nature portfolio

Peer Review File

Major human schistosome species express different glycans with immunological and diagnostic implications.

Corresponding Author: Professor Cornelis Hokke

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript described the characterization of glycans in N- and O-glycans in glycoproteins and in glycosphingolipids from parasites in Schistosoma haematobium and S. mansoni. The results indicate differences in glycosylation, also during the stages of development, that may relate to pathology and immunogenicity. The differences in glycosphingolipids between the two species were substantial, with differences apparent in fucosylation, glucuronic acid, and sequences. The work is interesting at many levels and represents one of the most advanced studies on glycans in these parasites conducted thus far. The studies, however, are highly descriptive, and yet not all linkages are defined, and the sequences are largely predicted. A key aspect of the study is the potential biological relevance of the glycans, which is provided through glycan microarrays, in which selected glycans were printed and examined for recognition by antibodies in sera from young patients with parasitic infections. The results indicate selective recognition of glycans from each species. The descriptive nature of the work overall is excellent and provides new information about developmental and species-specific difference in glycosylation. However, there are aspects to the study in terms of biological impact that are incremental in several ways and somewhat lacking in terms of novel insights beyond the glycan structural differences. Several of the major points of concern are listed below.

• The core structure of the GSLs from S. haematobium were not fully characterized and an unknown hexose and linkage was identified.
• The precise linkages of monosaccharides in the glycans were not identified, so this raises questions about whether there are many isomers of such structures in regard to linkage, and the enzymes that might be important in generating them.
• While the studies on antigenic nature of glycans is interesting, it is already well known from many prior studies that glycans from these parasites are antigenic and recognized by antibodies (IgG/IgM) in sera from infected individuals. It is not clear from the result whether each of the glycans is recognized by unique antibodies or that different epitopes shared between glycans are being recognized. These results in this manuscript are certainly well done and provide additional information, but much of the conclusions are consistent with prior efforts in the field.
• The overall results do not provide specific diagnostic or immunogenicity information relative to the glycan structures, but rather is a broad survey of the glycome. This is not a criticism, per se, since such detailed information is certainly important in directing future work perhaps in this direction. Already, a lot of glycans in these parasites are known to be antigenic in infected individuals, as the parasite synthesized glycans largely difference in most respects from their mammalian hosts. The present study adds to that diversity.
• The relationship between the glycome and the genome in terms of enzymatic machinery to synthesize these glycans is somewhat speculative as no detailed genetic information is provided in this regard.
• The parasites are derived from hamsters, and not from people, obviously, but that does raise the question as to whether the host might influence the production of glycans by the parasite. No information in this regard is provided or discussed.
• The antigenic nature of the glycans in hamsters is not discussed either, and that might be very interesting in terms of differences in mammalian responses to infection and glycan antigenicity.
• The lack of information about glycoproteins and glycoproteomics is striking in this regard, as one might expect that from such a huge diverse set of glycans they might be distributed somewhat uniquely among glycan species, as the authors indicate from prior studies on Omega-1.
• There are multiple monoclonal antibodies to the glycan determinants identified in table 2 for example. Did the authors examine the recognition of the relevant glycans under study for their binding to such antibodies, which would be an additional verification or potential validation of structure?

• Although the authors raise great interest in the presence of glucuronic acid in glycans from S. haematobium, such residues are found also in S. mansoni, and thus perhaps not unique to either. In addition, the finding of glucuronic acid in glycans has been known for some time. Prior studies on the glucuronic acid residues in glycans from S. mansoni and studies on chemical synthesis and immunogenicity of such glycans are not discussed [see for example, Halkes et al PMID: 9741076].
• The expression of glycans within the parasites are not known, as the total homogenates were used, and questions as to whether glycans are potentially expressed on the worm surface, secretions, or within other organs in some specific manner are not discussed.
• The results are discussed as being quantitative but it is unclear what is meant by that, as no specific quantitation is provided. Perhaps differences in abundance is what is meant in this context, and even then it might be 'relative abundance'. This reviewer is not certain of what is meant exactly. Given the glycans are fluorescently labeled, it might be possible to actually define the quantity of glycans. MALDI-TOF information is certainly not quantitative in its own right.
• There appear to be some significant differences in responses of individual sera from different donors, but that is not clear or well described in the study. The potential cause of that is unclear. Are the individuals infected with more than one parasite or type of parasite? The donor information in that regard is not clear.

