

**Isomeric separation and recognition of anionic and zwitterionic N-glycans from royal jelly glycoproteins**

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*Running title:*

Royal jelly N-glycome

*Abstract:*

Royal jelly has received attention due to its necessity for the development of queen honeybees as well as claims of benefits on human health; this product of the hypopharyngeal glands of worker bees contains a large number of proteins, some of which have been claimed to have various biological effects only in their glycosylated state. However, although there have been glycomic and glycoproteomic analyses in the past, none of the glycan structures previously defined would appear to have potential to trigger specific biological functions. In the current study, whole royal jelly as well as single protein bands were subject to off-line LC-MALDI-TOF MS glycomic analyses, complemented by permethylation, Western blotting and arraying data. Similarly to recent in-depth studies on other insect species, previously overlooked glucuronic acid termini, sulphation of mannose residues and core  $\beta$ -mannosylation of the N-glycans were found; additionally, a relatively rare zwitterionic modification with phosphoethanolamine is present, in contrast to the phosphorylcholine occurring in lepidopteran species. Indicative of tissue-specific remodelling of glycans in the Golgi apparatus of hypopharyngeal gland cells, only a low amount of fucosylated or paucimannosidic glycans were detected as compared to other insect samples or even bee venom. The unusual modifications of hybrid and multiantennary structures defined here may not only have a physiological role in honeybee development, but represent epitopes recognised by pentraxins with roles in animal innate immunity.

*Dedicated to Dr. Hubert Paschinger (1941-2011), chemist, lover of art and music and avid beekeeper.*

## Introduction

Royal jelly is a product of the hypopharyngeal and mandibular glands of worker honeybees (1); its natural role is as the food source for worker and drone larvae for the first three days, but induces queen development when fed beyond that time and remains, thereafter, the food for a queen's entire life. The observed fertility and longer lifespan of queens, as compared to worker bees, has been correlated with this special diet. However, royal jelly has attracted especial attention due to its putative effects on human health. Indeed, it has been used since ancient times as a food supplement or cosmetic and is considered to have a range of antibiotic, anti-inflammatory and other health-relevant effects (2), although these are controversial and allergic reactions are also known (3). Royal jelly contains a wide range of compounds including carbohydrates, lipids, vitamins and proteins, many of which are post-translationally modified (4,5). Due to their multiple and variable monosaccharide units and their size (typically 2000 Da), N-glycans represent the most diverse and complex set of protein modifications known (6) and modulate a wide range of biological processes.

On the basis of proteomic and glycoproteomic studies of royal jelly, up to 100 different proteins have been identified including the 'major royal jelly proteins' (MRJPs). Of these, MRJP1 (also known as apisin or royalactin) has a controversial role in queen development (7,8), but is reported to extend life-span in other invertebrates (9). Furthermore, MRJP1 and MRJP2 (apalbunin2) have been shown to have antihypertensive and antibiotic activity in their glycosylated state (10). Although the sites of glycosylation on MRJPs have been analysed, there is less information regarding the structures of the glycans on the specific glycoproteins. While mass spectrometric analyses (11) indicated potentially complex N-glycosylation of MRJP2, HPLC and NMR data showed the presence of oligomannosidic and hybrid glycans (12,13) on monomeric and oligomeric forms of MRJP1. There have also been a series of glycan analyses on royal jelly as a whole, culminating in a report that Gal $\beta$ 1,3GalNAc $\beta$ 1,4GlcNAc units are detectable on N-glycans and that there is a relevant galactosyltransferase activity (14-17). However, none of the hybrid or biantennary glycans described were especially unusual and so it was concluded that a role for these glycans in the biological activity of royal jelly is unlikely.

Our recent data have proven that insect glycosylation is more complicated than previously thought and that a range of anionic and zwitterionic modifications is present on dipteran and lepidopteran N-glycans (18,19). Therefore, we have reappraised the N-glycome of royal jelly using an off-line LC-MALDI-TOF MS workflow; we have also examined the glycosylation of individual major royal jelly proteins and have immobilised N-glycan pools in probably the first study to ever test interactions of natural insect glycans in an array format. A significant proportion of structures was thereby found to be of either the hybrid or complex type with up to three antennae. About 4% of the total N-glycans carry novel combinations of glucuronic acid, sulphate,  $\beta$ -mannose and phosphoethanolamine, whereby these epitopes have potential for physiological activity in both insects and humans.

## Experimental Procedures

*Enzymatic release and purification of N-glycans:* Royal jelly (Wald und Wiese, Vienna, Austria; ca. 9 g wet weight per glycan preparation) was suspended in water and heat-treated before proteolysis with thermolysin (Promega, Madison, USA) (20) followed by cation exchange (Dowex AG50, Bio-Rad; elution with 0.5 M ammonium acetate, pH 6) and gel filtration (Sephadex G25; GE Healthcare) chromatography of the proteolysate. Thereafter, N-glycans were released from glycopeptides using peptide:N-glycosidase F (PNGase F, 3 U; Roche) at pH 8 as previously described (20), with a subsequent digestion of the remaining glycopeptides using native PNGase A (0.15 mU; Roche) after adjusting to pH 5; the result was a combined PNGase F/A digest. Alternatively, in a second preparation, the PNGase F-released glycans were isolated from the glycopeptides by Dowex AG50 chromatography and the latter were treated with recombinant PNGase Ar (15 U; New England Biolabs, Ipswich, USA); in this case, the pools of PNGase F- and PNGase Ar-released glycans were separately analysed. After another round of cation-exchange chromatography (Dowex AG50; flow-through), the glycans were subject to solid-phase extraction on non-porous graphitised carbon (SupelClean ENVICarb; Sigma-Aldrich, Merck) as described (20); the ‘neutral’ and ‘anionic-enriched’ fractions were then eluted with 40% (v/v) acetonitrile and 40% (v/v) acetonitrile containing 0.1% trifluoroacetic acid (v/v) respectively. The pools of glycans were subject to MALDI-TOF MS before fluorescent labelling by reductive amination using 2-aminopyridine (PA; Sigma-Aldrich) (20). Similar results were obtained for two different lots of royal jelly from the same supplier. For the workflow in schematic form, refer to the **Scheme** in the Supplement.

*Permetylation:* The neutral and anionic N-glycans (70% of each pool from one PNGase F-released preparation) were separately permethylated as previously reported (21) with slight modifications. Briefly, the samples were dried in a glass tube prior to permethylation with 0.2 ml of a slurry of ground NaOH pellets in dimethyl sulphoxide and 0.1 ml of ICH<sub>3</sub>. The reaction mixture was shaken for 4 h at 4°C and then quenched on ice with 0.2 ml of cold water, followed by neutralization with 30% aqueous acetic acid, and then applied to a pre-equilibrated C18 solid phase extraction column. Hydrophilic salts and contaminants were step-wise washed off with 3 ml each of water, 2.5% and 10% (v/v) acetonitrile. Subsequently, permethylated N-glycans were eluted serially with 3 ml each of 25% and 50% (v/v) acetonitrile.

*HPLC fractionation:* Complete pyridylaminated N-glycomes (10% of the neutral and 90% of the anionic pools) were fractionated by reversed-phase HPLC (Ascentis Express RP-amide; 150 x 4.6 mm, 2.7 µm; Sigma-Aldrich) and a gradient of 30% (v/v) methanol (buffer B) in 100 mM ammonium acetate, pH 4 (buffer A) was applied at a flow rate of 0.8 ml/min as follows: 0-4 min, 0% B; 4-14 min, 0-5% B; 14-24 min, 5-15% B; 24-34 min, 15-35% B; 34-35 min, return to starting conditions (20). The RP-HPLC column was calibrated daily in terms of glucose units using a pyridylaminated dextran hydrolysate and the degree of polymerisation of single standards was verified by MALDI-TOF MS. Selected RP-HPLC fractions were subject to HIAx-HPLC as a second dimension as described (20) with an IonPac AS11 column (Dionex, Sunnyvale, USA; 4 x 250 mm, combined with a 4 x 50 mm guard column). A two solvent gradient with buffer A (0.8 M ammonium acetate, pH 3) and buffer B (80% acetonitrile; LC-MS grade) was applied at a flow rate of 1 ml/min: 0-5 min, 99% B; 5-50 min, 90% B; 50-65

min, 80% B; 65–85 min, 75% B. Detection for both columns was by fluorescence (Shimadzu RF-20A XS detector; excitation/emission at 320/400 nm). All manually-collected HPLC glycan fractions were lyophilised, re-dissolved in water and analysed by MALDI-TOF MS and MS/MS.

**Glycan mass spectrometry:** Monoisotopic MALDI-TOF MS was performed using an Autoflex Speed (Bruker Daltonics, Bremen) instrument in either positive or negative reflectron modes with 6-aza-2-thiothymine (ATT; Alfa-Aesar, Thermo Scientific) as matrix. MS/MS was in general performed by laser-induced dissociation of the  $[M+H]^+$  or  $[M-H]^-$  pseudomolecular ions (except for permethylated structures analysed as  $[M+Na]^+$  with 2,5-dihydroxybenzoic acid as matrix); typically 2000 shots were summed for MS (reflector voltage, lens voltage and gain respectively 27 kV, 9 kV and 2217 V) and 4000 for MS/MS (reflector voltage, lift voltage and gain respectively 27 kV, 19 kV and 2174 V). Spectra were processed with the manufacturer's software (Bruker Flexanalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and 3 for MS/MS (four-times smoothed). Glycan spectra were manually interpreted on the basis of the masses of the predicted component monosaccharides, differences of mass in glycan series, fragmentation patterns, comparison with co-eluting structures from other insects and nematodes and chemical treatments or exoglycosidase digestions (see also pages S2–S4 of the Supplement). Negative-ion mode LC-MS<sup>n</sup> of a 2D-HPLC-enriched glycan was performed as previously described, using a 5  $\mu$ m porous graphitised carbon column (10 cm  $\times$  150  $\mu$ m) coupled to a Thermo Scientific LTQ ion trap mass spectrometer (22); refer also to page S5 of the Supplement for further details. A list of theoretical *m/z* values for each glycan composition is presented in the Supplementary Table 1; mzXML files of raw MS/MS data are available as supplementary information.

**Enzymatic and chemical treatments:** Glycans were treated, prior to re-analysis by MALDI-TOF MS, with  $\alpha$ -fucosidase (bovine kidney from Sigma-Aldrich),  $\alpha$ -mannosidases (jack bean from Sigma-Aldrich, *Aspergillus*  $\alpha$ 1,2-specific from Prozyme, Hayward, USA, and *Xanthomonas*  $\alpha$ 1,2/3-specific from New England Biolabs),  $\beta$ -galactosidase ( $\beta$ 1,3-specific from New England Biolabs),  $\beta$ -glucuronidases (*E. coli* from Megazyme, Bray, Ireland, and *Helix pomatia* from Sigma-Aldrich; desalted and concentrated before use),  $\beta$ -N-acetylhexosaminidases (jack bean from Sigma-Aldrich, *Xanthomonas*  $\beta$ 1,2-specific *N*-acetylglucosaminidase from New England Biolabs, *Streptomyces*  $\beta$ 1,3/4-specific *N*-acetylhexosaminidase (chitinase) from New England Biolabs or in-house produced recombinant forms of *Caenorhabditis elegans* HEX-4 specific for  $\beta$ 1,4-linked GalNAc residues or *Apis mellifera* FDL specific for the product of GlcNAc-transferase I (23)) in 50 mM ammonium acetate, pH 5, at 37 °C overnight (except for pH 6.5 in the case of HEX-4, pH 7 in the case of *E. coli*  $\beta$ -glucuronidase or an incubation time of only 3 hours in the case of FDL); these incubations were performed in PCR tubes with a final volume of 3  $\mu$ l (for further details about conditions and specificities, refer to the supplement). Hydrofluoric acid was used for removal of phosphoethanolamine or  $\alpha$ 1,3-linked fucose (20). As appropriate, treated glycans were re-chromatographed by RP-HPLC to ascertain retention time shifts prior to MALDI-TOF-MS; otherwise, an aliquot (generally one-fifth) of any digest was analysed by MALDI-TOF-MS without further purification.

