N-glycosylation Site Analysis Reveals Sex-related Differences in Protein N-glycosylation in the Rice Brown Planthopper (*Nilaparvata lugens*)

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In Brief
N-glycosylation sites were analyzed for proteins from the adult morphs of the rice brown planthopper (*Nilaparvata lugens*). The results showed that protein N-glycosylation is sex-related in this pest insect, and sex-specific glycoproteins which can play a role in insect reproduction were identified. At the same time differences in N-glycan composition were detected for glycoproteins shared across sexes.

Graphical Abstract

Highlights
- N-glycosylation site analysis of the hemipteran pest insect *Nilaparvata lugens*.
- Differential N-glycosylation of proteins is observed between male and female adults.
- Sex-specific glycoproteins are involved in insect reproduction.
N-glycosylation Site Analysis Reveals Sex-related Differences in Protein N-glycosylation in the Rice Brown Planthopper (Nilaparvata lugens)*

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Glycosylation is a common modification of proteins and critical for a wide range of biological processes. Differences in protein glycosylation between sexes have already been observed in humans, nematodes and trematodes, and have recently also been reported in the rice pest insect Nilaparvata lugens. Although protein N-glycosylation in insects is nowadays of high interest because of its potential for exploitation in pest control strategies, the functionality of differential N-glycosylation between sexes is yet unknown. In this study, therefore, the occurrence and role of sex-related protein N-glycosylation in insects were examined. A comprehensive investigation of the N-glycosylation sites from the adult stages of N. lugens was conducted, allowing a qualitative and quantitative comparison between sexes at the glycopeptide level. N-glycopeptide enrichment via lectin capturing using the high mannose/paucimannose-binding lectin Concanavalin A, or the Rhizoctonia solani agglutinin which interacts with complex N-glycans, resulted in the identification of over 1300 N-glycosylation sites derived from over 600 glycoproteins. Comparison of these N-glycopeptides revealed striking differences in protein N-glycosylation between sexes. Male- and female-specific N-glycosylation sites were identified, and some of these sex-specific N-glycosylation sites were shown to be derived from proteins with a putative role in insect reproduction. In addition, differential glycan composition between males and females was observed for proteins shared across sexes. Both lectin blotting experiments as well as transcript expression analyses with complete insects and insect tissues confirmed the observed differences in N-glycosylation of proteins between sexes. In conclusion, this study provides further evidence for protein N-glycosylation to be sex-related in insects. Furthermore, original data on N-glycosylation sites of N. lugens adults are presented, providing novel insights into planthopper's biology and information for future biological pest control strategies. Molecular & Cellular Proteomics 19: 529–539, 2020. DOI: 10.1074/mcp.RA119.001823.

Nilaparvata lugens Stål (Hemiptera: Delphacidae) or the brown planthopper (BPH)1 is one of the most notorious pest insects in the rice producing countries of Asia (1). Being a sap-sucking insect, N. lugens causes direct damage to the rice plant by feeding on the phloem. In addition, BPH can also act as a vector for viruses causing indirect plant damage (2). N. lugens exhibits wing polymorphism, and adults appear either as short-winged or long-winged morphs. Whereas the short-winged morph is flightless and specialized in reproduction, the long-winged morph has a fully developed flight apparatus, allowing it to migrate and infest new rice fields (3). Recently, an extensive N-glycome analysis for the different life stages of N. lugens revealed that protein N-glycosylation is sex-related in this insect (4). Female N-glycomes show a 10-fold increase in high mannose N-glycans compared with males, whereas in adult males, mono- and difucosylated paucimannose N-glycans are more abundant. Furthermore, male glycoproteins can carry complex N-glycans with terminal galactose (Gal), which was not detected in females. The functionality of this differential N-glycosylation between sexes is yet unknown. Therefore, it is of interest to identify the protein moieties carrying these differential carbohydrate structures in N. lugens males and females. Because male and female adults can occur either as long-winged or short-winged morphs, it is also of interest to investigate whether protein N-glycosylation is wing type-dependent.

Using lectin enrichment, glycoproteins from several insect species have already been described (5–7), but little information is available on the actual insect N-glycosylation sites.
Furthermore, all these studies focused on adult insects without distinguishing for sexes or wing forms. Here, the N-glycosylation sites from *N. lugens* adults, comprising both sexes and wing types, were studied using the N-glyco-FASP method as developed by Zielinska et al. (10). This technique was recently optimized for insect studies by our group (8), and N-glycosylation sites from insects belonging to different insect orders were analyzed and compared. Two lectins, Concanavalin A (ConA) and *Rhizoctonia solani* agglutinin (RSA), were used for the enrichment of peptides carrying specific carbohydrate structures. ConA specifically interacts with high mannose and paucimannose N-glycans, whereas RSA binds to complex N-glycans carrying terminal Gal and N-acetylgalactosamine (GalNAc) residues. Previous glycomics studies revealed that especially high mannose and paucimannose N-glycans occur in insects, together comprising over 90% of the total N-glycan pool, whereas complex glycans represent less than 10% of the total amount of detected N-glycans (4, 11–15). Enrichment of N-glycopeptides with ConA or RSA will thus allow enriching for abundant and rather scarce N-glycopeptides, respectively.