Reviewer #2

(Remarks to the Author)
Nature Commun Petralia

Overall, the manuscript is of interest due to the variations in glycan structures between the two Schistosoma species as well as due to the glycan array data. The data is new, the insights into S. haematobium are novel. The methodology is typical of that from the authors' laboratory. With such a large dataset, it is not possible to assess all files, but I have a number of questions.

Page 6/8 - does the removal by SpHex suggest a chondroitin-type and/or CAA-type terminus, or should the terminal HexNAc be undefined "white"? In the Excel files, then there are 'mixed' blue/yellow squares for GlcNAc or GalNAc, but "white" undefined hexose residues. It could be mentioned on page 8 that a HexA has more-or-less the same delta m/z (176) as methylhexose, a modification not unknown in helminths or snail hosts.

Figure 3D - none of the higher Mr glycans above m/z 2300 are annotated, are they triantennary - although a composition is given in one of the Excel files. Inspection of the corresponding mzxml files on glycopost suggests the m/z window was not wide enough to detect the entire range as an obvious fucose series suddenly is cut of at m/z 3500. (In the Excel file cut off at m/z 3321.)

Figure 4 - it is a bit strange that there is a gap of some 1200 amu between the largest and second largest glycan (although there are traces apparently of intermediate peaks shown in the Excel file) - this assumes an almost 'quantitative' complete biosynthetic modification once the second 'core 2-like' arm is generated.

Page 15 - the authors say "once validated" without briefly stating at this point what they mean (except when the reader digs into the supplement).

Methods - replace MQ by water - other manufacturers also have machines capable of generating pure water.

Ref 84 - check author names, perhaps for McShaneb and Doddb, b is a superscripted indication of affiliation. Ref 90 is the same as Ref 29.

Figure S2 - what is meant by "purified glycans", "purified fraction" ... if it corresponds to a certain HPLC fraction, then this should be stated in terms of fraction number or time. Three decimal places for standard MALDI is over-exact - also in the Excel files. Are all the annotated m/z in the supplementary figures actually glycans or glycan fragments or just what the software annotates without human interpretation? What is the blank spectrum for the glucuronidase? The enzyme seems particularly full of contaminants.

Figure S5 - not all the glycosidic bonds are easily seen.

Figure S8 - refers to a previous study for the O-glycans - however, the basic characteristics of these samples should be stated here (e.g., also AA labelled?). Which entire O-glycan pools are meant in the methods? Were the concentrations defined on the basis of comparison to a standard?

Excel file "631032_0_data_set_11107602_sz8r9r" - the "accuracy" varies between the N-and O-glycans - was the MALDI calibrated with an external standard on a daily basis? Are the "null" masses 'junk'? Perhaps there are limitations to what the rather old Glycoworkbench program can find. For the O-glycans, there is a mix of H and Na forms - some have only an H form, which is suspicious - considering the Na-rich release method, finding only Na or having both H and Na seems logical, rather than having some glycans solely as protonated forms.

Excel file "631032_0_data_set_11107609_sz8r9r" - although the glycan fractions were printed in triplicate, I guess only mean values are shown. No individual data points are shown in the relevant supplementary figures S8-S10. What are the corresponding wet or dry weights of the numbers of eggs, worms, etc (methods and legend to Figure S8)?

The Excel files should be headed in the file with the same Table number as in the text, considering the renumbering by the editorial management system.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript has addressed several key concerns, namely characterization of the core structure of the GSLs, clarified the limitations of their studies in regard to linkages of sugars, expanded on their insights into the differential antibody responses to glycans previously observed versus their current observations, addressed the limited nature of the study's insights regarding linkages to the genome, clarified the nature of glycosylation in worms isolated from hamsters compared to human hosts, addressed the limitation of the study in regard to lack of glycoproteomic information, provided additional information in Figure S9 regarding structure of glycans recognized by antibodies, revised their discussion regarding the presence of glucuronic acid in glycans, the lack of quantitation of glycan expression, which is a common limitation of current technologies, and the individualistic nature of immune responses to glycan antigens.