*Western blotting:* Prior to SDS-PAGE, resuspended royal jelly was precipitated (mixed with a five-fold volume excess of methanol), incubated at -80 °C for one hour, centrifuged at 4 °C, 21 000 g and dissolved in a reducing sample buffer. After electrophoresis (10 µg/lane) and blotting to a nitrocellulose membrane, the following reagents for detection of glycan epitopes were employed: anti-horseradish peroxidase (Sigma-Aldrich; 1:10000 diluted in Tris buffered saline with 0.05% Tween and 0.5% BSA, to detect core α1,3-fucose (24)) and serum amyloid P protein (Fitzgerald, Acton, USA; 1:200, to detect phosphoethanolamine (25)) as well as C-reactive protein (MP Biochemicals, Santa Ana, USA; 1:200, which binds preferentially to phosphorylcholine (25)) followed by the relevant peroxidase-conjugated secondary antibodies and development with SigmaFAST 3,3'-diaminobenzidine tetrahydrochloride (19). Other glycan determinants were detected with biotinylated forms of *Aleuria aurantia*, wheat germ and peanut agglutinins (Vector Labs, Burlingame, USA; 1:1000) followed by phosphatase-conjugated anti-biotin (Sigma-Aldrich; 1:10,000) and development with SigmaFAST BCIP/NBT (26).

*Protein-specific glycan analysis:* After performing SDS-PAGE (10 µg/lane) and staining with Coomassie Blue, protein bands were excised and washed/destained (twice with 50% acetonitrile in water and successively once with 1:1 0.1 M ammonium bicarbonate/acetonitrile and 100% acetonitrile only) prior to drying (26). The proteins in the gel pieces were reduced for one hour at 56 °C with 10 mM dithiothreitol and alkylated for 45 minutes with 55 mM iodoacetamide in the dark. After a second round of washing (as above), the gel pieces were dried, covered with a 1:2 mixture of ammonium bicarbonate/trypsin (100 ng/µl) and incubated at 37°C overnight. The (glyco)peptides were extracted with a mixture of acetonitrile/water/trifluoroacetic acid (660:330:1 (v/v/v)), dried and dissolved in water before MALDI-TOF MS using either α-cyanocinnamic acid (ACH) or 6-aza-thiouridine (ATT) as matrices, using similar mass spectrometer settings as above. Tryptic peptide fingerprint data were analysed using the Mascot webserver (version 2.6.0) and the Swissprot database (release 2017\_06) as described on page S5 of the Supplement. For protein-specific glycan analysis, the (glyco)peptides were first heat-treated to inactivate trypsin and 90% of the samples were subject to PNGase Ar treatment (2.5 U; New England Biolabs) in 20 mM ammonium acetate, pH 5 overnight at 37°C. Whereas the deglycosylated peptides were analysed by MALDI-TOF MS, the released N-glycans were purified using two different columns packed respectively with Lichroprep C18/Dowex AG50 and non-porous graphitized carbon/Lichroprep C18. The C18/AG50 column was washed with 2% acetic acid and 60% isopropanol; after equilibration of the column with 2% acetic acid, the N-glycans were acidified with 10% acetic acid before application. The N-glycans were eluted (three column volumes of 2% acetic acid) prior to use of the carbon/C18 column, which was pre-washed with 100% acetonitrile and equilibrated with water. The N-glycans (combined neutral and anionic pools) were then eluted with 40% acetonitrile containing 0.1% trifluoroacetic acid. After drying, the glycans were fluorescently labelled by reductive amination using 2-aminopyridine and then analysed with MALDI-TOF MS and HPLC as above.

*Immobilisation of N-glycans:* Free N-glycans of the neutral pool were modified reductively with 2-amino-N-(2-amino-ethyl)-benzamide (AEAB; excitation/emission of 330/420 nm) as described by Song (27). Briefly, the dried N-glycan pool was dissolved in 0.35 M AEAB and 1 M NaCNBH<sub>3</sub> and incubated for 2 h at 65°C. To remove the excess AEAB, the labelled glycans were precipitated three times with 100% acetonitrile followed by solid phase extraction on

LC-NH<sub>2</sub> (Supelco, Sigma-Aldrich) normal phase material. Successful derivatisation of the N-glycans was determined by MALDI-TOF MS and HPLC (also to normalise the concentration of glycans on the basis of fluorescence intensity). The glycan pool was mixed 1:1 with spotting buffer (300 mM sodium phosphate pH 7.5, 0.005% Tween-20) then spotted ( $n = 10$ ) by non-contact printing (Flexarrayer S1; Scienion, Berlin, Germany) onto NHS-derivatised Nexterion H glass slides (Schott, Jena, Germany). After 16 h of hybridisation, slides were blocked (50 mM ethanolamine in 50 mM sodium borate, pH 9.0) for 1 h at RT, washed (TBS + 0.05% Tween-20, TBS, and H<sub>2</sub>O) and dried (28). The slides were incubated with (i) biotinylated forms of peanut agglutinin, wheat germ agglutinin or concanavalin A (VectorLabs; 10 µg/ml or 5 µg/ml in TBS + 0.05% Tween-20 + 1% BSA, i.e., TTBSA) followed by incubation with anti-biotin FITC conjugate (Sigma-Aldrich) (28), (ii) serum amyloid protein (amyloid P component from human serum, SAP; Fitzgerald, diluted 1:200 in TTBSA) followed by incubation with anti-amyloid P IgG from rabbit (Calbiochem, Merck; in TTBSA) and finally anti-rabbit IgG AlexaFluor-647 conjugate (Invitrogen, Carlsbad, USA; in TTBSA) or (iii) anti-L2/HNK-1 (clone 412; diluted 1:1000 in TTBSA) followed by incubation with anti-mouse IgG AlexaFluor-647 conjugate (Invitrogen; in TTBSA). Slides were scanned with an Agilent G2565AA Microarray Scanner (multiple photomultiplier tube (PMT) gain values from 10-100%) and raw fluorescence values (green for FITC and red for AlexaFluor-647) were used to calculate (in Excel) the mean and standard deviation from all ten spots. The negative controls (either only TBS-Tween, no primary lectin or pentraxin for respectively 'background' and 'no lectin/pentraxin' controls) show fluorescence due to either the AEAB label itself or non-specific binding of the fluorescent secondary antibodies. Galactosidase (recombinant *Aspergillus niger* β1,3/4-specific) and glucuronidase (*Helix pomatia* β-specific) treatments of AEAB-labelled glycans were respectively performed in solution before printing or directly on the printed slides prior to probing with lectins or antibodies. For further details, refer to pages S6 and S7 of the Supplement.

## Results

### *Strategy for the N-glycan analysis:*

Analysis of glycan-based post-translational modifications of proteins remains a challenge and experience has shown that a thorough analysis of unknown invertebrate glycomes requires multiple fractionation steps in order to prevent suppression effects and to enable separation of isomeric/isobaric structures of the same or similar mass (29). Thus, we first separated the free N-glycans of honeybee royal jelly into 'neutral' and 'anionic' pools, prior to fluorescent labelling, fractionation on an RP-amide HPLC column (calibrated in terms of glucose units) and MALDI-TOF-MS/MS in combination with chemical and enzymatic treatments (20). Based on the fluorescence intensities of the two glycan pools, it is estimated that at least 3% of the N-glycans are 'anionically' modified.

The initial screen, prior to HPLC, of the pyridylaminated pools by MALDI-TOF MS indicated that the neutral pool contained, in accordance with previous studies (14), a range of oligomannosidic and potentially hybrid or complex structures as judged by mass (**Supplementary Figure 1**). On the other hand, MALDI-TOF MS of the anionic pool suggested the presence of glycans potentially modified with sulphate or glucuronic acid, neither of which have been reported for royal jelly glycans, but of a type familiar from our recent data on dipteran and lepidopteran N-glycomes (18,19). However, unusually for N-glycans from an

insect source, fucosylation was not obvious from the complete spectra despite using both PNGase F and PNGase A in series to release the glycans. Relatively low amounts of core mono- and difucosylation were found only upon HPLC analysis of the glycans; also, paucimannosidic glycans ( $\text{Man}_{2-4}\text{GlcNAc}_2$ ), generally dominant in insect glycomes (18,30), were present at only a low level.

The subsequent RP-amide fractionation was necessary to resolve the fine detail of the N-glycome of royal jelly; nearly 70 structures were detected in the neutral pools (see **Figure 1**) with a partial overlap with the over 80 structures in the various anionic-enriched pools; in total, over 100 different compositions could be verified (see the **Supplementary Table 1** for the theoretical *m/z* values). Considering a widely-held belief that MALDI-TOF-MS of non-permethylated glycans leads to ‘in source’ artefacts, the major neutral RP-HPLC peaks were subject to both NP-HPLC (i.e., HIAx; **Figure 2**) and permethylation; also, aliquots of the whole N-glycome were permethylated (**Supplementary Figures 2 and 3**). The results of these additional procedures confirmed the validity of our general approach. Furthermore, the analysis was extended to the individual glycoproteins by using Western blotting and tryptic peptide mapping as well as release of the N-glycans from the single protein bands followed by fluorescent labelling and HPLC fractionation.

#### *Oligomannosidic N-glycans:*

In two independent preparations, the most abundant N-glycans in royal jelly were found to be, according to mass and retention time data (**Figure 1** and **Supplementary Figures 1 and 2**), either the typical ‘Golgi-processed’ isomer of  $\text{Man}_5\text{GlcNAc}_2$  or  $\text{Man}_9\text{GlcNAc}_2$  (respectively eluting at 7.2 or 5.5 g.u. with *m/z* 1313 or 1961). The MALDI-TOF MS analysis of individual HPLC fractions revealed the significant presence of a number of other oligomannosidic structures ( $\text{Man}_{6-8}\text{GlcNAc}_2$ ) as well as one other isomer of  $\text{Man}_5\text{GlcNAc}_2$  (6.4 g.u.) and low amounts of paucimannosidic glycans ( $\text{Man}_{2-4}\text{GlcNAc}_2$ ). In total, around 20 distinct pauci- and oligomannosidic isomers can be proposed on the basis of MS/MS data, comparisons to retention times of such glycans from other species (19,31) and selected  $\alpha$ -mannosidase digests (see, e.g., **Supplementary Figure 4**). Comparison of the MALDI-TOF MS data on the major RP-amide HPLC fraction with HIAx size fractionation (**Figure 2**) and permethylation (data not shown) prove that no ‘in source’ fragmentation of oligomannosidic N-glycans occurs in our MALDI-TOF MS analyses.

#### *Neutral hybrid and complex N-glycans:*

About 50 of the proposed royal jelly neutral glycans were predicted on the basis of mass to contain three or more *N*-acetylhexosamine residues; the initial mass spectrometric screen of the neutral pool revealed a rich set of hybrid and complex structures ranging from  $\text{Hex}_3\text{HexNAc}_3$  (*m/z* 1192) through to  $\text{Hex}_5\text{HexNAc}_6$  (*m/z* 2125; **Supplementary Figure 1**). The more in-depth analysis of the neutral HPLC fractions (**Figure 1**) showed the presence of further masses including minor amounts of triantennary  $\text{Hex}_{3-6}\text{HexNAc}_{7-8}$  (*m/z* 2004, 2166, 2329, 2532 and 2694) and fucosylated complex  $\text{Hex}_{3-4}\text{HexNAc}_{5-6}\text{Fuc}_1$  (*m/z* 1744, 1906, 1947 and 2109) structures, the latter being some of the few fucosylated glycans found upon PNGase F release. MS/MS of glycans of *m/z*  $\geq 1395$  generally revealed B-fragments at *m/z* 407 and/or 569 ( $\text{Hex}_{0-1}\text{HexNAc}_2$ ), which was taken as an indication for the presence of LacdiNAc units (**Figure 2** and **Supplementary Figures 5-6**).