Our experiments allowed both a qualitative as well as a quantitative comparison of the N-glycosylation sites between the two sexes and wing types of *N. lugens* adults. For a selection of proteins, the proteomic data were validated at transcript level, using complete adult insects as well as adult tissues. Our comprehensive overview of N-glycosylation sites in *N. lugens* adults can help to address the importance of protein N-glycosylation in adult sexes and wing types and provides novel insights into planthopper’s biology. Furthermore, this information will have to be considered when assessing the possibilities of protein N-glycosylation disruption as a novel strategy to control insect pests.

**EXPERIMENTAL PROCEDURES**

**Experimental Design and Statistical Rationale—**N-glycosylation sites were analyzed for *N. lugens* adult morphs; short-winged males (SM), short-winged females (SF), long-winged males (LM) and long-winged females (LF). Protein extracts were prepared in three biological replicates for each adult morph. Glycopeptides were enriched from these protein extracts, and for each biological replicate the eluent from three filter units was pooled. All samples were purified on C18 Ziptips (10 µl, Millipore) prior to analysis on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For each sample, 10 µl was first loaded on a trapping column (made in-house, 100 µm internal diameter (I.D.) × 20 mm, 5 µm beads C18 Reprosil-HD; Dr. Maisch, Ammerbuch-Entringen, Germany). After flushing from the trapping column, the sample was loaded on an analytical column (made in-house, 75 µm I.D. × 400 mm, 1.9 µm beads C18 Reprosil-HD; Dr. Maisch). Peptides were loaded with loading solvent (0.1% TFA, 2% ACN) and eluted with a non-linear 150 min gradient of 2–56% solvent B (0.1% formic acid in water/acetonitrile, 20/80 (v/v)) at a flow rate of 250 nL/min, followed by a 10 min wash reaching 99% solvent B and re-equilibration with solvent A (0.1% formic acid in water). The column temperature was kept constant at 50 °C (CoControl 3.3.05, Sonation). The mass spectrometer was operated in data-dependent, positive ionization mode, automatically switching between MS and MS/MS acquisitions. The source voltage was set to 2.8 kV and the capillary temperature was 250 °C. One MS1 scan (m/z 375–1500, AGC target 3E6 ions, maximum ion injection time of 60 ms) acquired at a resolution of 60,000 (at 200 m/z) was followed by up to 16 tandem MS scans (resolution 15,000 at 200 m/z, AGC target 1E5 ions, maximum ion injection time of 80 ms, isolation window of 1.5 m/z, fixed first mass of 145 m/z, spectrum data type: centroid) of the most intense ions fulfilling predefined selection criteria (underfill ratio 2%, intensity threshold 1.3E4, exclusion of unassigned, singly and >7 charged precursors, peptide match preferred, exclude isotopes on, dynamic peptide signals).

**Mass Spectrometry—**Samples were purified on C18 Ziptips (10 µl, Millipore) prior to analysis on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For each sample, 10 µl was introduced into an LC-MS/MS system through an Ultimate 3000 RSLC nano LC (Thermo Fisher Scientific). The sample mixture was first loaded on a trapping column (made in-house, 100 µm internal diameter (I.D.) × 20 mm, 5 µm beads C18 Reprosil-HD; Dr. Maisch, Ammerbuch-Entringen, Germany). After flushing from the trapping column, the sample was loaded on an analytical column (made in-house, 75 µm I.D. × 400 mm, 1.9 µm beads C18 Reprosil-HD; Dr. Maisch). Peptides were loaded with loading solvent (0.1% TFA, 2% ACN) and eluted with a non-linear 150 min gradient of 2–56% solvent B (0.1% formic acid in water/acetonitrile, 20/80 (v/v)) at a flow rate of 250 nL/min, followed by a 10 min wash reaching 99% solvent B and re-equilibration with solvent A (0.1% formic acid in water). The column temperature was kept constant at 50 °C (CoControl 3.3.05, Sonation). The mass spectrometer was operated in data-dependent, positive ionization mode, automatically switching between MS and MS/MS acquisitions for the 16 most abundant peaks in a given MS spectrum. The source voltage was set to 2.8 kV and the capillary temperature was 250 °C. One MS1 scan (m/z 375–1500, AGC target 3E6 ions, maximum ion injection time of 60 ms) acquired at a resolution of 60,000 (at 200 m/z) was followed by up to 16 tandem MS scans (resolution 15,000 at 200 m/z, AGC target 1E5 ions, maximum ion injection time of 80 ms, isolation window of 1.5 m/z, fixed first mass of 145 m/z, spectrum data type: centroid) of the most intense ions fulfilling predefined selection criteria (underfill ratio 2%, intensity threshold 1.3E4, exclusion of unassigned, singly and >7 charged precursors, peptide match preferred, exclude isotopes on, dynamic peptide signals).

1 The abbreviations used are: BPH, brown planthopper; ABC, ammonium bicarbonate; ASA, *Allium sativum* agglutinin; ConA, Concanavalin A; FASP, Filter-aided sample preparation; Gal, galactose; GalNAc, N-acetylgalactosamine; GO, Gene Ontology; PBS, phosphate buffered saline; PNGase A, Peptide-N-glycosidase A; LF, long-winged female; LFQ, label-free quantification; LM, long-winged male; RSA, *Rhizoctonia solani* agglutinin; SF, short-winged female; SM, short-winged male.
exclusion time of 12 s). The HCD collision energy was set to 28% Normalized Collision Energy and the polydimethylcyclosiloxane background ion at 445.12002 Da was used for internal calibration (lock mass).