Overall, the revised manuscript provides excellent information about glycan structures in these parasites, as well as developmental and species-specific difference in glycosylation.

Reviewer #2

(Remarks to the Author)
Petralia revised

Overall, the manuscript is of interest due to the variations in glycan structures between the two Schistosoma species as well as due to the glycan array data. The data is new, the insights into S. haematobium are novel. The methodology is typical of that from the authors' laboratory. With such a large dataset, it is not possible to assess all files, but spot checks still result in some questions. The authors have revised their manuscript, adding further data and explanations as well as making corrections. My line numbers refer to the 'tracked changes' version which follows the revised manuscript in the single "manuscript" pdf.

Heading - line 77 - "chains" or "oligomers" rather than "stretches"

Line 93 - if both a general and a specific b-galactosidase worked, then the linkage is Galb1,4Glc and if the a-Gal is not a1,4, then it can be hypothesised that it is Gala1,3Galb1,4Glc (Gala1,6 in an animal would probably be unusual). Possibly in vitro a1,3-galactosylation of lactose with a commercially available enzyme to generate an alternative standard would resolve this in the absence of enough material for linkage analysis - alternatively Elictyl actually sell Isoglobotriose.

Line 108 - specify S. pneumoniae in the text, not just the table.

Line 370 - certainly one alpha and one beta galactosyltransferase

Comment 2: The S/N ratio of 4 and a 1% intensity as a cut-off for defining glycan structures seems arbitrary. Can these larger glycan structures be annotated by careful manual interpretation or are the MS2 spectra uninterpretable? Also, it should be possible to detect and fragment glycans above m/z 3500 using at least the Rapiflex mentioned in the methods. The result of these omissions is an underestimation of the glycomic potential of the organism. Testing interactions of the long LN repeats with either mammalian lectins or antibodies would be of interest; again, it is the authors' assumption that they will be immunologically invisible, which if true is also of interest in terms of 'glycan mimickry'.

Comment 8: Importing from Glycoworkbench does have the tendency to make slightly blurry pictures and one has to really zoom in to distinguish the rather small alphas and betas, which could be added rather with a drawing program for legibility.

Comment 9: It is indeed good that the authors found the mistakes in the table due to misassignments as protonated ions. The m/z 738 glycan shown in Tab S4 is not found in both replicates, but m/z 779, 1402, 1432, 1475 found in one replicate are not in Tab S4, while m/z 1636 is in both replicates, but is not in Tab S4. Is Tab S4 meant to be a consensus set - if so, then m/z 738 should not feature if it was only found in one experiment, while m/z 1636 should be included. Without me checking: has the Table S1 - Raw MALDI-TOF-MS data been cross-checked with the Tabs S2-S4?

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have again revised their manuscript, adding some 'zoomed' spectra for the higher molecular weight range. Unfortunately, their attempt at another preparation did not work well as the 'only for referees' supplement shows a spectrum full of polyhexose contaminants. Nevertheless a statement could be included in the legend to Supp 6 that: "In experiments with total eggs, there were indications of higher molecular weight N-glycans of approximately 4000 Da, whereby no interpretable MS2 could be obtained to verify whether these had either a fourth antenna or extra LacNAc repeats." As previous indicated, a perfect "corroboration" for the GSL core would be to do PGC-nano-LC-MS with isoglobotriose from Elictyl. Line 585 - does the Rapiflex® have a 1 kHz Smartbeam II laser or a 10 kHz one?