In order to resolve the individual neutral glycans in terms of the modifications and isomeric status, selected HPLC fractions were treated in a targeted manner with a number of

exoglycosidases. For glycans containing four or five hexose residues, a major question was as to whether these hybrid structures were terminally galactosylated as previously suggested (14). However, isomers of the same observed masses (e.g., two isomers each of Hex<sub>4-5</sub>HexNAc<sub>4</sub> with *m/z* 1557 and 1719) were identified by their different 2D-HPLC elution and MS/MS properties (**Figure 2**). Man<sub>4-5</sub>-based hybrid glycans lost maximally three residues with jack bean  $\alpha$ -mannosidase (dependent on the efficiency of removal of the ‘core’  $\alpha$ 1,6-mannose), whereas the pattern of  $\beta$ 1,3-specific galactosidase and HEX-4  $\beta$ 1,4-specific *N*-acetylgalactosaminidase (23) sensitivity depended on the isomer (**Figure 2** and **Supplementary Figure 4**). Successful galactosidase digestion correlated with the distinct presence of an MS/MS fragment at *m/z* 569, whereas HEX-4 sensitivity was observed for glycans with fragments at *m/z* 407 (**Figure 2 C-R**). The presence of either a fourth/fifth mannose or a galactose substitution of antennal GalNAc was corroborated by MS/MS of permethylated glycans showing Hex<sub>4-5</sub>HexNAc<sub>2</sub> Y-fragments at *m/z* 1361 and 1565 or Hex<sub>0-1</sub>HexNAc<sub>1-2</sub> B-fragments at *m/z* 486, 527 and 731 (**Supplementary Figure 3 A and B**). For those hybrid glycans containing a sixth mannose (one isomer each of *m/z* 1881 and 2043; 6.4 g.u.), four mannose residues were lost upon jack bean mannosidase digestion, while a single one was removed by an  $\alpha$ 1,2-specific mannosidase (data not shown).

In the case of biantennary glycans, the definition of which antenna was elongated was based on the retention time (isomers with longer lower arms, i.e., on the  $\alpha$ 1,3-mannose, elute earlier (32)), fragmentation pattern and enzyme sensitivity (**Supplementary Figure 5**), whereby the FDL *N*-acetylglucosaminidase is specific for removal of unsubstituted  $\beta$ 1,2-linked GlcNAc from the lower arm (23). As for the hybrid glycans, the longer antennae (with *m/z* 569 B-fragments for, e.g., isomers of *m/z* 1760) were digested by a combination of  $\beta$ 1,3-galactosidase and HEX-4 as for the hybrid forms, while glycans with a terminal GalNAc were sensitive to HEX-4 alone (with *m/z* 407 B-fragments as for isomers of *m/z* 1598). The cumulative data show that LacdiNAc (GalNAc $\beta$ 1,4GlcNAc), with or without a  $\beta$ 1,3-linked galactose cap, is a frequent motif of royal jelly N-glycans and that galactose was present on HexdiNAc units.

In the case of triantennary glycans eluting at 11 g.u. (see **Figure 1**), a combination of galactosidase and specific hexosaminidase digests together with reinjection onto the HPLC column revealed that there could be a ‘free’ GlcNAc on the upper  $\alpha$ 1,6-mannose and two branches on the lower  $\alpha$ 1,3-mannose (**Supplementary Figure 6 A-E**). Upon treatment with *Xanthomonas*  $\beta$ 1,2-specific *N*-acetylglucosaminidase, removal of the GlcNAc on the  $\alpha$ 1,6-arm resulted in conversion of the *m/z* 1030 Y-fragment to one at *m/z* 827; the third antenna is based on a  $\beta$ 1,4-linked GlcNAc as shown by co-elution (9.5 g.u.) of the *m/z* 1598 galactosidase/HEX-4 digestion product of the 11 g.u. fraction with a degalactosylated bovine fetuin N-glycan. Later eluting glycans (12 g.u.) having two or three fully galactosylated antennae displayed intense MS/MS fragment ions at *m/z* 1233 or 1395 resulting from loss of the ‘heavy’  $\alpha$ 1,3-arm as indicated (**Supplementary Figure 6 F and G**). All defined hybrid, bi- and triantennary structures were the basis for various anionic/zwitterionic-modified glycans subsequently found in the anionic pool.

#### *Core mannosylated N-glycans:*

A hallmark of many invertebrate N-glycans, when pyridylaminated, is the presence of an *m/z* 446 (Fuc<sub>1</sub>GlcNAc<sub>1</sub>-PA) fragment indicative of core fucosylation (see below); recently, in an oyster and a marine snail (22,33) we have found glycans with an *m/z* 462 fragment (Hex<sub>1</sub>HexNAc<sub>1</sub>-PA). In the snail this fragment was abolished upon  $\beta$ -mannosidase digestion

of relevant glycans; this rules out that this is a Y-fragment of an endoglycosidase digestion product, which would elute before 5 g.u. rather than at approximately 8 g.u.. In the present study, the *m/z* 462 fragment was also found for the first time in an insect species and could also be verified by the MS/MS pattern of 2D-HPLC-purified glycans followed by glycosidase digestion of selected structures including one carrying antennal glucuronic acid (**Figure 3** and **Supplementary Figure 7**). Initially, we presumed that the  $\beta$ -mannose in royal jelly would also be 1,3-linked to the proximal GlcNAc as in molluscs (22), but parallel re-injection experiments with two RP-HPLC columns (**Supplementary Figure 7 A and B**) as well as the LC-MS<sup>n</sup> fragmentation pattern (**Figure 3 C and D**) indicated that the royal jelly *m/z* 1557 mannosylated structure was not identical to that from the snail; thus, we conclude that the  $\beta$ -mannose in royal jelly glycans is 1,6-linked.

#### *Zwitterionic N-glycans:*

Upon analysis of the individual ‘neutral’ fractions, it was observed that some glycans could be detected in both positive and negative ion modes and had compositions suggestive of a modification with a moiety of 123 Da, which is reminiscent of the presence of phosphoethanolamine on N-glycans from *Trichomonas vaginalis* or *Penicillium* species (34,35). The most distinct fragment ions (**Figure 4** and **Supplementary Figure 8 and 9**) were previously unknown ones at *m/z* 1153, 1315, 1477, 1518 and 1639 in positive mode (Hex<sub>2-5</sub>HexNAc<sub>3-4</sub>PE<sub>1</sub>-PA Y-fragments) or at *m/z* 528/530 and 690/692 in negative/positive modes (Hex<sub>0-1</sub>HexNAc<sub>2</sub>PE<sub>1</sub> B-fragments, which reflect the antennal capping by LacdiNAc with or without galactose). In order to resolve isomers, HIAx separation of the RP-HPLC 6.6-6.8 g.u. was performed (**Supplementary Figure 8**). Thereafter, it was found that the Hex<sub>4-5</sub>HexNAc<sub>4</sub>PE<sub>1</sub> isomers (*m/z* 1678/1680 and 1840/1842) display different MS/MS spectra due to variations in the positions of galactose and mannose residues, similarly to the ‘parental’ later-eluting non-zwitterionic glycans (compare with **Figure 2**). The position of the phosphoethanolamine on ‘internal’ GlcNAc, rather than on GalNAc, became obvious upon removal of antennal galactose and GalNAc residues as judged by the alterations in the B-fragments, i.e., conversion to *m/z* 325/327 or 528/530 (**Figure 4 A-F** and **Supplementary Figure 9**).

Further proof for the presence of the phosphodiester was provided by sensitivity towards hydrofluoric acid (**Figure 4 H-K**) and a subsequent shift to higher RP-HPLC retention time correlating with the loss of the hydrophilic moiety (**Figure 4 G**). Thereby, rather than the phosphorylcholine substitution found on N-glycans from two lepidopteran species (19), we conclude that a number of hybrid and complex glycans in royal jelly are modified with phosphoethanolamine, a moiety which also decorates in 6-linkage internal GlcNAc residues of dipteran glycolipids (36-38). Despite an in-depth examination and the ability to find phosphoethanolamine-modified glycans when permethylating *Penicillium* glycans (unpublished data) which display a 30% degree of such structures, no zwitterionic glycans were found upon permethylation of the royal jelly glycome, which may be due to losses of these low-abundance structures (<0.5%) during the chemical or separation procedures; thus, the off-line LC-MALDI approach was necessary to detect this class of glycans, which were not detected in any spectrum of an entire glycome (Supplementary Figures 1 and 2).

#### *Glucuronylated N-glycans:*

One of the key steps in our analyses to reveal the fine details of royal jelly glycans was the separation on graphitised carbon of the neutral from the anionic species. Thus, after

pyridylation, the anionic pools (approximately 3% of the total) obtained from different preparations were separately applied to the RP-amide HPLC column (**Figure 5**); obvious was the presence of glycans modified with glucuronic acid, which (as in our previous studies of insect N-glycans (18,19)) could be detected in both positive and negative ion modes, but fragment best in the positive mode to yield MS/MS fragments at, e.g., *m/z* 542 and 745 (HexA<sub>1</sub>Hex<sub>1</sub>HexNAc<sub>1-2</sub>; see **Figures 6-8** as well as **Supplementary Figures 6H-K and 10-12**). On this basis, mono-, di- and triantennary glycans with up to three glucuronic acid residues were identified in the PNGase F-released anionic pool (Hex<sub>3-6</sub>HexNAc<sub>4-8</sub>Fuc<sub>0-1</sub>GlcA<sub>1-3</sub>; **Figure 5A**). Glucuronylated glycans were also found upon permethylation of the anionic pool and displayed relevant HexA<sub>1</sub>Hex<sub>1</sub>HexNAc<sub>0-2</sub> fragments at *m/z* 476, 704 and 949 indicative of linear antennae (**Supplementary Figures 2C and 3F**).

Verification that the 176 Da modification is glucuronic acid rather than methylhexose was also offered by sensitivity to  $\beta$ -glucuronidases of three HPLC fractions containing monoglucuronylated hybrid and biantennary glycans; this treatment resulted in a shift to later RP-amide retention times (typically an increase of about 1 g.u.; see chromatograms in **Figure 6** and **Supplementary Figure 10**), loss of the negative ion mode signal and alterations in the MS/MS spectra (i.e., loss of the *m/z* 542 and 745 fragments and increased intensities at *m/z* 366 and 569; **Figure 6 H-K** and **Supplementary Figure 10 A-L**). As deglucuronylation resulted in retention times and MS/MS spectra akin to those for glycans found in the neutral pools, it is assumed that the structures with antennal Gal $\beta$ 1,3GalNAc $\beta$ 1,4GlcNAc underlie the glucuronylated forms.

#### *Glucuronylated N-glycans modified with phosphoethanolamine:*

A further set of modifications is represented by glucuronylated structures also carrying phosphoethanolamine present in the anionic pool. Such glycans elute one HPLC fraction earlier than the ‘parent’ structure lacking the zwitterion; hydrofluoric acid treatment to release the phosphoethanolamine results in a shift backwards in terms of retention time (**Figure 6A**). This treatment also results in alterations in the MS/MS spectra (i.e., losses of negative or positive mode HexA<sub>1</sub>Hex<sub>1</sub>HexNAc<sub>2</sub>PE<sub>1</sub> B-fragments at *m/z* 866/868 and appearance of positive ones at *m/z* 745; **Figure 6 B-G** and **Supplementary Figure 11 A-D**) indicative of removal of phosphoethanolamine (123 Da) from glucuronylated antennae. As judged by re-application onto HPLC, phosphoethanolamine-containing structures account for some 5% of the anionic pool.

#### *Sulphated N-glycans:*

In previous studies on dipteran and lepidopteran glycomes, the sulphated N-glycome within the anionic pool was dominated by fucosylated structures such as Hex<sub>3</sub>HexNAc<sub>2-3</sub>Fuc<sub>1</sub>S<sub>1</sub> (*m/z* 1213 and 1416); these were not present in royal jelly. Rather non-fucosylated Hex<sub>3</sub>HexNAc<sub>3-4</sub>S<sub>1</sub> (*m/z* 1270 and 1473; see **Figure 5**) were the major sulphated glycans detected, reflecting the general lack of fucose in royal jelly. Serial digestions of larger structures (Hex<sub>3-4</sub>HexNAc<sub>4-5</sub>S<sub>1</sub>) with  $\beta$ 1,3-galactosidase and jack bean  $\beta$ -hexosaminidase removed the antennae; as only one underlying mannose was sensitive to both the unspecific jack bean  $\alpha$ -mannosidase and  $\alpha$ 1,3-specific mannosidase, it was possible to define the  $\alpha$ 1,6-mannose as being sulphated (**Figure 7A-F**). Confirming our assignments were the typical negative mode fragments at *m/z* 606, 768 and 971 (Hex<sub>2-3</sub>HexNAc<sub>1-2</sub>S<sub>1</sub>), as seen for other cases of sulphation of mannose residues, including glycans of the same elution time previously identified in lepidopteran species (18,19). Finally, upon using solid phase extraction

following permethylation of the anionic pool, it was also possible to detect certain sulphated glycans; MS/MS thereof supported the conclusion that the  $\alpha$ 1,6-linked mannose is sulphated (**Supplementary Figures 2B and 3C**). Otherwise, sulphated forms of the fucosylated and glucuronylated glycans were only observed in the off-line LC-MS analysis (see below).