Data Analysis—Raw data from each LC run were searched with MaxQuant software (version 1.5.8.3) (20) against the unannotated N. lugens proteome (27,571 entries, BioProject: PRJNA177647) (21) with a precursor mass tolerance set to 20 ppm for the first search and to 4.5 ppm for the main search. Trypsin/P was selected as enzyme setting, allowing for two missed cleavages, and cleavage was allowed when arginine or lysine was followed by proline. S-carbamidomethylation of cysteines was set as a fixed modification, whereas methionine oxidation, acetylation of the protein N terminus and asparagine deamidation were set as variable modifications (supplemental Fig. S1). The false discovery rate for peptide, protein, and site identification was set to 1%, and the minimum peptide length to 7. The minimum score threshold for both modified and unmodified peptides was set to 40, and known contaminants were excluded. Match between runs was set to True. The presence of remaining glycopeptides was assessed by tracing glucan specific oxonium ions. For each sample we extracted the trace in MS2 for the masses 163.06 and 204.09 which are specific for the fragmentation of glycopeptides containing hexoses and N-acetylhexosamines, respectively. These raw traces are depicted in supplementary (supplemental Fig. S1).

Collected data were analyzed using a KNIME workflow (version 3.3.2) (22). First, former N-glycopeptides were identified as peptides containing one or more N-glycosylation site(s), either N-X(-S, -T, -N-X(-V or N-X(-C, where the asparagine residue at position 1 in the N-glycosylation sequon is deamidated, and X represents any amino acid apart from proline. Only N-glycopeptides that were detected in at least two biological replicates were withheld for further analysis. Glycopeptides were grouped by protein to assess the number of least two biological replicates were withheld for further analysis. Glycopeptides were grouped by protein to assess the number of N. lugens adults were annotated by means of the Drosophila melanogaster proteome (UniProt: 21,979 entries) using BLAST+ (version 2.2.3.1, E-value threshold = 0.00001) (23).

Gene Ontology (GO) was studied for all annotated N-glycopeptides using the online tool from UniProt (www.uniprot.org). For the ConA and RSA unique N-glycopeptides, gene ontologies were analyzed using the online database DAVID (24), allowing the visualization of overrepresented cellular components and biological processes in relation to the D. melanogaster proteome. The molecular weight from glycopeptides was estimated using the online ‘compute pi/Mw’ tool from ExPASy (https://web.expasy.org/compute_pi/). After log2 transformation, the label-free quantification (LFQ) intensities of the identified glycopeptides were compared between groups (sex and wing form) using Two-way ANOVA to reveal proteins with a significantly different intensity (\( p < 0.001 \)) using the “peptides” file from MaxQuant in Perseus software version 1.6.2.2. The ratios for the (male/female) of the log2 transformed LFQ intensities of the proteins identified in the shotgun proteome analysis were calculated as well, and proteins detected with higher intensity in males or females were selected as regulated.

Dissection of Adult Tissues—Adult insects (SM and SF) were collected and kept on ice. Insects were dissected in phosphate buffered saline (PBS) under a microscope (Leica) using forceps. Collected tissues (head, gut, ovaries, testes) were rinsed three times in PBS to minimize hemolymph contamination, and were subsequently collected in an Eppendorf tube containing 600 \( \mu l \) of lysis buffer with \( \beta \)-mercaptoethanol (for RNA extraction, Qiagen RNeasy mini kit) or in 600 \( \mu l \) PBS (for protein extraction) on ice. Tissues were collected from 50 male and 50 female insects.

Far-Western Blot Analysis—Protein extracts from complete adult insects (LF, LM, SF, SM; 50 \( \mu g \)) were loaded on two SDS-PAGE gels. One gel was used for Coomassie Brilliant blue staining, the other one for blotting. After blotting, the PVDF membrane was blocked overnight with 5% (w/v) bovine serum albumin. Then, the PVDF membrane was incubated for 3 h with ConA-solution (5 \( \mu g/ml \)). Subsequently, the blot was incubated for 1 h with primary antibody (anti-ConA, 1:4000), secondary antibody (goat-anti-rabbit, 1:20,000) and peroxidase-antiperoxidase (1:400). Finally, the glycoproteins were detected with 3,3’-diamino-benzidine staining.

Gene Expression Analysis—Approximately 15 mg of adult insects and insect tissues (head, gut, ovaries, testes) were collected for RNA extraction. RNA was isolated using Qiagen RNeasy mini kit according to the manufacturer’s protocol. The TURBO DNA-free Kit (Ambion, Life Technologies, Carlsbad, CA) was used to remove trace quantities of DNA. cDNA was synthesized with SuperScript IV First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) using 1 \( \mu g \) of total RNA. The cDNA was diluted 1:10 for subsequent real-time qPCR, which was performed using GoTaq qPCR Master Mix (Promega, Madison, WI) and Bio-Rad CFX 96 Connect robot. The program for thermal cycling included 10 s denaturation at 95 °C, 30 s annealing/extension at 60 °C with melt curve analysis at the end of the run. NlRps15 and NlActin were used as reference genes. Detailed information on primer sequences and amplification efficiency can be found in supplementary Table S1. The whole experiment was performed in four independent biological replicates for complete adult insects and in three independent biological replicates for adult tissues. Relative mRNA expression of the target genes was quantified by the BioRad CFX Manager software. Statistical analysis was conducted in qbase+ (Biogazelle, Zwijnaarde, Belgium). Primers for qPCR were designed using Primer 3 (http://bioinfo.ut.ee/ primer3-0.4.0/).