Revised Manuscript NCOMMS-25-53181A: Response to Reviewer Comments

Reviewer #1

This manuscript described the characterization of glycans in N- and O-glycans in glycoproteins and in glycosphingolipids from parasites in *Schistosoma haematobium* and *S. mansoni*. The results indicate differences in glycosylation, also during the stages of development, that may relate to pathology and immunogenicity. The differences in glycosphingolipids between the two species were substantial, with differences apparent in fucosylation, glucuronic acid, and sequences. The work is interesting at many levels and represents one of the most advanced studies on glycans in these parasites conducted thus far. The studies, however, are highly descriptive, and yet not all linkages are defined, and the sequences are largely predicted. A key aspect of the study is the potential biological relevance of the glycans, which is provided through glycan microarrays, in which selected glycans were printed and examined for recognition by antibodies in sera from young patients with parasitic infections. The results indicate selective recognition of glycans from each species. The descriptive nature of the work overall is excellent and provides new information about developmental and species-specific difference in glycosylation. However, there are aspects to the study in terms of biological impact that are incremental in several ways and somewhat lacking in terms of novel insights beyond the glycan structural differences. Several of the major points of concern are listed below.

We sincerely thank the reviewer for their constructive comments. The feedback has contributed to strengthening the manuscript by tightening loose ends of our structural study and improving the data presentation of our glycan microarray dataset. We also appreciated the insights highlighting important directions for future research. Below we provide a point-by-point response to the comments and questions raised.

1. **The core structure of the GSLs from S. haematobium were not fully characterized and an unknown hexose and linkage was identified**

   We acknowledge that the undefined trihexosyl core structure of *S. haematobium* GSL glycans represented a notable gap in our study. We are grateful to the reviewer for giving us the opportunity to further characterize this glycan class. To do so, we used a UHPLC-based method optimized for the detection of short, labeled oligosaccharides to address previous limitations of our workflow. With this experimental platform in combination with exoglycosidase digestions, we were able to determine that the ceramide-linked glycan portion of *S. haematobium* GSL glycans is built on a sequence of Galα-Galβ-Glcβ1-Cer. This new data has been included in the manuscript as Figure S4. These additional results are now described **L.90***, discussed **L.341(342), L.356(359)-362(365)** and the UHPLC method is detailed **L.634(648)-647(661)** (M&M section).

   **NB: Please note that line numbers refer to the version of the manuscript without track changes, while numbers in brackets correspond to the version with track changes —except when they are identical in both versions.*

2. **The precise linkages of monosaccharides in the glycans were not identified, so this raises questions about whether there are many isomers of such structures in regard to linkage, and the enzymes that might be important in generating them.**

   While it is true that the precise linkages were not elucidated for every single glycan structure, our workflow provides substantial information in that regard. With the exception of the *O*-glycans for which we primarily relied on informed assumptions and analogies with *S. mansoni*, exoglycosidase digestions provided linkage information (at least in terms of α or β anomers) for monosaccharides in most GSL and *N*-linked glycans. Special attention was given to elucidating the linkages in glycan structures that diverge from those already characterized in *S. mansoni*, in particular the acidic GSL glycans detected in both *S. haematobium* and *S. mansoni* eggs, the GSL glycans of *S. haematobium* cercariae containing branched galactose residues, or the trihexosyl core of *S. haematobium* GSL glycans. PGC-nano-LC-MS/MS was employed to resolve some of the isomeric complexity of the GSL glycans. Our analysis clearly shows that isomeric variants are frequent in schistosomes, and we do not claim to have completed the daunting and perhaps impossible task of elucidating each glycan structure at the isomeric level. We hope to have now clarified this for the reader in the expanded discussion section (**L.342(343)-348(351)**).
   The reviewer raises an interesting point by linking the observed structural diversity to the enzymatic machinery responsible for glycan synthesis. As further discussed in question 5, our current understanding of the enzymatic pathways involved in glycan synthesis in *Schistosoma* species remains extremely limited, and the present observations suggest that the underlying biosynthetic machinery must indeed be highly complex and diverge significantly from known mammalian pathways.