*Core mono- and di-fucosylated anionic N-glycans:*

In the combined PNGase A and F-released neutral pools or when PNGase F alone was employed, only traces of a variety of singly fucosylated glycans were detected (**Figure 1**); however, most fucosylated glycans were glucuronylated and therefore present in the anionic pool (**Figure 5**), including the largest structures observed in this study, i.e., triantennary monofucosylated forms without and with sulphate (*m/z* 3366 and 3446 as [M-H] $^-$ ). The presence of a core  $\alpha$ 1,6-fucose on these can be inferred from their release by PNGase F and late elution time as well as by the *m/z* 446 Y-fragment absent after bovine  $\alpha$ -fucosidase treatment (**Supplementary Figure 12 F-J and K-N**). However, when the glycans were released from the second sample of royal jelly using PNGase Ar, we noted traces of glycans with two or three fucose residues in the anionic pool (**Figure 5B**). A key Y-fragment of *m/z* 592 (Fuc<sub>2</sub>GlcNAc<sub>1</sub>-PA; **Figure 8** and **Supplementary Figures 11 E and H and 12 A-E**), as well as a degree of anti-horseradish peroxidase staining (**Supplementary Figure 13A**), indicates that there are core  $\alpha$ 1,3/ $\alpha$ 1,6-difucosylated N-glycans; these glycans elute earlier than those carrying a single core  $\alpha$ 1,6-fucose. The third fucose residue is present on the antennae as seen, e.g., on bee venom glycoproteins (39), and is defined by Y-fragments such as *m/z* 1630, 1792 and 1954 (Hex<sub>3-5</sub>HexNAc<sub>3</sub>Fuc-PA) or losses of 891 mass units from the parent; hydrofluoric acid treatments and relevant shifts to later elution time or alterations in MS/MS spectra confirmed the presence of core and/or antennal  $\alpha$ 1,3-fucose (**Figure 8 and Supplementary Figure 11 E-J**). Another isomeric trifucosylated variation, due to a single fucose on the core and two antennal fucoses, was found in the PNGase F-released pool (compare the two glycans of *m/z* 2916; **Supplementary Figure 12 E and I**). Some of the fucosylated/glucuronylated glycans even contained phosphoethanolamine (**Figure 8E** and **Supplementary Figure 12 B and G**).

Rare, but detectable, were fucosylated glycans containing both glucuronic acid and sulphate observed as [M-H] $^-$  ions in negative mode and [M-SO<sub>3</sub>+H] $^+$  in positive mode (**Figure 7 G-J**); here also the fragmentation patterns indicate the sulphation of an  $\alpha$ -mannose, as serial losses of an glucuronic acid antenna and then of sulphate are observed in negative mode MS/MS spectra, whereas the positive mode MS/MS show the nature of the ‘backbone’ core fucosylated structure (**Figure 7 K-N**). Smaller sulphated difucosylated glycans akin to those in High Five cells (19) were also detected (*m/z* 1359 and 1562; **Figure 8 A**) and one fucose could be removed from these with hydrofluoric acid; as this treatment is also known to remove phosphoesters (29) but not sulphate (40), the resistance of the 80 Da moiety is another confirmation that these glycans are sulphated.

*Analysis of protein-specific glycosylation and binding to glycan arrays:*

To test whether there are variations in the glycosylation of individual glycoproteins in royal jelly, SDS-PAGE was performed prior to either Western blotting or release of glycans from trypsin-treated gel bands. In terms of blotting, we used reagents capable of binding core  $\alpha$ 1,3-fucose, zwitterionic or antennal modifications. Thereby, the presence of core  $\alpha$ 1,3-fucose and of phosphoethanolamine as judged by the glycomic data correlates with the

staining with anti-horseradish peroxidase and serum amyloid P; correspondingly the binding to C-reactive protein, which recognises preferentially phosphorylcholine over phosphoethanolamine (25), is low (**Supplementary Figure 13A**). Also, reactivity towards fucose-specific *Aleuria aurantia* lectin, HexNAc-specific wheat germ agglutinin and Gal $\beta$ 1,3GalNAc-specific peanut agglutinin (41) was detected. The AEAB-labelled neutral and anionic pools of royal jelly N-glycans (**Figure 9A**) were immobilised and specifically recognised in microarray format by peanut agglutinin (42), serum amyloid P (25) and an anti-L2/HNK-1 antibody (43,44), whereby the reactivity to peanut agglutinin and anti-L2/HNK-1 was reduced upon respective  $\beta$ -galactosidase or  $\beta$ -glucuronidase treatment (**Figure 9B** and **Supplementary Figure 14**) and is compatible to the MS-based glycomic analyses.

For analysis of protein-specific glycosylation, the five most dominant Coomassie Blue-stained protein bands were excised as indicated and subject to trypsin peptide mass fingerprinting to verify their identity (coverage of 29-46%; **Supplementary Figure 13 B-D** and **Supplementary Table 2**); MRJP1 is the major band, MRJP2 the secondmost dominant component and MRJP3 present in three protein bands, consistent with its heterogeneity as reported in the literature (45). We could detect forms of the arginine-containing peptide 136-146 from MRJP2 modified by complex N-glycans (**Figure 9C**). The tryptic peptides were treated with PNGase Ar to release the N-glycans and these protein-specific glycans were separately pyridylaminated, HPLC fractionated and analysed by MALDI-TOF MS and MS/MS (**Supplementary Figure 15**). Considering the difference in the amount of each protein in the sample, it is not possible to conclude that there is much variation in the types of glycans on the individual glycoproteins. Each protein (MRJP1, MRJP 2 and MRJP 3) is modified with a range of glycans despite having each a limited number ( $n = 1-3$ ) of glycosylation sites with the most dominant glycan being Man<sub>9</sub>GlcNAc<sub>2</sub> (in terms of MALDI-TOF MS as well as fluorescence intensity at 5.7 g.u.); on the other hand, the heterogeneous sets of hybrid and complex glycans probably account for 50% of the structures as is the case in the overall royal jelly glycome. The ability to detect phosphoethanolamine (correlating with the binding to serum amyloid P), glucuronic acid and core  $\beta$ -mannose in the MRJP1 glycome (**Supplementary Figure 15 D-K**) is possibly a result of this protein being the most abundant.

## Discussion

### *N-glycan analysis of insects:*

The Insecta is one of the most diverse classes of invertebrates with about thirty different orders, four of which contain the vast majority of insect species: Coleoptera (beetles), Diptera (true flies), Hymenoptera (wasps, bees and ants) and Lepidoptera (moths and butterflies). Amongst these, scientifically, economically and medically significant species include mosquitoes (e.g., *Anopheles* and *Aedes* spp.) which transmit pathogens, the model fruitfly *Drosophila melanogaster* and the honeybee *Apis mellifera* as well as *Trichoplusia ni* and *Spodoptera frugiperda* whose cell lines are used for baculovirus-based production of recombinant proteins. N-glycan structures from all these species have been described, but until recently only 'simple' paucimannosidic and oligomannosidic glycans were reported (30,46,47) with the exception of the more complex fucosylated antennae of bee venom glycoproteins (39). Of other insects, there are singular studies on glycans or glycoproteins isolated from the silkworm *Bombyx mori* (48,49), the flour beetle *Tribolium castaneum* (50)

and the locust *Locusta migratoria* (51), of which the latter was most noteworthy as aminoethylphosphonate was detected as an antennal modification.

The most recent data indicate that the N-glycomic potential of insects is indeed underestimated. For many years, a major question (now resolved (52)) was whether insects sialylate their glycoconjugates, but perhaps the search for sialylated structures distracted attention from considering other anionic modifications. Our workflow based on separating the neutral and anionic fractions by solid phase extraction on graphitised carbon followed by off-line MALDI-TOF MS (in both modes) is relatively unbiased, circumvents suppression-dependent underestimation of anionic modifications and allows for further chemical or enzymatic treatments in order to confirm fine details of glycan structures (20,29). Not only does our approach result in detection of typically one hundred different glycans, but has vastly increased the range of structures now known in different species including molluscs, nematodes, slime moulds and insects (18,19,22,33,53,54). We have also examined binding of three lectins (Con A, PNA and WGA), one antibody (anti-L2/HNK-1) and one pentraxin (serum amyloid P) to the neutral and anionic N-glycomes of royal jelly in a microarray format.

#### *The variety of N-glycans in royal jelly:*

Some of the basic backbones of the neutral glycans were shown to be biantennary, others hybrid: isomeric variation could be detected due to the 1D- or 2D-HPLC separation approach. In terms of the neutral N-glycome, our data refines previous interpretations of those structures beyond the oligomannosidic, specifically showing that terminal galactosylation in royal jelly is in the context of LacdiNAc units, resulting in the previously-reported ‘pseudo’ T-antigen Gal $\beta$ 1,3GalNAc $\beta$ 1,4GlcNAc (16), but not as Gal $\beta$ 1,3GlcNAc as proposed in two other studies (15,55); our data is also compatible with the presence of terminal LacdiNAc as well as of  $\beta$ 1,4-linked branching GlcNAc (15,56). The galactosylation of LacdiNAc, rather than also of GlcNAc, is a hallmark of royal jelly as opposed to honeybee larvae (unpublished data), whereas the venom is well known for both core and antennal  $\alpha$ 1,3-fucose (39). Interestingly, we detect core  $\beta$ -mannosylation in an insect for the first time, whereas fucosylation is at a low level in royal jelly as compared to other insect glycans.

In the present study on royal jelly, the variety of N-glycan modifications is again larger than previously appreciated and this ‘expansion’ is primarily due to the detection of some new and unusual anionic and zwitterionic structures (summarised in **Figure 10**). We have detected anionic hybrid and bi/tri-antennary glycans with sulphate, phosphoethanolamine and glucuronic acid singly or in combination, some of which also have multiple fucose residues; indeed, unlike previously-defined di- and trifucosylated glycans from insects (19,39), all such structures in the royal jelly glycome were glucuronylated and/or sulphated. While phosphoethanolamine has been found on wasp and mosquito O-glycans (18,57) and insect glycolipids (38), this is the first demonstration of this modification on any N-glycan from an animal species. Otherwise, phosphoethanolamine is a component of trichomonad, fungal and bacterial N-glycans (34,35,58), protist and bacterial lipid-linked oligosaccharides (59,60) and glycosylphosphatidylinositol membrane anchors from various species (61); sulphation of mannose is known in, e.g., slime moulds and insects (18,54,62), but sulphates are linked to other monosaccharides in mammalian glycoconjugates (e.g., Gal on thyroglobulin (63), GalNAc on pituitary glycoproteins (64), GlcNAc in the context of sulpho-Lewis X (65) and GlcA of the HNK-1 epitope (66)). In all animals, glucuronic acid is a

component of chondroitin and heparan sulphates (67), whereas it is also present in mammals within the extended matriglycan chains on dystroglycan (68) as well as on various neural N-glycans (66); the binding of some anti-L2/HNK-1 monoclonals to blowfly glycoproteins and glycolipids, presumed to be due to non-sulphated GlcA (69), would correlate with our structural and array data for royal jelly N-glycans.