RESULTS

Far-Western Blot Analysis Reveals Differences in Protein N-glycosylation Between Adult Sexes and Reproductive Tissues—To evaluate whether differences in protein glycosylation are present between wing forms and/or adult sexes, as was previously observed at the N-glycome level (4), far-Western blot analysis was conducted using anti-ConA as the primary antibody to visualize glycoproteins carrying high mannose and/or paucimannose N-glycans. First, complete protein extracts for the adult morphs were studied (supplemental Fig. S2A). Coomassie Brilliant blue staining revealed very similar protein patterns for all adult morphs. After anti-ConA staining, visual differences between males and females can be observed, whereas no such differences were noted between wing forms. Next, a comparative analysis was made between extracts from ovaries and testes to examine whether the observed differences at the level of the complete insect arise from differences in the reproductive tissues (supplemental Fig. S2B). Indeed, the male and female reproductive tissues show a completely different pattern of high mannosylated and/or paucimannosylated glycoproteins, with more glycoproteins observed in ovaries.

In summary, these observations indicate that there are clear differences at the glycoprotein level between adult sexes, but not between wing forms, and that ovaries contain more glycoproteins compared with testes.
Enrichment of N-glycosylation Sites from N. lugens Adults—To further compare protein glycosylation between adult sexes and wing forms of *N. lugens*, a comprehensive N-glycosylation site analysis was conducted for the different adult morphs. Complete protein extracts were enriched for glycopeptides using the lectins ConA and RSA to select peptides carrying high mannose/paucimannose N-glycans and complex N-glycans, respectively. After deglycosylation these peptides were analyzed using mass spectrometry to identify the N-glycosylation sites. ConA enrichment resulted in the detection of over 1200 N-glycosylation sites, derived from about 600 different glycoproteins (Table I). Enrichment with RSA led to the identification of over 300 N-glycosylation sites, from close to 200 glycoproteins. In total, over 1300 N-glycosylation sites were identified in *N. lugens* adults (Fig. 1). Almost 40% or 528 N-glycosylation sites were detected in all adult morphs and thus were present in both sexes and both wing morphs. On the total number of N-glycosylation sites, 216 (or 16%) were identified both after enrichment with ConA and RSA (supplemental Table S2). These results indicate the micro-heterogeneity of N-glycan structures in *N. lugens* proteins, where certain N-glycosylation sites can carry either high mannose/paucimannose N-glycans or complex N-glycans. A complete list of all identified N-glycosylation sites is provided in supplemental Table S3.

GO terms were analyzed using the online tool from UniProt to assess the cellular localization of the identified glycoproteins and the biological processes in which they are involved. Glycoproteins from *N. lugens* adults are mostly present in membranes and the extracellular space, as is expected for N-glycosylated proteins, and play a role in diverse biological processes such as metabolic, developmental and reproductive processes. Glycopeptides enriched with different lectins, hence decorated with different glycan structures, were most likely derived from glycoproteins with differential functions. Based on GO analysis using DAVID to obtain an overview of the overrepresentation of cellular localization and biological processes, glycoproteins carrying high mannose and/or paucimannose N-glycans were found to be mostly membrane proteins that are involved in proteolysis (GO:0006508, *p* = 2.2E-4) and cell adhesion (GO:0007155, *p* = 7.4E-7), whereas glycoproteins decorated with complex glycans are more involved in transmembrane transport (GO:0055085, *p* = 6.0E-2) and are enriched in membranes and lipid particles.

Sex-specific Glycopeptides Reveal a Putative Role for Glycosylation in Reproduction—Quantitative comparisons of glycopeptides between sexes and wing forms were made based on log2 transformed LFQ intensities to evaluate the putative involvement of protein N-glycosylation in sex- and wing type-specific processes. Glycopeptides detected with differential intensity (*p* < 0.001) between the two sexes were abundant for both lectins, with 68 glycopeptides enriched with ConA, and 33 with RSA (Fig. 2). Glycopeptides carrying complex N-glycans were mostly male-specific (29 male, 4 female), whereas there was a more equal sex distribution for high mannosylated and/or paucimannosylated glycopeptides (38 male, 30 female). These sex-specific glycopeptides were, among others, derived from proteins important for male (seminal fluid protein) and female reproduction (putative vitellogenin receptor, vitellogenin-like protein, Nasrat). Glycopeptides detected with differential intensity between the two wing forms were scarce (1 detected for ConA and 4 for RSA). The complete list of glycopeptides with differential intensity between either sexes or wing forms is provided in supplemental Table S4.

A comparison can also be made between the information obtained from the mass spectrometry data and the far-Western blotting analyses. Given that the far-western blots visual-

| Sample | Identified proteins | Enriched glycosites | Enriched glycopeptides | Enriched glycoproteins |
|--------|--------------------|---------------------|------------------------|------------------------|
|        |                    | ConA RSA | ConA RSA | ConA RSA | ConA RSA |
| LF     | 2251               | 870 124  | 863 119  | 501 96    |
| LM     | 2242               | 1012 225 | 997 214  | 532 161   |
| SF     | 2421               | 679 232  | 677 217  | 411 158   |
| SM     | 2807               | 905 249  | 893 237  | 507 179   |
| Total  | 3322               | 1269 315 | 1216 297 | 606 205   |
|        |                    |          | 1368      | 1352      |
|        |                    |          | 658       |           |