3. **While the studies on antigenic nature of glycans is interesting, it is already well known from many prior studies that glycans from these parasites are antigenic and recognized by antibodies (IgG/IgM) in sera from infected individuals. It is not clear from the result whether each of the glycans is recognized by unique antibodies or that different epitopes shared between glycans are being recognized. These results in this manuscript are certainly well done and provide additional information, but much of the conclusions are consistent with prior efforts in the field.**

   We do agree with the reviewer that many of our findings regarding anti-glycan antibody responses corroborate observations already made in the context of *S. mansoni* infections. We hope to have acknowledged this at **L.334-335** by stating "These antibodies are partly directed to the same antigenic glycan elements as anti-glycan antibodies in *S. mansoni* infection sera". Although the antigenicity of several schistosome glycans was previously known, this study is the first to investigate the antigenicity of *S. haematobium* glycans, and importantly, the first to address differences in term of anti-glycan antibody responses between individuals infected with different *Schistosoma* species.

   Although certain antibodies might be specific for entire glycan structures, it is clear that many serum antibodies from *Schistosoma*-infected individuals rather bind to glycan motifs or "glycotopes". This was shown in previous studies with binding to synthetic glycan elements (neoglycoconjugates) printed

on glycan microarrays (*ref#13-15, 19 in manuscript*). In the present work, antibodies in serum from *S. haematobium*-infected individuals bind to *S. mansoni* GSL glycans of compositions $H_1N_{4-7}F_{2-8}$ while *S. haematobium* does not synthesize such structures, constituting a clear example of cross-reactive glycotopes. In this example, the antibodies raised against the multifucosylated glycans of *S. haematobium* cercariae likely bind the multifucosylated *S. mansoni* GSL glycans due to the shared DF/TF glycotopes - see discussion **L.380(388)-385(393).**

4. **The overall results do not provide specific diagnostic or immunogenicity information relative to the glycan structures, but rather is a broad survey of the glycome. This is not a criticism, per se, since such detailed information is certainly important in directing future work perhaps in this direction. Already, a lot of glycans in these parasites are known to be antigenic in infected individuals, as the parasite synthesized glycans largely difference in most respects from their mammalian hosts. The present study adds to that diversity.**

   We agree with the reviewer's analysis of the present work and the fact that such studies are necessary steps towards exploiting the antigenicity of parasite glycans for diagnostics or vaccines.

5. **The relationship between the glycome and the genome in terms of enzymatic machinery to synthesize these glycans is somewhat speculative as no detailed genetic information is provided in this regard.**

   While high-quality nuclear genome assemblies are now available for *S. mansoni* and *S. haematobium*, at this stage, the relationship between the glycome and the genome is indeed highly speculative, with the exception of highly conserved *N*-glycan structures. The majority of the enzymes involved in glycan synthesis and their complex regulation remain uncharacterized (mentioned **L.362(365)-364(367)** *with ref #27*), particularly those responsible for the synthesis of antigenic, non-mammalian motifs (mentioned **L. 389(397)-390(398)** for DF/TF). We have chosen to limit ourselves to brief discussion points as providing any further information in that direction would require functional characterization of predicted glycogenes *via* challenging studies beyond our current scope.

6. **The parasites are derived from hamsters, and not from people, obviously, but that does raise the question as to whether the host might influence the production of glycans by the parasite. No information in this regard is provided or discussed.**

   It is fair to question the influence of the host on the parasite glycan synthesis, as the parasite material in this study was obtained from hamsters. Hamsters are not known to be a host of *S. mansoni* or *S. haematobium* in nature, but they are fully permissive to infections with these human schistosomes. It is not known or expected that the enzymatic machinery of the parasite rapidly adapts to a different host, as host-specificity is observed for most other definitive and intermediate (snail) hosts, making drastic variations in glycan expression in the hamster host unlikely. Moreover, the glycan array results clearly demonstrate that infected (human) individuals have antibody responses to the glycans isolated from schistosomes obtained from hamsters, confirming that the glycans expressed by schistosomes obtained from hamsters accurately reflect those expressed by schistosomes infecting a human host.

7. **The antigenic nature of the glycans in hamsters is not discussed either, and that might be very interesting in terms of differences in mammalian responses to infection and glycan antigenicity**.