Previously, a minor MRJP2 homologue was concluded to have Hex<sub>9</sub>HexNAc<sub>2</sub> on one glycosylation site and Hex<sub>4-5</sub>HexNAc<sub>3-4</sub> on the other (11) and here we obtained a similar result for the second site, while the N-terminal glycosylation site of MRJP1 is apparently modified by Hex<sub>3-5</sub>HexNAc<sub>4-6</sub> and the other two sites by oligomannosidic structures (16), which may be due to differential exposure to Golgi processing enzymes. Due to the off-line LC-MS approach, we have revealed a heterogeneity in the protein-specific glycosylation pattern of MRJP1 and MRJP2 previously not observed by MS or other analyses. The protein-specific glycans identified reflect the higher amount of Man<sub>9</sub>GlcNAc<sub>2</sub> in the second preparation as compared to the first (compare Supplementary Figure 1 A and B); this could be due to variations in hypopharyngeal gland glycan processing in individual bees or the presence of α-mannosidase, a proteomically-determined royal jelly component (5). Furthermore, the 3D-structures of the major glycoproteins present in royal jelly may play a role in determining the accessibility of glycans to Golgi enzymes and so effect the overall status of the secreted glycome. It is also of interest that some of these royal jelly proteins are also present in honeybee venom and, in recombinant form, are recognised by the IgE of allergic patients (70), whereas MJRP1 in honey possesses glycan-dependent IgE epitopes (71). On the other hand, in contrast to our data showing a low degree of core α1,3-fucosylation, Western blotting with an anti-plant-glycoprotein antiserum was considered negative in a previous study (14), whereas wheat germ and peanut agglutinin binding (respectively indicative for terminal HexNAc and Galβ1,3GalNAc modifications) to royal jelly proteins was positive (the latter correlating with the glycan array data). Certainly, modifications with fucose, glucuronic acid, core mannose or phosphoethanolamine were previously not defined on single royal jelly glycoproteins.

In contrast to mosquitoes and lepidopterans (18,19), sulphation and glucuronylation were almost mutually exclusive in royal jelly, with the latter again being the dominant 'charged' modification; parallel (unpublished) analyses of honeybee venom and larvae indicate that these glycans are different in terms of the degree and type of antennal modifications, which suggests that royal jelly glycans are the product of a tissue-specific glycosylation pattern. Thereby, whereas fucosyltransferases (FucTA, FucTC and FucT6) are predicted to be expressed at low levels in hypopharyngeal glands, other gland-specific transcription events may be responsible for the increase in anionic/zwitterionic glycans. Indeed, FucTA core α1,3-fucosyltransferase mRNA levels are seemingly far lower in hypopharyngeal as compared to venom glands (72).

#### *Potential biological relevance of royal jelly N-glycans:*

As anionic and zwitterionic moieties have different biophysical properties and so be differently recognised as compared to neutral saccharides, it is to be expected that they have distinct bioactivities. For instance, sulphation of mannose of the N-glycans of a lepidopteran protein has been claimed to have a pro-inflammatory role in leukocyte activation in dermatitis caused by setae of a tropical moth (62), while in various species, sulphation of glycosaminoglycans, Lewis X or terminal GalNAc is associated with processes such as blood clotting, morphogenesis, cell adhesion or hormone clearance (73-76).

Furthermore, the glucuronic acid-containing L2/HNK-1-type epitope (present in non-sulphated form on royal jelly glycans as verified by the array data in **Figure 9B**) may be recognised by the human cytokine IL-6, so having relevance to interleukin signalling (77), as well as by HMGB1 or amphotericin (78) which is also known to interact with Toll-like receptors (79). Phosphoethanolamine is one of the ligands for serum amyloid P protein, a pentraxin known to bind, e.g., bacteria, but also to activate complement (80) and here we show by blotting and array experiments that glycoproteins and the anionic N-glycans thereof in particular in royal jelly are indeed recognised by serum amyloid P (**Figure 9B** and **Supplementary Figure 13A**); it would certainly be of interest to know which ligands are recognised by insect members of the pentraxin family (81), including a homologue in the honeybee (gene LOC102656294), as such proteins from another arthropod (the horseshoe crab) do bind zwitterions (82). Phosphoethanolamine is the non-methylated form of phosphorylcholine, which is present on N- and O-glycans of insect cell lines used for recombinant protein production (19,83), but which is also known to possess immunomodulatory function as a modification of nematode glycoproteins (84). Thus, although there are no systematic studies as to the protein ligands or receptors for such moieties, for each ‘charged’ modification of royal jelly N-glycans there is potential for bioactivity, immunoreactivity or immunoactivity.

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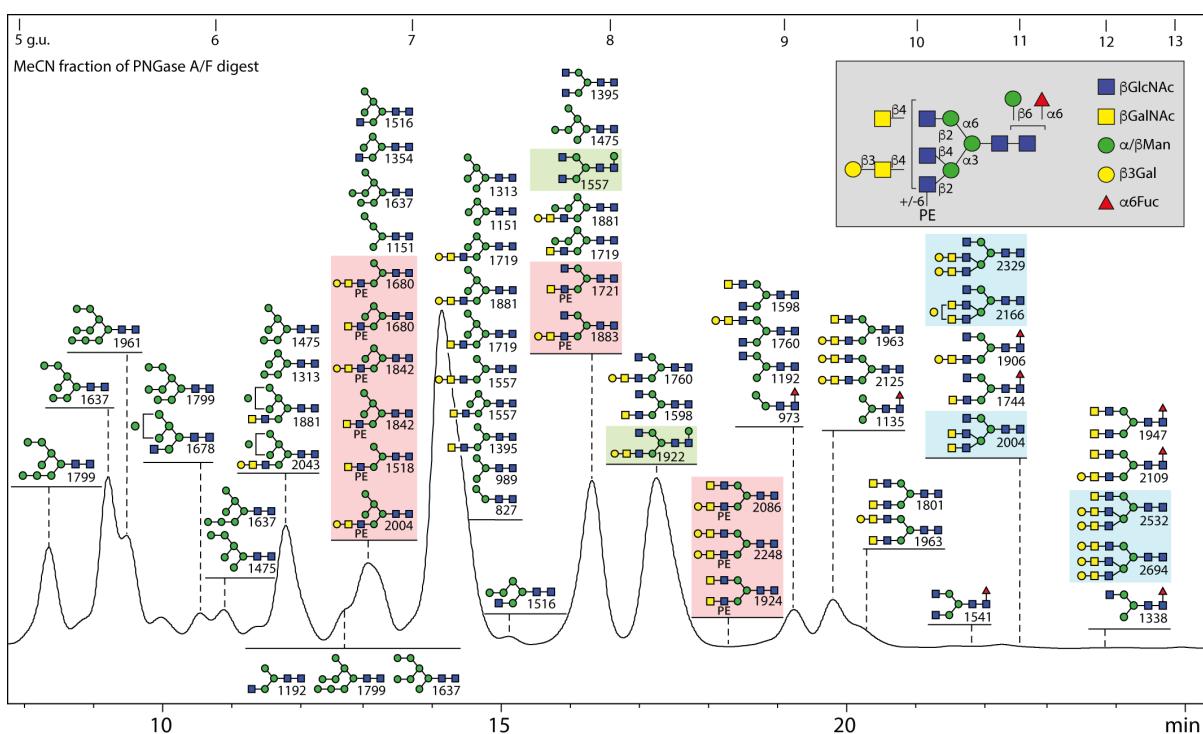
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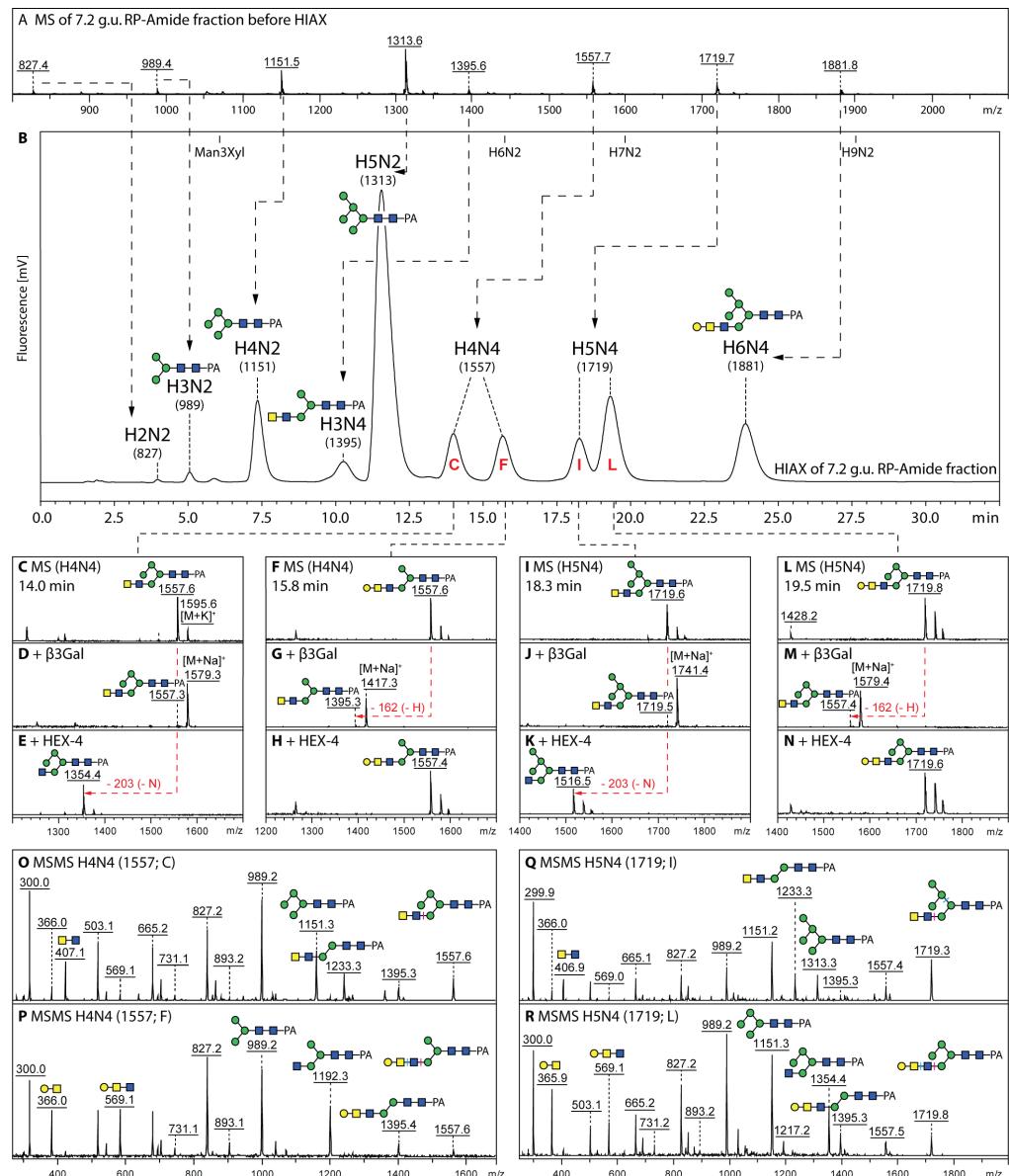
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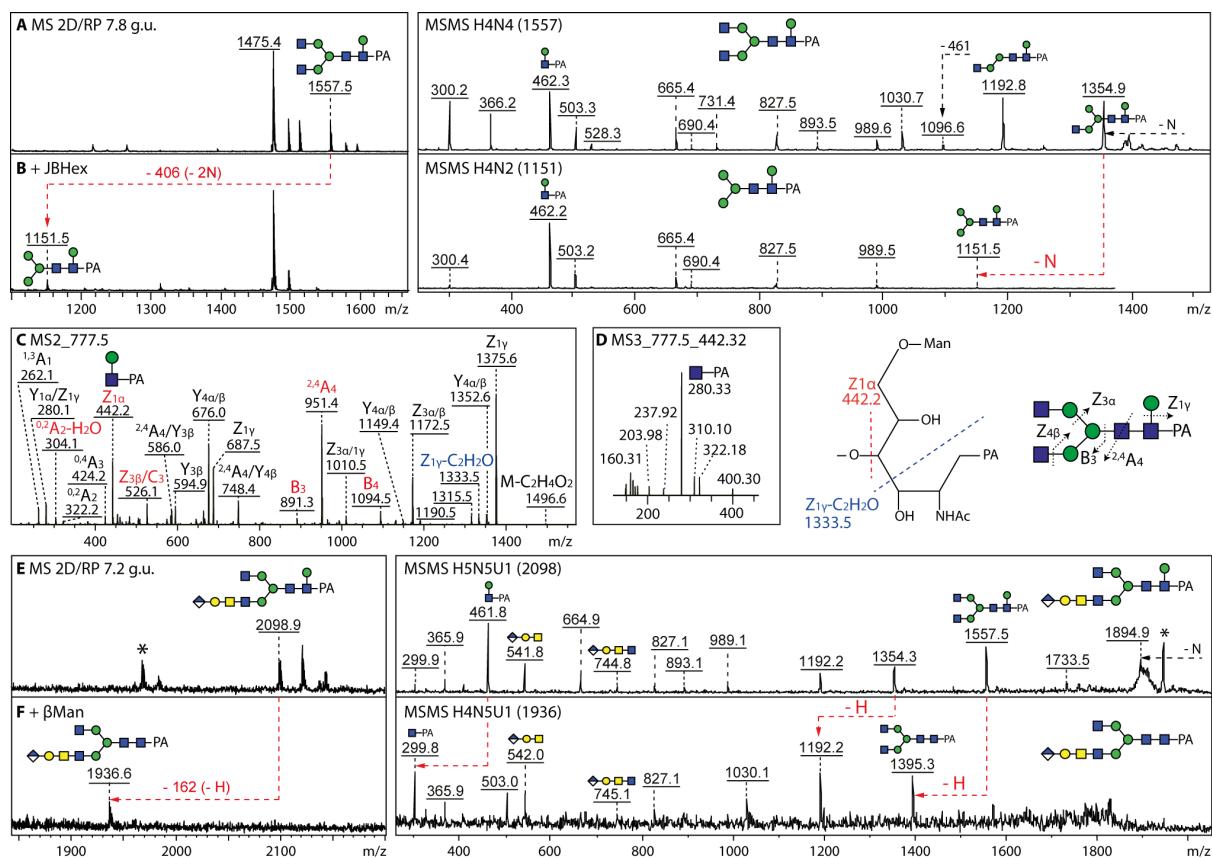
**Figure 1: RP-HPLC fractionation of the neutral N-glycans from royal jelly.** Glycans were released by PNGase F and A prior to solid phase extraction (to separate the neutral and anionic glycans), pyridylation and finally fractionation on an RP-amide column (refer to Supplementary Figure 1 for overall MALDI-TOF MS profiles). The chromatogram is annotated with glycan structures (each with the positive mode *m/z* value for the protonated ion in order of abundance for each fraction) according to the Symbolic Nomenclature for Glycans (circles, galactose or mannose; squares, GalNAc or GlcNAc; triangles, fucose; PE, phosphoethanolamine; see also inset for symbols and linkage information). The HPLC column was calibrated in terms of glucose units (g.u.). Oligomannosidic glycans are annotated by comparison to previous studies on insect glycomes using the same column (unusual isomers were also subject to specific  $\alpha$ -mannosidase digestion), whereas other glycans are defined on the basis of MS/MS and digest data. The PNGase F alone and PNGase Ar (after F) digests of the second preparation yielded qualitatively similar chromatograms, but  $\text{Man}_9\text{GlcNAc}_2$  was more dominant than  $\text{Man}_5\text{GlcNAc}_2$  (see also Supplementary Figure 1 A and B). The hymenoptera-specific zwitterionic,  $\beta$ -mannosylated and triantennary glycans are highlighted in red, green or blue boxes; the presence of phosphoethanolamine (rather than phosphorylcholine) and of  $\beta$ -mannose, but the relative lack of fucose, contrasts to the N-glycomes of lepidopteran species (19).