**Fig. 1. Distribution of N-glycosylation sites in the different adult morphs of *N. lugens***. N-glycosylation sites were identified from *N. lugens* adult morphs using mass spectrometry. The Venn diagram shows the distribution of the N-glycosylation sites over the different adult morphs; long-winged females (LF, 913), long-winged males (LM, 1,083), short-winged females (SF, 784), and short-winged males (SM, 1,004).
ized the high mannosylated and/or paucimannosylated glycopeptides, only the sex-related glycopeptides detected with ConA can be evaluated. The 68 sex-related glycopeptides were derived from 50 unique glycoproteins; among which 13 female-specific and 37 male-specific glycoproteins. The molecular weights of these 50 glycoproteins were calculated based on their amino acid sequences, allowing to determine the molecular weights of the non-glycosylated proteins. Taking into account that the molecular weight of the high mannose N-glycan GlcNAc$_2$Man$_9$ is $\sim$2 kDa, the molecular weight of each glycoprotein was estimated (taking into account the number of identified N-glycosylation sites for each protein) (supplemental Table S5). For the males, a faint band around 38 kDa can be observed in complete insects and is also clearly present in the testes, but not observed in females or ovaries. In testes, two other distinct bands are visible at 25 kDa and 65 kDa. All these polypeptides react with ConA and could possibly correspond to the identified male-specific glycopeptides.

Fig. 2. Heatmap of sex-specific glycopeptides. Quantitative comparison based on LFQ intensities between male and female N. lugens adults using Two-way ANOVA in Perseus. For each adult morph, three biological replicates were analysed. Glycopeptides with a statistical different intensity ($p<0.001$) between sexes are visualized for glycopeptides enriched with ConA (left) and RSA (right) and clustered by sample. The protein accession is represented of the protein from which the glycopeptide is derived. Protein sequences are available on ftp://parrot.genomics.cn/gigadb/pub/10.5524/100001_101000/100139/. Accession numbers that occur more than once, indicate multiple glycosylation sites on the given protein. The scale represents the Z-score of the log2 LFQ intensities.
coproteins James bond (37 kDa), Dumpy (26 kDa) and Amino
acid transporter (67 kDa), respectively. For females, the high
number of glycoproteins visualized in the ovaries is promi-
nent, with most glycoproteins appearing at molecular weights
ranging from 130 to 180 kDa, whereas no proteins smaller
than 37 kDa were detected. These observations correspond
well to the female-specific glycoproteins identified after mass
spectrometry analysis, which are almost all glycoproteins with
a molecular weight of over 100 kDa. At present, only assump-
tions can be made with respect to interpretation of the bands
detected on the western blots and the molecular masses of the
glycoproteins. Further post-translational processing of the
proteins could occur, which will affect their electrophoretic
behavior on SDS-PAGE. Therefore, future experiments should
focus on the identification and characterization of the ConA
reactive glycoproteins.

Comparison of Glycopeptides Between Adult Males and
Females Reveals Altered Glycosylation of Proteins Shared
Across Sexes—Complete protein extracts were analyzed us-
ing mass spectrometry for the different adult morphs of N.
lugens, and this shotgun proteome analysis resulted in the
identification of over 3300 proteins (Table I). The log2 trans-
fomed LFQ intensities from the proteins identified and quan-
tified in both male and female adult samples were compared
between the sexes. Further comparison with the identified
sex-specific glycopeptides allowed to determine whether dif-
fferences between sexes on the glycopeptide level resulted
from differential glycosylation between the sexes or from dif-
fences in protein levels. The 99 sex-specific glycopeptides
were derived from 69 unique glycoproteins, of which 49 (71%)
were also present in the shotgun proteomics data. For 22 of
these glycoproteins, no statistically significant differences
($p > 0.05$) could be observed between the protein intensities
from male and female samples, indicating that the observed
differences on the glycopeptide level result from differential
glycosylation between sexes (Table II). Up-regulation of the
protein in either males or females was observed for 13 glyco-
proteins (Table II), whereas complete absence in either of the
two sexes was observed for 14 other glycoproteins. After
correction of the regulation of the protein, differential levels of
glycopeptides between sexes observed for these 27 proteins
could be assigned to the (up)regulation of the protein. All
glycoproteins involved in the reproductive process belong to
this latter group.

Transcript Expression Analyses Confirm Observed Differ-
ences Between Sexes On N-glycopeptide Level—Striking dif-
ferences in protein N-glycosylation were observed between
sexes, whereas there were no obvious discrepancies between
wing forms. Both sex-specific glycoproteins as well as pro-
teins that are differentially glycosylated in the two sexes were
identified. To confirm this observation, transcript expression
was studied for adult morphs and adult tissues (head, gut,
ova ries and testes) for 5 selected glycoproteins. These glyco-
proteins were identified by glycopeptides only detected in
either male or female adults or with a statistically different
glycopeptide intensity ($p < 0.001$) between sexes, after en-
richment with ConA, RSA or both (Table III). Transcript anal-
ysis from complete adult insects showed no differences be-
tween wing forms, because both long- and short-winged
adults cluster together and no statistical differences could be
observed (One-way ANOVA, $n = 4$) (supplemental Fig. S3).
Two transcripts were found to be female-specific; Nasrat ($p =
9.36E-10$) and Flyers-cup ($p = 1.97E-7$), whereas James bond
was found to be male-specific ($p = 3.20E-8$) (Fig. 3). The other
two transcripts showed no significant differences between
males and females at the level of the whole insect.