   Comparing glycan antigenicity across different mammalian species is indeed of major interest. While studying antigenicity in hamsters may yield interesting insights, it is even more relevant in species resistant to schistosome infections for exploiting glycan antigenicity in therapeutic applications for human schistosomiasis. Previous studies have notably identified specific anti-glycan antibody responses associated with infection clearance in monkey species resistant to *Schistosoma* infection (*refs#14, 51, 52 in manuscript*). We, however, believe that this constitutes a distinct research question that falls outside the scope of the present study.

8. **The lack of information about glycoproteins and glycoproteomics is striking in this regard, as one might expect that from such a huge diverse set of glycans they might be distributed somewhat uniquely among glycan species, as the authors indicate from prior studies on Omega-1.**

   The reviewer raises a valid point and highlights an important direction for future research. Since glycoproteomic analyses rely on robust glycan databases, we hope that the present comprehensive glycomic study will contribute to such efforts. We have edited the discussion section (**L.435(448)-449(462)**) to share this perspective with the reader.

9. **There are multiple monoclonal antibodies to the glycan determinants identified in table 2 for example. Did the authors examine the recognition of the relevant glycans under study for their binding to such antibodies, which would be an additional verification or potential validation of structure?**

   We did incubate the constructed glycan microarrays with several mAbs binding to specific glycan motifs. We show the results for four of these mAbs in Figure S9 (previously Figure S8) and Table S6 of the manuscript namely 273-3F2-A (anti-LDN); 291-2G3-A (anti-LeX); 291-5D5-A (anti-F-LDN(-F)) and 100-4G11 (that binds to the trimannosyl branching of *N*-glycans). We conducted this experiment primarily for validation of the array print quality, but it also provided confirmation of the structural assignment. These results are presented in Figure S9, where we briefly mentioned that mAb binding was consistent with predicted glycan structures in the array fractions, confirming the expression of putative LeX, LDN and fucosylated LDN motifs. In the revised manuscript, we added the sentence **L.276-279** in the main text for the reader's reference and we incorporated mAb binding results side by side with the structural assignment in Table S2 (GSL glycans) and S3 (N-glycans). We thank the reviewer for the suggestion, allowing a more effective presentation of the data.

10. **Although the authors raise great interest in the presence of glucuronic acid in glycans from *S.haematobium*, such residues are found also in *S. mansoni*, and thus perhaps not unique to either. In addition, the finding of glucuronic acid in glycans has been known for some time. Prior studies on the glucuronic acid residues in glycans from S. mansoni and studies on chemical**

synthesis and immunogenicity of such glycans are not discussed [see for example, Halkes et al PMID: 9741076].

The fact that glucuronic acid (GlcA) is clearly not unique to schistosome glycans is indeed worth noting, and we hope to have done so in the discussion **L.398(407)-411(414)**. In summary, GlcA in helminth glycans has been observed so far:

- As part of the repeated [-6(GlcAβ1-3)GalNAcβ1-] units of schistosomal CAA (*ref #56 in manuscript*)
- As a terminal residue capping LDN in *S. haematobium* adult worm GSL glycans (this study) & in the *N*-linked and GSL glycans of other helminths (*refs #62-64 in manuscript*)
- As a subterminal residue capped with HexNAc and associated with different fucosylation patterns in *S. haematobium* and *S. mansoni* egg GSL glycans (this study)

Thus, the present work adds to our knowledge of acidic glycans in parasitic helminths by describing novel GlcA-containing structures in schistosomes. Importantly, these different contexts of GlcA-containing motifs greatly matter for their immunogenicity. In the case of CAA, the glycosaminoglycan-like molecule is bound specifically by antibodies in serum from *Schistosoma*-infected individuals making it a relevant pan-schistosome diagnostic target (*ref #19 in manuscript*). We believe that the quoted study together with the added sentence **L.413(422)-415(425)** should effectively address previous work on immunogenicity of CAA as suggested here by the reviewer.
We would like to emphasize, however, that the serum antibody responses targeting the CAA molecule differ from those binding to GlcA-containing GSL glycans, in line with their vastly different structures. Based on previous work, it is known that several repeated units, and thus several GlcA residues are needed for antibody binding to synthetic CAA, while clearly, a single GlcA residue is sufficient to create an immunodominant epitope targeted by serum antibodies in the context of the GSL glycans. The differential recognition of this epitope by IgG from *S. haematobium* and *S. mansoni*-individuals discussed **L. 415(425)-423(433)** adds to the importance of the context of GlcA within the glycans for their specific antigenicity.