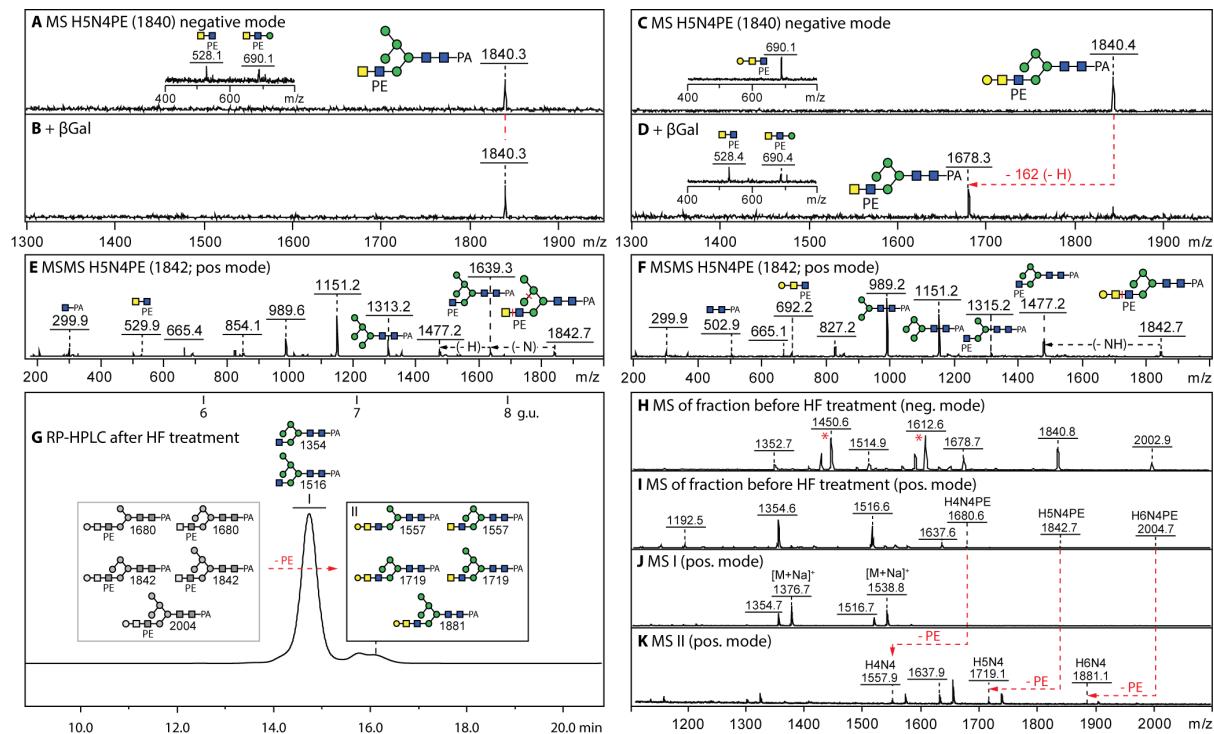
**Figure 2: Isomeric separation of hybrid N-glycans by two-dimensional HPLC.** The major RP-HPLC fraction (7.2 g.u.) was analysed by MALDI-TOF MS (**A**) and subfractionated by HIAx-HPLC (**B**); thereby, the HIAx column resolves glycans not just on the basis of mass, but even with a different isomeric composition of mannose and galactose residues. This fractionation results in peaks containing single glycans with no evidence of in source MS fragmentation with even the fluorescent intensities correlating to those for the MALDI-TOF MS data. The pairs of isomeric glycans ( $\text{Hex}_{4-5}\text{HexNAc}_4$  with  $m/z$  1557 and 1719; C/F and I/L) display contrasting susceptibilities to  $\beta 1,3$ -specific galactosidase (**D, G, J and M**) and  $\beta 1,4$ -N-acetylgalactosamine-specific HEX-4 treatments (**E, H, K and N**) as well as apparently different MS/MS spectra (**O-R**) for the undigested glycans. The  $m/z$  407 B-fragments correlate with terminal LacdiNAc (O and Q), but those at  $m/z$  569 with the galactosylated version (P and R), whereas the pairs of isomers also differ in the pattern of  $m/z$  989/1151/1313 Man<sub>3-5</sub>-based Y-fragments. Based on these data the presence of LacdiNAc motifs with or without a terminal  $\beta 1,3$ -galactose substitution can be demonstrated. Refer to Supplementary Figure 4 for further data on digestion of hybrid glycans, including  $\alpha$ -mannosidase or combined galactosidase/HEX-4 treatment of the 7.2 g.u. fraction.



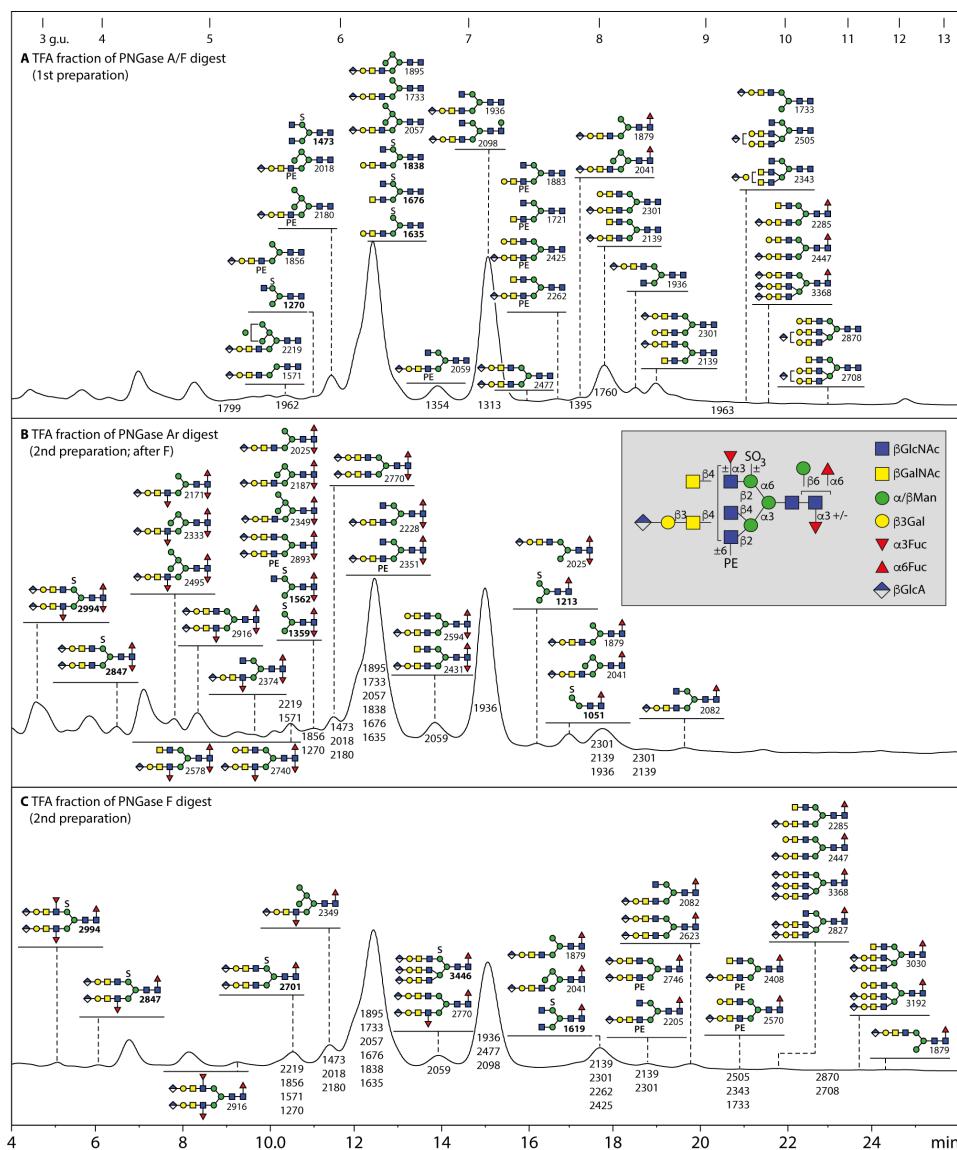
**Figure 3: Core  $\beta$ 1,6-mannose as a modification of 2D-HPLC-fractionated royal jelly glycans.** (A and B) The  $m/z$  1557 glycan ( $\text{Hex}_4\text{HexNAc}_4\text{-PA}$ , 7.8 g.u.; see Figure 1) was subject to MALDI-TOF MS/MS before and after removal of the terminal GlcNAc residues with jack bean  $\beta$ -hexosaminidase (JBHex); the  $m/z$  462 Y-fragment is indicative of the  $\beta$ -mannose modification as verified by  $\beta$ -mannosidase digestion. (C and D) LC-MS<sup>2</sup> and LC-MS<sup>3</sup> analyses of  $\text{Hex}_4\text{HexNAc}_4\text{-PA}$  ( $m/z$  777.5 as  $[\text{M}-2\text{H}]^{2-}$ ) confirm hexosylation of the reducing terminal GlcNAc residue, but the presence of an  $m/z$  1333.5 MS<sup>2</sup> fragment and the absence of a  $m/z$  221 MS<sup>3</sup> fragment contrast with the previously-published data on an isomeric glycan from a marine snail (22); on the basis of the LC-MS data as well as the RP-HPLC comparisons (Supplementary Figure 7), we conclude that the royal jelly core mannose is  $\beta$ 1,6-linked to the proximal GlcNAc. (E-F) The  $m/z$  2098 anionic glycan (7.2 g.u.; see Figure 5) was analysed by MALDI-TOF MS/MS before and after  $\beta$ -mannosidase treatment which resulted in loss of the  $m/z$  462 fragment, thereby confirming the core modification, while the glucuronylated  $m/z$  542 and 745 fragments indicate the presence of a terminal glucuronic acid in this structure; asterisks indicate ions of unknown origin. It is to be noted that  $\beta$ -mannosylation has only a minor effect on N-glycan RP-amide HPLC elution properties as compared to the non- $\beta$ -mannosylated structure, thus 2D-HPLC was necessary prior to the presented digests.



