele studies van N-glycosylatie in de kastanjebruine rijstmeelkever. We ontdekten dat mRNA niveaus van de genen die betrokken zijn bij het N-glycosylatie proces kunnen veranderen tijdens de ontwikkeling van het insect en dat de veranderingen in de N-glycaansamenstelling (beschreven in **Hoofdstuk 4**) het gevolg zijn van een wisselende expressie van deze genen gedurende het pop- en adultstadium. Analyse van de fenotypes die bekomen werden nadat deze genen met behulp van RNA interferentie werden gesilenced wees er bovendien op dat glycosylering van eiwitten cruciaal is voor larvale groei en overleving, terwijl de eerste stappen van N-glycaan prescessing noodzakelijk zijn voor de overgang van het larvale stadium naar het popstadium. Bovendien onthulde silencing van de genen die betrokken zijn in verdere stappen van de pathway een tot nu toe onbekende rol voor N-glycosylatie in de ontwikkeling van adulten, met name in de vorming van de vleugels en poten. Uiteindelijk toonde deze data aan dat post-embryonische ontwikkeling en de metamorfose van *Tribolium castaneum* afhankelijk is van correcte N-glycaan verwerking en dat verstoring van deze pathway via RNAi een effectieve strategie kan zijn in de bestrijding van dit plaaginsect.

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Master of Science in Applied Biotechnology (Magister Inżynier)
Biotechnology - Interfaculty Studies, Agricultural University in Krakow, Poland
Specialization: Plant Biotechnology

September 2002 – June 2005:

Secondary education
Piarist Fathers Secondary School in Krakow, Poland

PUBLICATIONS

Walski, T., Smargiasso, N., Christiaens, O., De Pauw, E., Van Damme, E. J.M., & Smagghe, G. (2016) Differential N-glycan processing is required for successful metamorphosis in the pest beetle *Tribolium castaneum. (in press)*

Walski, T., Van Damme, E. J. M., & Smagghe, G. (2014). Penetration through the peritrophic matrix is a key to lectin toxicity against *Tribolium castaneum*. *Journal of Insect Physiology*, *70*, 94-101.

Kaszycki, P., **Walski, T**., Hachicho, N., & Heipieper, H. J. (2013). Biostimulation by methanol enables the methylotrophic yeasts *Hansenula polymorpha* and *Trichosporon* sp. to reveal high formaldehyde biodegradation potential as well as to adapt to this toxic pollutant. *Applied Microbiology and Biotechnology*, *97*, 5555-5564.

RESEARCH EXPERIENCE

October 2010 – December 2015:

PhD research project: "Glycosylation in *Tribolium castaneum*: composition, physiological significance and exploitation for pest control".

under the supervision of Prof. Dr. Guy Smagghe and Prof. Dr. Els JM Van Damme. Agrozoology Lab, Department of Crop Protection and Biochemistry and Glycobiology Lab, Department Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Belgium

Main activities:

- Gene expression analysis using qRT-PCR
- RNA interference (dsRNA design, production, microinjection)
- Insect phenotype and behavior analysis
- Protein purification and analysis (affinity, ion exchange, size-exclusion chromatography)
- Protein analysis (SDS-PAGE, Western blot)
- Maintenance of insect cell cultures
- In vivo and in vitro toxicity assays
- Confocal microscopy of insect cells and tissues
- Glycan isolation and analysis (HPLC, MALDI-TOF-MS)

Teaching and supervision:

- Guidance for new PhD students: Ying Shen and Freja Scheys (2014-2015)
- Master student: Elena Suarez-Barcena (2013-2014)
- Bachelor students: Lars Rosseel, Sander Vandamme, Joren Vandecasteele, Wouter Vanrolleghem (2014)

September 2012 – December 2012:

Research stay in the Glycobiology lab of Prof. Erika Staudacher

Analysis of protein linked glycosylation.

under the supervision of Prof. Erika Staudacher, Glycobiology Division, Biochemistry Division, Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria

Main activities:

- Purification of proteins and glycans
- Ion exchange and size exclusion chromatography
- HPLC analysis of glycans
- Mass spectrometry of N-glycans