Because no differences on transcript level were observed
between wing types, expression in adult tissues was only eval-
uated for short-winged insects. For all studied transcripts, dif-
fferences between tissues were observed (One-way ANOVA,
$n = 3$) (Fig. 4). For the female-specific transcripts, expression of
Flyers-cup was high in all female tissues ($p = 2.60E-4$), whereas
Nasrat was found to be ovary-specific ($p = 4.97E-6$). For two
other transcripts, the expression was the highest in the testes;
James bond ($p = 4.97E-6$) and Aminopeptidase ($p = 2.19E-4$).
CGS276 was expressed at the highest levels in the male gut
($p = 1.56E-3$) and in testes.

4. DISCUSSION

Sex-related N-glycosylation has already been reported in
several organisms, including humans (25), parasitic nema-
todes (26) and trematodes (27). Recently, differences in pro-	ein N-glycosylation between sexes were also observed for
the first time in an insect species, the pest insect N. lugens (4).
All these studies only focused on the N-glycomes from males
and females, whereas here we opted to identify the proteins
carrying these differential carbohydrate structures to unravel
the relevance of these observed differences between sexes at
the glycoprotein level. Experiments were conducted in N.
lugens, a hemipteran model insect, where male and female
adults can appear as two wing morphs; the flightless short-
winged morph and the migratory long-winged morph. N-gly-
cosylation sites were identified from the different adult
morphs, including both sexes and wing types, allowing for the
evaluation of the putative involvement and significance of
protein N-glycosylation in sex- and wing type-specific pro-
cesses.

Far-Western blotting experiments were first conducted to
test the hypothesis that protein N-glycosylation can be sex-
and/or wing type-dependent in N. lugens adults. Complete
protein extracts from the adult insects as well as male and
female reproductive tissues were investigated. Obvious dif-
fferences were observed in the presence of glycoproteins
between males and females, independent of their wing form.
Furthermore, clear differences between glycoproteins present
in ovaries and testes were visualized, where ovaries seem to
contain a high amount of high mannosylated and/or pauci-
mannosylated glycoproteins. This Western blotting result is a
TABLE II

Sex-specific glycopeptides result both from increased glycosylation of their corresponding proteins as well as from increased protein levels in one the sexes

| Sex          | Accession     | Protein description                        | Male/Female         |
|--------------|---------------|--------------------------------------------|---------------------|
| Female       | NLU001824.1   | Putative vitellogenin receptor             | ND Male             |
| Female       | NLU012863.1   | Uncharacterized protein                    | ND Female           |
| Female       | NLU006222.1   | Female sterile (1) Nasrat                   | ND Male             |
| Female       | NLU004755.1   | Flyers-eup                                 | 0.75                |
| Female       | NLU019204.1   | Vitellogenin-like                          | 0.82                |
| Female       | NLU011477.1   | Vitellogenin-like                          | 0.86                |
| Female       | NLU011555.1   | Uncharacterized protein                    | 0.94                |
| Female       | NLU024350.1   | Uncharacterized protein                    | 0.96                |
| Female       | NLU010113.1   | Alpha-mannosidase                          | 0.96                |
| Female       | NLU020778.1   | Transmembrane GTPase Marf                  | 1.00                |
| Female       | NLU002989.3   | Receptor expression-enhancing protein      | 1.02                |
| Male         | NLU026415.2   | Maltase A8                                 | 0.99                |
| Male         | NLU001320.1   | LD23292p                                   | 1.00                |
| Male         | NLU002176.1   | Aminopeptidase                             | 1.00                |
| Male         | NLU023838.1   | Maltase A3                                 | 1.00                |
| Male         | NLU027145.1   | CG5276                                     | 1.00                |
| Male         | NLU014188.3   | Amino acid transporter                     | 1.01                |
| Male         | NLU003276.1   | Cardinal                                   | 1.01                |
| Male         | NLU008295.1   | RNA-binding protein cabeza-like            | 1.01                |
| Male         | NLU011751.3   | RH72323p                                   | 1.01                |
| Male         | NLU007664.2   | UDP-glucuronosyltransferase                | 1.01                |
| Male         | NLU001964.1   | Basigin                                    | 1.01                |
| Male         | NLU024229.1   | Neurotactin                                | 1.02                |
| Male         | NLU016734.1   | Kazachoe                                   | 1.03                |
| Male         | NLU020709.1   | Dipeptidase                                | 1.03                |
| Male         | NLU011804.1   | RE14947p                                   | 1.04                |
| Male         | NLU005014.1   | Neural lazarillo                           | 1.05                |
| Male         | NLU011657.1   | Spactzle-processing enzyme                 | 1.07                |
| Male         | NLU017497.1   | Carboxypeptidase B-like                    | 1.11                |
| Male         | NLU001528.1   | Uncharacterized protein                    | 1.12                |
| Male         | NLU001918.1   | Uncharacterized protein                    | 1.12                |
| Male         | NLU027835.1   | Dumpy                                      | 1.12                |
| Male         | NLU024819.2   | RE52890p                                   | 1.17                |
| Male         | NLU012529.1   | Uncharacterized protein                    | 1.18                |
| Male         | NLU021589.1   | Uncharacterized protein                    | 1.23                |
| Male         | NLU002851.1   | Seminal fluid protein                      | 1.23                |
| Male         | NLU012421.1   | Carboxylic ester hydrolase                 | 1.23                |
| Male         | NLU014493.1   | Carboxylic ester hydrolase                 | 1.32                |
| Male         | NLU003617.2   | ATPase ASNA1 homolog                       | ND Female           |
| Male         | NLU003798.1   | Kekkon-2                                   | ND Female           |
| Male         | NLU003811.1   | Transmembrane 9 superfamily member         | ND Female           |
| Male         | NLU006117.1   | Esterase P                                 | ND Female           |
| Male         | NLU012109.1   | Uncharacterized protein                    | ND Female           |
| Male         | NLU012692.1   | Nuclear factor NF-kappa-B p110 subunit     | ND Female           |
| Male         | NLU014036.1   | Uncharacterized protein                    | ND Female           |
| Male         | NLU018639.1   | Vesicular acetylcholine transporter        | ND Female           |
| Male         | NLU020349.1   | LD28763p                                   | ND Female           |
| Male         | NLU020863.1   | Thioredoxin reductase I                    | ND Female           |
| Male         | NLU028220.1   | Amino acid transporter                     | ND Female           |