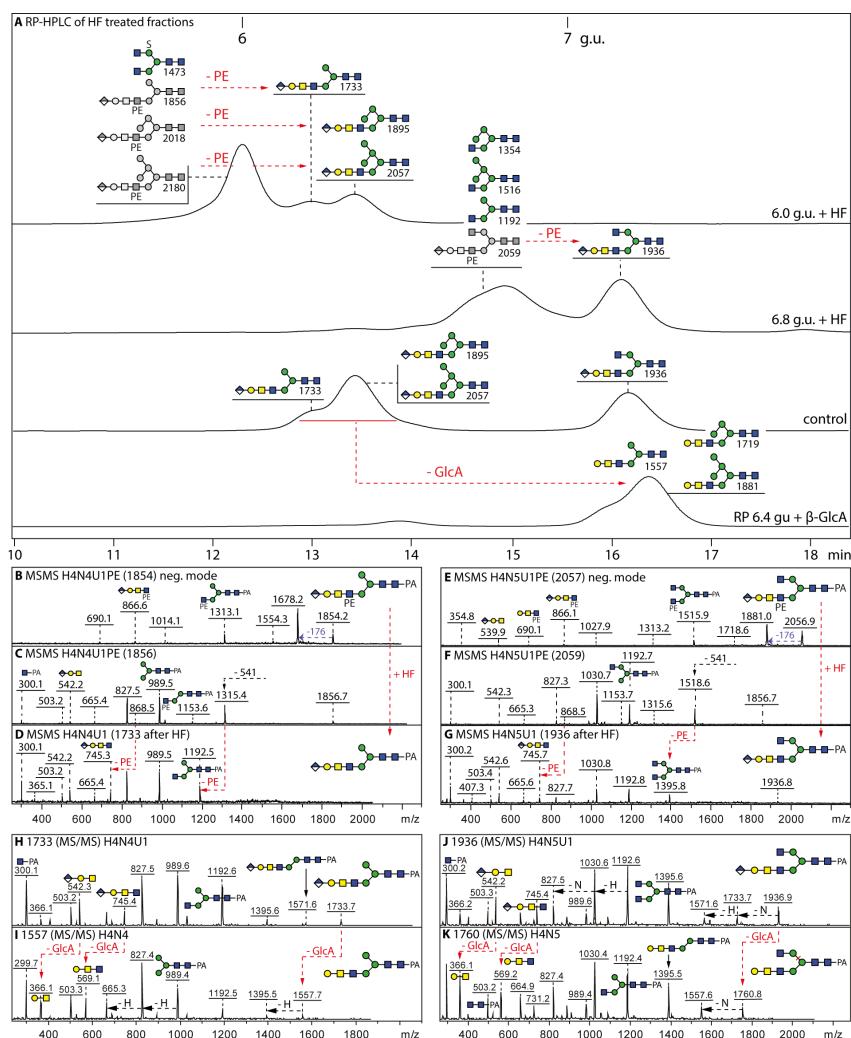
**Figure 4: MALDI-TOF MS analysis of phosphoethanolamine-modified N-glycans. (A-F)** Analysis of two 2D-HPLC separated glycans of  $m/z$  1840/1842 (Hex<sub>5</sub>HexNAc<sub>4</sub>PE<sub>1</sub>; for the HIAx chromatogram, see Supplementary Figure 8) showing negative mode spectra before and after treatment with  $\beta$ 1,3-specific galactosidase ( $\beta$ 3Gal), which (together with the effects of HEX-4  $\beta$ -N-acetylgalactosaminidase and jack bean  $\alpha$ -mannosidase; see Supplementary Figure 9 A-N) aid definition of the isomeric positions for mannose or galactose; insets show a region of the negative mode MS/MS spectrum highlighting changes in the phosphoethanolamine-containing B-fragments. **(G-K)** RP-amide HPLC and MALDI-TOF MS of the 6.6-6.8 g.u. fraction after hydrofluoric acid treatment showing the shift to later elution times upon loss of phosphoethanolamine of a small proportion (10%) of glycans in this particular fraction (peak II with smaller  $m/z$ ), while the elution position of the major  $m/z$  1354/1516 glycans (peak I) remains unaffected; the phosphoethanolamine-modified glycans are observable in negative and positive modes, while the simple hybrid structures are also detected as adducts in negative mode (indicated as \*). Alterations in MS due to digestion are indicated with red arrows; greyscale annotations in panel G indicate original structures before removal of phosphodiesters. For further MS/MS data, refer to Supplementary Figures 8 and 9.



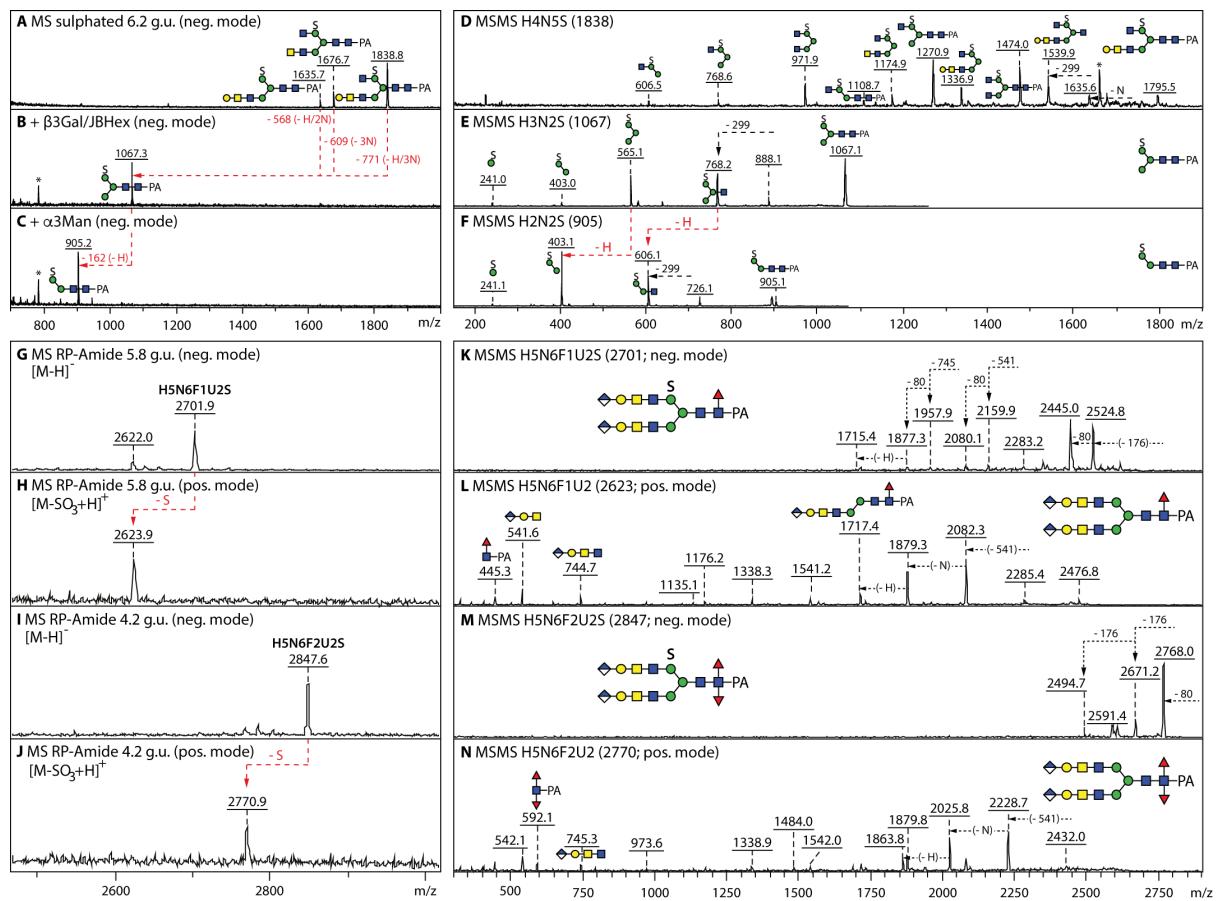
**Figure 5: RP-HPLC fractionation of the anionic N-glycans from royal jelly.** Glycans were released by **(A)** PNGase F and A, **(B)** PNGase Ar after PNGase F or **(C)** PNGase F alone prior to solid phase extraction to separate the neutral and anionic glycans, pyridylation and finally separation on an RP-amide column. The chromatograms are annotated with glycan structures (each with the positive mode  $m/z$  value for glucuronylated glycans or the negative mode  $m/z$  value, in bold, for sulphated structures) according to the Symbolic Nomenclature for Glycans (circles, galactose or mannose; diamonds, glucuronic acid; squares, GalNAc or GlcNAc; triangles, fucose; PE, phosphoethanolamine; S, sulphate; see inset for symbol and linkage information). The HPLC column was calibrated in terms of glucose units (g.u.). The chromatogram A (of the PNGase F/A-released anionic glycans) is annotated with all structures determined in that pool, whereas chromatograms B and C (i.e., the PNGase Ar and PNGase F released anionic glycans from a second preparation) highlight the fucosylated glycans (the elution positions of other detected glycans being indicated by the  $m/z$  values alone); in the case of PNGase Ar release, the low abundance fucosylated structures can be core difucosylated and even carry one Lewis-like antennal fucose, whereas PNGase F can release glycans with two antennal ( $\alpha 1,3$ -) and one core ( $\alpha 1,6$ -) fucose residues.