11. **The expression of glycans within the parasites are not known, as the total homogenates were used, and questions as to whether glycans are potentially expressed on the worm surface, secretions, or within other organs in some specific manner are not discussed.**

We agree with the reviewer's rightful comment that points out one of the limitations of our study. The present work is indeed not informative in terms of localization of the identified glycans, although the glycan microarray results indicate that most structures must be sufficiently exposed to trigger antibody responses in infected individuals. While interesting, the question of the spatial distribution of glycan falls outside the scope of this study, which aims to characterize the glycan repertoire of *S. haematobium* and provide insights into their antigenicity. This point echoes question #8: as for glycoproteomics, we believe that it is a critical area for future investigation, with the potential to significantly advance our understanding of glycan function. Thus, we hope to have tackled this in the paragraph we added **L.435(448)-449(462)** discussing future research directions and proposing potential analytical approaches and tools that could be employed to investigate these aspects in greater depth.

12. **The results are discussed as being quantitative but it is unclear what is meant by that, as no specific quantitation is provided. Perhaps differences in abundance is what is meant in this context, and even then it might be 'relative abundance'. This reviewer is not certain of what is meant exactly. Given the glycans are fluorescently labeled, it might be possible to actually define the quantity of glycans. MALDITOF information is certainly not quantitative in its own right.**

We fully agree with the reviewer's comment and understand their concern. Different approaches were taken with respect to (relative) quantitation, depending on glycan classes.

- For the *O*-glycans, all data are MALDI-based, and *S. haematobium* and *S. mansoni* samples were not processed side-by-side. Instead, the comparison relies on previously published *S. mansoni* data (*ref #9 in manuscript*). The same analytical workflow as in this previous study was applied to *S. haematobium* in the present work, and the identified *O*-glycans harbor similarities with those of *S. mansoni*. Although MALDI-TOF-MS is not quantitative, as rightfully pointed out by the reviewer, it still provided a sense of relative abundance based on signal intensity, which we have used cautiously to complement our qualitative observations.

- For the *N*-linked and GSL glycans, on the other hand, *S. haematobium* and *S. mansoni* samples were processed side-by-side in our analytical workflow, allowing for a more robust comparison. Importantly, in addition to MALDI-TOF-MS, these samples were also analyzed using LC platforms, providing us with quantitative data:
  - During the glycan microarray construction, *N*-linked and GSL glycans underwent UHPLC fractionation, and the amount of glycan collected was estimated by comparison with a RNAse B standard (as described in Figure S9). The resulting quantitation was used to build the glycan library for the microarray construction and confirmed our structural observations. For instance, although similar numbers of eggs were used as starting material for both species, over 300pM of acidic GSL glycans were isolated from *S. haematobium* eggs distributed across 16 fractions (Table S5). In contrast, the 12 acidic fractions obtained from *S. mansoni* eggs total approximately 150pM of glycans, a direct consequence of the smaller amount of acidic glycans in *S. mansoni*.
  - We presented the marked quantitative differences observed in the egg GSL glycans in Figure 2. We used PGC-nano-LC-MS to assess the differences regarding the expression of fucosylated motifs (right panel "Fucosylation of major egg GSL glycans). To estimate the relative proportion of fucosylated and acidic GSL glycans in the total GSL pool (left panels: "Fucosylated" and "Acidic"), we used intensity signals in MALDI-TOF spectra, as specified in the figure legend. These results were confirmed beforehand using UHPLC (data made available to the reviewers in Figure R1) as we acknowledge the inherently non-quantitative nature of MALDI-TOF-MS.

We hope that we have clarified the approaches to quantitation used in this MS-based study, and that the reviewer finds our rationale justified.