The column Male/Female contains the ratios of the log2 transformed LFQ intensities from the proteins identified in the shotgun proteome samples from male and female adults. The 95% confidence interval is [0.93; 1.07], where <0.93 means upregulated in females compared to males (yellow), and >1.07 means upregulated in males compared to females (blue). ND male–not detected in male samples, ND female–not detected in female samples.
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**FIG. 3.** Clustergram of transcript expression of sex-specific glycoproteins from *N. lugens* adult morphs. Transcript levels for 5 selected glycoproteins were analysed in *N. lugens* adult insects (long-winged females - LF, long-winged males - LM, short-winged females - SF, short-winged males - SM). NIRps15 and NIActin were used as reference genes. Statistical differences were evaluated using One-way ANOVA test (n=4). The data are shown in a hierarchy based on the degree of similarity of expression for different samples and targets; upregulation (red), downregulation (green) or no regulation (black).

**TABLE III**
Selection of sex-specific glycoproteins for transcript expression analysis

| *N. lugens* | *D. melanogaster* | Protein name | Sex specificity | Lectin | Sex-specific N-glycosites |
|-------------|------------------|--------------|----------------|--------|--------------------------|
| NLU002176.1 | Q8INH5           | Aminopeptidase | Male           | RSA    | 1                        |
| NLU004755.1 | Q9V3X1           | Flyers-cup    | Female         | ConA,RSA | 7                      |
| NLU006222.1 | Q76904           | Nasrat        | Female         | ConA   | 2                        |
| NLU020486.2 | Q9VCY7           | James bond    | Male           | ConA   | 2                        |
| NLU027145.1 | Q9VGN8           | CG5276        | Male           | ConA   | 1                        |

**Fig. 4.** Transcript expression of sex-specific glycoproteins in tissues from *N. lugens* short-winged adults. Transcript levels for 5 selected glycoproteins were analysed in *N. lugens* short-winged adult tissues (head, gut, ovaries, testes). NIRps15 and NIActin were used as reference genes. The bar chart shows the average ± standard deviation (n = 3). Statistical differences were evaluated using One-way ANOVA with post-hoc Tukey test, and are marked with a different letter (a, b, c, d).
first indication that there is no differential glycosylation between wing types, but protein N-glycosylation is sex-related in *N. lugens* adults and could play a role in (female) reproduction.

Glycopeptides were enriched by the N-glyco-FASP method using two lectins interacting with different N-glycan structures, allowing for the further identification of the N-glycosylation sites. ConA specifically interacts with high mannose and paucimannose N-glycans, whereas RSA binds to complex N-glycans with terminal Gal or GaINAc residues. In total, over 1300 N-glycosylation sites were identified, most of them via enrichment with ConA. This result is not surprising because high mannose and paucimannose N-glycans together make up >90% of the insect N-glycan profile (4, 15, 28, 29). Overlap between the datasets obtained after enrichment with ConA and RSA indicates that certain glycoproteins may occur in different glycoforms, suggesting micro-heterogeneity of N-glycan structures in *N. lugens* proteins.

The N-glycosylation sites identified were derived from glycoproteins playing a role in diverse biological processes such as metabolic, developmental and reproductive processes, demonstrating the importance of protein N-glycosylation in *N. lugens* adults. Interestingly, N-glycosylation sites that were uniquely identified after enrichment with ConA or RSA were derived from glycoproteins with differential functions. Glycoproteins decorated with high mannose and/or paucimannose N-glycans are mostly membrane proteins that are involved in adhesion and proteolysis. Although glycoproteins carrying complex N-glycans, as enriched with RSA, play a role in transmembrane transport, and are present in lipid particles and membranes. A similar observation was made in *Spodoptera* midgut cells; high and oligomannose N-glycans appeared to be more abundant in the basal region of the cells, which is involved in cell-cell and cell-matrix interactions (30). On the other hand, glycans with terminal Gal/GalNAC residues were more abundant in the apical region of the columnar midgut cells, the primary site for many physiological, biological and biochemical interactions (e.g. release of digestive enzymes, uptake of nutrients). Furthermore, the addition of carbohydrate structures to proteins can influence their properties (31), and thus the biological activity of a particular protein can differ between the glycosylated and non-glycosylated form, as well as between different glycoforms of the same glycoprotein. For example in *Drosophila*, diminished N-glycosylation of the protein chaoptin resulted in an abolished activity of the protein (32), whereas sialylation of the voltage gated K⁺ channel SBb demonstrated that sialylation may increase gating efficiency (33).