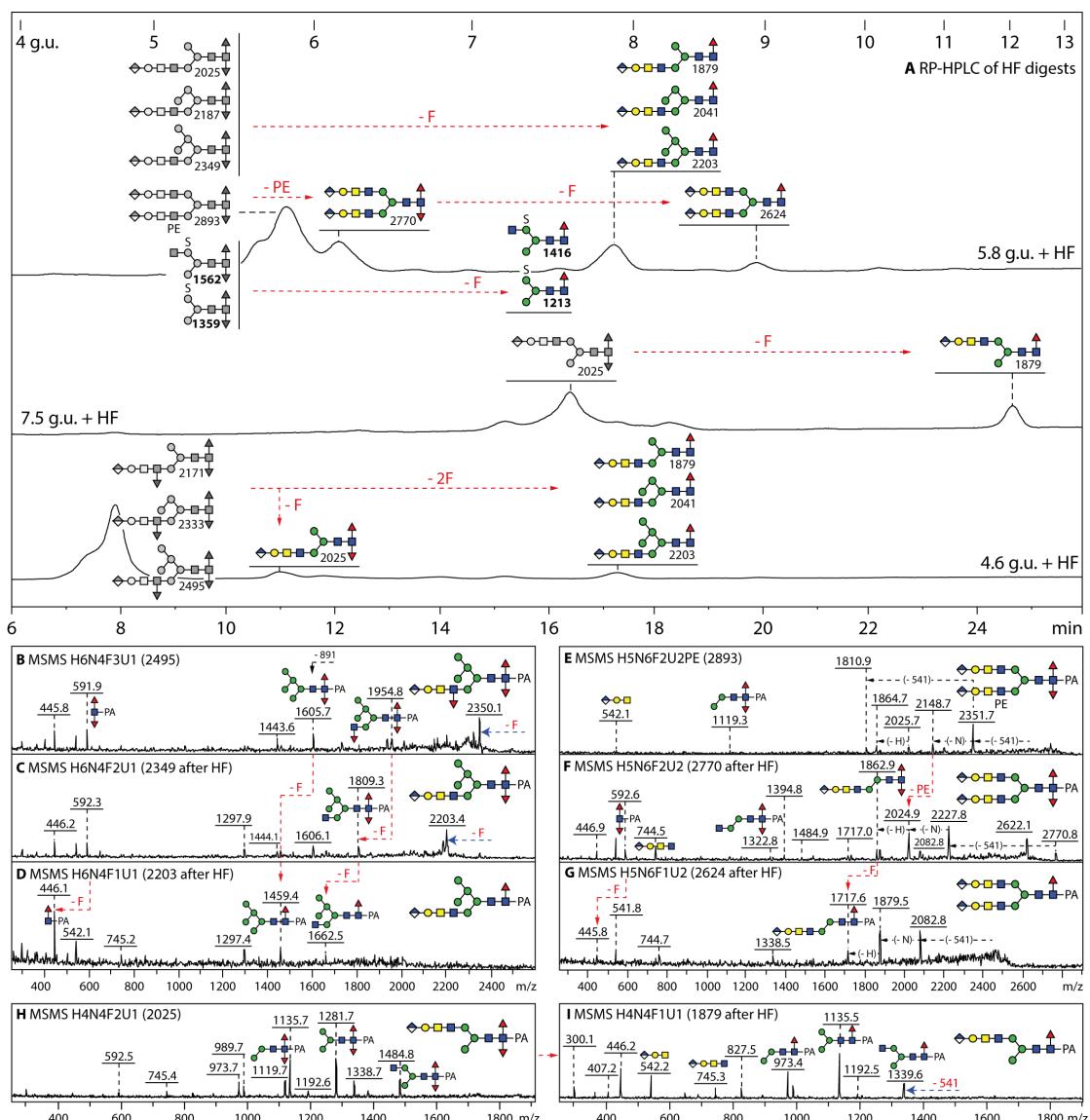
**Figure 6: RP-HPLC and MS/MS analysis of glycans modified with both glucuronic acid and phosphoethanolamine.** (A) RP-amide chromatograms of the two hydrofluoric acid (HF) treated 6.0 and 6.8 g.u. fractions are shown in comparison to a control co-injection of aliquots of 6.4 and 7.2 g.u. glucuronylated glycans. The effect of *E. coli*  $\beta$ -glucuronidase on the retention time of the 6.4 g.u. control glycans, corresponding to the HF products of the 6.0 g.u. fraction, is also indicated; see Supplementary Figures 10 and 11 for further MS and HPLC data on glucuronidase and HF digests. (B-G) The MALDI-TOF MS/MS of untreated glycans in negative (B and E) and positive modes (C and F) and of the HF-treated products (positive mode only; D and G) highlight changes in the fragmentation patterns due to removal of phosphoethanolamine residues (loss of  $m/z$  868 fragments correlating to the later retention times; indicated with red arrows). (H-K) Positive mode MALDI-TOF MS/MS of the 6.4 and 7.2 g.u. glucuronylated glycans before and after  $\beta$ -glucuronidase digestion indicating loss of the  $m/z$  542 and 745 fragments and appearance of ones at  $m/z$  366 and 569. For space reasons, the phosphoethanolamine group is depicted underneath the antenna, although a 6-linkage is assumed based on analogy to insect glycolipids (36-38); the greyscale structures indicate the original elution times, those in colour the products'. Based on these analyses, glycans carrying both phosphoethanolamine and glucuronic acid are estimated to represent 30% of these particular anionic fractions. It is to be noted that sulphated glycans (e.g.,  $m/z$  1473) are resistant to hydrofluoric acid treatment, unlike those modified with phosphomono- or diesters.



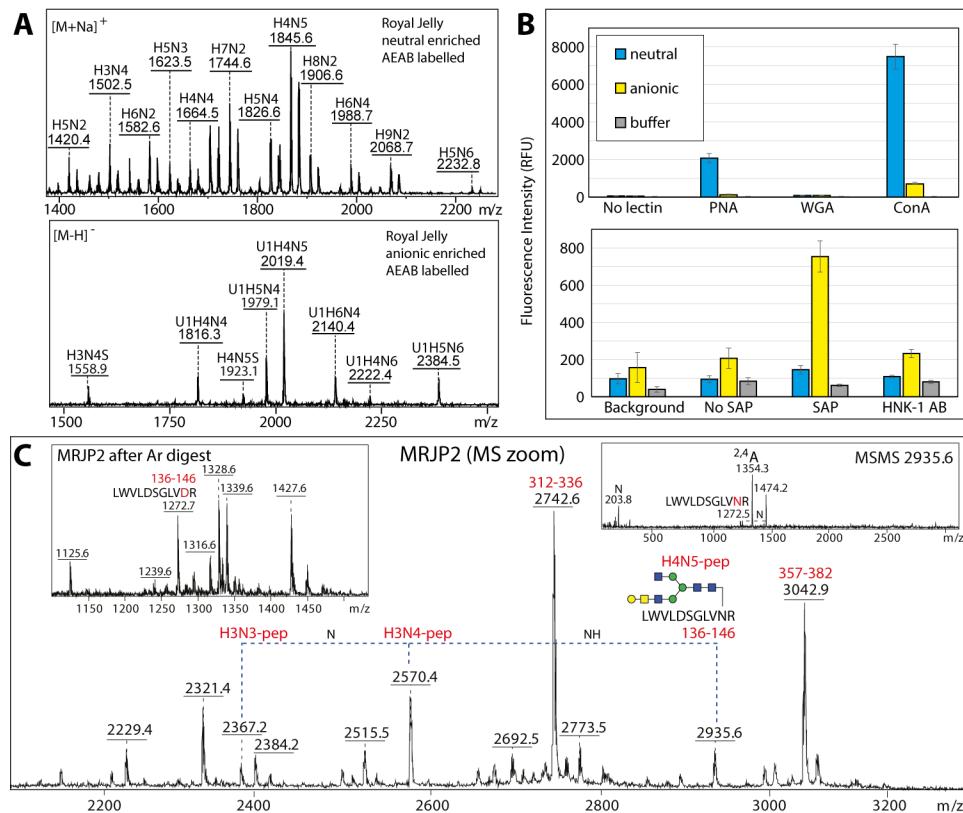
**Figure 7: Glycosidase digestion and MALDI-TOF MS/MS analysis of sulphated N-glycans.** **(A-C)** Negative mode MALDI-TOF MS of the 6.2 g.u. anionic fraction before and after combined  $\beta$ 1,3-galactosidase ( $\beta$ 3Gal) and jack bean hexosaminidase (JBHex) digestion and subsequent  $\alpha$ 1,3-mannosidase ( $\alpha$ 3Man) treatment; the removal of galactose and HexNAc residues results in a single  $m/z$  1067 product from which the  $\alpha$ 1,3-mannose can be digested, thereby localising the sulphate to the  $\alpha$ 1,6-mannose as in other monosulphated glycans from insects. **(D-F)** Negative mode MALDI-TOF MS/MS of a sulphated glycan and of its digestion products. **(G-N)** MS and MS/MS in negative and positive modes of sulphated glycans modified with also fucose and glucuronic acid; in contrast to the ability of phosphorylated glycans to ionise in both positive and negative modes (54), sulphated structures are detectable in the negative mode as  $[M-H]^-$  and the indicated in-source fragmentation in positive ion mode results in the presence of an ion of 78 mass units lower than the negative ion detected in the same fraction. MS/MS of the  $[M-SO_3+H]^+$  ion aided definition of the core modifications ( $m/z$  446 and 592 mono- and difucosylated Y-fragments), while the sulphation of the 6-linked mannose is assumed by analogy to the smaller structures and to the permethylation data in Supplementary Figure 3.



**Figure 8: HPLC and MS/MS analysis of N-glycans modified with both glucuronic acid and fucose residues.** **(A)** RP-HPLC of three hydrofluoric acid treated anionic fractions (originally eluting at 4.6, 5.8 and 7.5 g.u.) highlighting the shifts to higher retention times due to the loss of core and antennal  $\alpha$ 1,3-fucose and of phosphoethanolamine (either -F, -2F or -PE; respective shifts of 2, 1 and 0.5 g.u. for the removal of core  $\alpha$ 1,3-fucose, antennal  $\alpha$ 1,3-fucose and phosphoethanolamine) (either -F, -2F or -PE; respective shifts of 2, 1 and 0.5 g.u. for the removal of core  $\alpha$ 1,3-fucose, antennal  $\alpha$ 1,3-fucose and phosphoethanolamine); the loss of antennal  $\alpha$ 1,3-fucose or phosphoethanolamine is more efficient, whereas the loss of the core  $\alpha$ 1,3-fucose is partial for the employed hydrolysis time. The greyscale structures indicate the original elution times, those in colour the products'; note that sulphated difucosylated glycans merely lose a fucose, but not the sulphate, as previously seen for the same structures isolated from High Five cells (19). **(B-G)** Positive mode MALDI-TOF MS/MS of a trifucosylated glycan and of a difucosylated phosphoethanolamine-modified glycan showing the spectra for the original glycan as well as the intermediate and final HF-hydrolysis products. **(H and I)** Positive mode MALDI-TOF MS/MS of a difucosylated glycan with an 'upper' arm before and after hydrofluoric acid treatment. The loss of core  $\alpha$ 1,3-fucose correlates with the absence of the difucose-containing  $m/z$  592 Y-fragment upon hydrofluoric acid treatment; see also further MS/MS in Supplementary Figure 11.



**Figure 9: (A)** MALDI-TOF MS of AEAB-labelled royal jelly neutral and anionic N-glycans; refer to Supplementary Figure 14 for further selected MS and MS/MS data. **(B)** The binding of peanut agglutinin (PNA) to neutral and anionic N-glycans and of the human pentraxin serum amyloid P (SAP) and the anti-L2/HNK-1 antibody clone 412 (HNK-1) to anionic N-glycans is proven by probing the AEAB-labelled N-glycan pools on NHS-modified glass slides (see corresponding MALDI-TOF MS); the chart indicates the uncorrected fluorescence values with the standard deviations (green or red channels; mean of 10 spots) as compared to concanavalin A (as a non-specific lectin) and to negative controls (spotting buffer or no primary lectin, antibody or pentraxin). The recognition by PNA, SAP and anti-L2/HNK-1 correlates, respectively, with the presence of 'pseudo' T antigen epitopes in both pools and of phosphoethanolamine and terminal glucuronic acid in the anionic pool as proven by the analyses of the PA-labelled glycans, but the binding of the glycans to wheat germ agglutinin (WGA) is minimal. As shown in Supplementary Figure 14, binding to PNA decreases and to WGA increases after  $\beta$ -galactosidase treatment, while binding to anti-L2/HNK-1 is  $\beta$ -glucuronidase sensitive. Refer to Supplementary Figure 13A for Western blotting data with PNA, WGA and SAP. **(C)** A zoomed-in region of the MRJP2 spectrum (see Supplementary Figure 13D) containing ions corresponding to a peptide modified with different complex N-glycans, including one with a T antigen epitope of the type recognised by PNA; the insets show respectively a region of the spectrum after PNGase Ar-treatment and one example MS/MS showing the  $^{2,4}$ A cross-ring GlcNAc-containing fragment for the  $m/z$  2935 glycopeptide. Raw mzXML files corresponding to the full MRJP2 peptide spectra before and after PNGase Ar treatment as well as two MS/MS glycopeptide spectra are included in the supplementary zip archive.



**Figure 10: Summary of the N-glycome of honeybee royal jelly.** The N-glycans are categorised on the basis of structural type with colour code indicative of either oligomannosidic, paucimannosidic, hybrid, biantennary and triantennary structures; a further highlighting is used for the  $\beta$ -mannose, sulphate, glucuronic acid or phosphoethanolamine motifs. Approximate abundances were calculated on the basis of HPLC and MS intensities. The order of abundance of the glycans decreases from the centre (with less intense background colours for the least abundant), whereby the oligomannosidic  $\text{Man}_{5-9}\text{GlcNAc}_2$  are estimated as accounting for 37% of the total N-glycome. For simplicity not all glycans of a certain type are shown, rather a representative major example, whereby, e.g., the biantennary class (27%) includes structures with Gal and GalNAc residues or the hybrid class (17%) pertains also to  $\text{Man}_{3-5}$ -based isomers without Gal/GalNAc modification.