October 2008 – June 2010:

Master thesis research project: "Changes in lipid membrane fluidity of non-conventional yeasts upon cellular stress induced by selected xenobiotics".

under the supervision of Dr. Paweł Kaszycki, Department of Biochemistry, Faculty of Horticulture, Agricultural University in Krakow, Poland

Main activities:

- Maintenance of yeast cell cultures
- Xenobiotic toxicity assays (phenol, formaldehyde)
- Quantification of xenobiotic biodegradation
- Lipid isolation and analysis by gas chromatography

July 2009:

Voluntary research assistant to a PhD student Jonas Van Hove

Project: Recombinant production of plant lectin.

Biochemistry and Glycobiology Lab, Department Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Belgium

Main activities:

- Molecular techniques (Gateway cloning, mini/midi prep, heat-shock transformation)
- Protein purification and characterization (Ni-affinity chromatography, Western blotting)

September 2008:

Voluntary research assistant to a PhD student Wojciech Siwek

Project: Recombinant production and crystallization of NIaIV restriction enzyme.

Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology, Warsaw, Poland

Main activities:

- PCR, cloning, preparation of competent E. coli cells and transformation,
- Plasmid isolation, agarose electrophoresis
- Protein purification (affinity and ion-exchange chromatography)

CONFERENCE PARTICIPATIONS

With oral presentation:

Walski, T., Smargiasso, N., De Pauw, E., Van Damme, E. J. M., Smagghe, G. (October, 2015). Disruption of N-glycosylation through RNAi: a promising approach for pest control. The 26th Joint Glycobiology Meeting, Lille, France.

Walski, T., Smargiasso, N., De Pauw, E., Van Damme, E. J. M., Smagghe, G. (August, 2015). Genes involved in N-glycosylation and their roles in growth, metamorphosis and fitness of the red flour beetle. Pan-American Society for Evolutionary Developmental Biology Inaugural Meeting, International *Tribolium* satellite meeting, Berkeley, USA.

Walski, T., Van Damme, E. J. M., Goodman, C., Smagghe, G. (December, 2013). Lectins, sugars & insects. 1st Brazil-Belgium conference of Innovative technologies applied to pest control, Pelotas, Brazil.

Walski, T., Van Damme, E. J. M., Goodman, C., & Smagghe, G. (May, 2013). Insecticidal effects of lectins against the red flour beetle, *Tribolium castaneum*. 65th International

Symposium on Crop Protection, Ghent, Belgium.

With poster:

Smargiasso, N., **Walski, T**., Van Damme, E., De Pauw, E., & Smagghe, G. (May, 2015). Study of N-linked glycosylation in different life stages of the red flour beetle (Tribolium castaneum). 63rd ASMS Conference on Mass Spectrometry and Allied Topics, St. Louis, USA.

Walski, T., Smargiasso, N., Staudacher, E., De Pauw, E., Van Damme, E. J. M., & Smagghe, G. (September, 2014). N-Glycosylation in different life stages of the red flour beetle, Tribolium castaneum.25th Joint Glycobiology meeting, Ghent, Belgium.

Walski, T., Staudacher, E., Van Damme, E. J. M., & Smagghe, G. (March, 2013). Sweet side of the beetle: Importance of glycosylation in Tribolium castaneum. iBeetle symposium, Göttingen, Germany.

PROFESSIONAL DEVELOPMENT

Chemical and Biological Tools for Crop Improvement, Symposium, 2015

Project Management Training, 2014

Advanced Academic English: Writing Skills Course, 2013

Effective Scientific Communication Course, 2012

SCHOLARSHIPS OBTAINED

Doctoral (PhD) Grant for Strategic Basic Research, 2011-2015

Agency for Innovation by Science and Technology in Flanders (IWT)

PERSONAL SKILLS AND COMPETENCES

Languages:

English (fluent spoken and written), German (basic), Polish (native speaker)

Computer and software skills:

Good command of Microsoft Office tools,

Experience in processing and quantitative analysis of images (ImageJ, Fiji)

Advanced knowledge of data analysis and visualization (SigmaPlot, SPSS, GraphPad)

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