A quantitative comparison of N-glycopeptides between the two sexes and wing types of *N. lugens* adults revealed the presence of almost 100 sex-specific glycopeptides, whereas wing type-specific glycopeptides were scarce. The sex-specific glycopeptides carried high mannose/paucimannose N-glycans, complex N-glycans or both. Glycopeptides carrying complex N-glycans were mostly male-enriched, which is in agreement with the presence of more Gal-terminal N-glycans in males (4). Comparative analyses between glycopeptides and the presence of glycoproteins in complete protein extracts allowed to determine whether differences between sexes at the glycopeptide level resulted from differences in glycoprotein levels or from differential glycosylation between the sexes. This comparison revealed that differences in protein glycosylation between sexes are caused for ~55% by differences observed at protein level, whereas 45% can be attributed to differential glycosylation. For example, glycoproteins involved in insect reproduction belong to the first category of proteins and are either unique for one sex or much higher expressed in one of both sexes. For males, only one glycoprotein (seminal fluid protein), both carrying high mannose/paucimannose N-glycans and complex N-glycans, was identified that plays a role in male reproduction. Insect seminal fluid proteins are transferred to females during mating and induce several physiological and behavioral post-mating changes in females (34). In contrast to males, several high abundant glycoproteins were identified in females, carrying high mannose/paucimannose N-glycans that are involved in female reproduction; putative vitellogenin receptor (35), vitellogenin-like protein (36), and Nasrat (37–39). The vitellogenin-like protein also carries complex N-glycans, next to the high mannose/paucimannose N-glycans. These findings are in line with the detection of a high amount of glycoproteins in ovaries by Western blotting analyses, and support the hypothesis that female adults obtain a female-unique N-glycan fingerprint with increased levels of high mannose N-glycans because of the presence of female-unique glycoproteins that play a role in female reproduction (4). Glycoproteins that are equally produced in both sexes, but show differential glycosylation between males and females are of high interest. Of these, 22 glycoproteins were identified of which two are yet uncharacterized. The other glycoproteins are putatively involved in metabolism, stress response and developmental processes. Further investigation of the function of these glycoproteins in male and female adults could indicate whether this differential protein glycosylation also results in a different biological function for these glycoproteins between sexes.

To verify the observations from the proteomic analysis, a selection of glycoproteins identified by male- and female-specific glycopeptides, carrying high mannose/paucimannose N-glycans and/or complex N-glycans, was also analyzed for transcript expression.

Three male-specific glycoproteins were selected: James bond, CG5276, and Aminopeptidase. For the first two male-specific glycoproteins it was previously shown that they could play a role in male reproduction. The fatty acid elongase James bond was already proven to be essential for sex pheromone synthesis and male fertility in *Drosophila* (40). CG5276 is a calcium-activated nucleosidase with apyrase activity from
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D. melanogaster, found on the cell surface and as a secreted protein (41). The expression of this enzyme in Drosophila salivary glands and testes suggests an important role in these organs, and a putative role in male reproduction. Aminopeptidase was previously shown to have metalloendopeptidase activity (42), but there is no evidence for a role in reproduction.

Two female-specific glycoproteins were further investigated: Nasrat and Flyers-cup. The role of Nasrat in female reproduction has already been extensively studied in D. melanogaster (35–37), and the protein was proven to be essential for the vitelline membrane formation. In D. melanogaster, Flyers-cup transcripts were detected in spermatids (43), and its involvement in memory formation has also previously been implicated (44). In contrast to Drosophila, Flyers-cup is much more abundant in Nilaparvata female adults compared with males, based on information from the shotgun proteome analysis. Expression of the selected glycoproteins was studied at transcript level for both complete adult insects and adult tissues. Expression analysis from complete adult insects confirmed the sex-specific expression as seen on glycopeptide level for Nasrat, Flyers-cup and James bond, whereas no differences in transcript levels were observed for aminopeptidase and CG5276. For Aminopeptidase, James bond and Nasrat, gene expression was statistically highest in the reproductive tissues, supporting a potential role for these proteins in Nilaparvata reproduction. CG5276 showed highest expressed in male gut and testes. Flyers-cup revealed a higher expression in all female tissues compared with the male tissues. The combination of transcript expression data from complete insects and adult tissues confirms that the observed sex-differences in N. lugens protein glycosylation are the result from both the presence of sex-specific glycoproteins as well as from differential glycosylation of proteins occurring in both male and female adults. Given the contradictions for the tissue specificity of Flyers-cup between D. melanogaster and N. lugens, it would be of interest to focus further experiments on the functionality of this protein in N. lugens.

In summary, our study presents original data on the N-glycosylation sites from N. lugens adults over sexes and wing types. Although almost no differences in N-glycopeptides were observed between wing types, several sex-specific glycopeptides have been identified. Next to the N-glycome, differences between males and females have now also been observed on N-glycosylation site level, strengthening the belief that protein N-glycosylation is sex-related in this pest insect. This sex-related protein glycosylation in N. lugens adults is believed to result from both the presence of sex-specific glycoproteins as well as from altered glycosylation of proteins shared across sexes. Furthermore, the discovery that several proteins involved in (female) reproduction are glycosylated implies that protein N-glycosylation can play a role during insect reproduction. Our research provides novel insights into planthopper’s biology, and offers information for future research on planthopper’s development, reproduction and potential control methods.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013657. All spectra and search results were uploaded to MS-Viewer with following accessions; ke88onqwl (shotgun samples), hgenowvedt (ConA-enriched samples), and gfrwdgysub (RSA-enriched samples).

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