13. **There appear to be some significant differences in responses of individual sera from different donors, but that is not clear or well described in the study. The potential cause of that is unclear.**

Are the individuals infected with more than one parasite or type of parasite? The donor information in that regard is not clear.

It is indeed possible that coinfections with other pathogens harboring cross-reactive glycans account for some of the observed individual-to-individual variations, and we cannot rule out infections with parasites other than schistosomes in the cohorts used in this study. However, the magnitude of individual-to-individual variations observed in the present work is consistent with previous glycan-microarray assisted studies. Specifically, controlled human schistosome infections (*ref #19 in manuscript*) comprise the most homogenous groups of human subjects for which antibody responses have been studied using glycan microarrays. Even in these healthy Dutch adult volunteers from non-endemic areas, similar variations in anti-glycan antibody responses were observed. Importantly, the impact of *S. haematobium* and *S. mansoni* infections eliciting strong anti-glycan antibody responses, is clearly discernible despite inter-individual variations.

Reviewer #2

Overall, the manuscript is of interest due to the variations in glycan structures between the two Schistosoma species as well as due to the glycan array data. The data is new, the insights into *S. haematobium* are novel. The methodology is typical of that from the authors' laboratory. With such a large dataset, it is not possible to assess all files, but I have a number of questions.

We would like to sincerely thank the reviewer for their careful and insightful review. We greatly appreciate their knowledgeable comments, keen eye for detail, and interest in our work. We have done our best to clarify all points raised and we hope to have made all necessary and appropriate changes.

1. **Page 6/8 - does the removal by SpHex suggest a chondroitin-type and/or CAA-type terminus, or should the terminal HexNAc be undefined "white"? In the Excel files, then there are 'mixed' blue/yellow squares for GlcNAc or GalNAc, but "white" undefined hexose residues. It could be mentioned on page 8 that a HexA has more-or-less the same delta m/z (176) as methylhexose, a modification not unknown in helminths or snail hosts.**

   By comparing the activity of β-HexNAcase with the activity of β-GlcNAcase, we determined the capping HexNAc in acidic egg GSL glycans to be mainly β1-4-linked GalNAc as these structures were mainly resistant to β-GlcNAcase, while β-HexNAcase could cleave off the terminal HexNAc residue, both for *S. haematobium* (Figure S2.C, 3rd panel *vs* 5th panel) and for *S. mansoni*, (Figure S2.E, 4th panel *vs* 5th panel). Unlike β-GlcNAcase, β-HexNAcase can digest β1-4 linked GalNAc (Table 1). We specified this in the text, **L.135 & L.166-168**.
   Using the same enzymatic investigation, we determined that GlcA could be linked to either GlcNAc or GalNAc in the backbone chain of *S. haematobium* egg GSL glycans (Figure S2.C, 9th panel *vs* 10th panel, **L.139-142**). A “white” HexNAc suggests that the nature of the HexNAc is unknown or has not been investigated, while in this case, we actually know that both β-linked GlcNAc and GalNAc occur. Thus, mixed blue/yellow square seemed more appropriate to convey this message. For convenience, we decided to represent the GalNAc-containing isomer in the manuscript main figures and to show

both possibilities in the supplementary table S2 as well as in Table 2, that has been updated to address this comment.

We believe that it may not be fully accurate to qualify the terminal acidic epitope characterized in schistosome GSL glycans of chondroitin-type and/or CAA-like. In the context of CAA, GlcA is branched in repeated [-6(GlcAβ1-3)GalNAcβ1-] units, differing from the linear GalNAcβ1-4GlcAβ-HexNAcβ-R sequence characterized in the GSL glycans. We hope to have clarified this by adding a sentence in the discussion **L393(400)-398(407)***. The absence of repeat or polymerization of the motif in the egg GSL glycans constitutes a major difference with CAA but also with glycosaminoglycans. These structural variations have major implications regarding the antigenicity of these acidic motifs as anti-CAA antibodies do not bind to the acidic GSL glycans (data not shown) and vice versa.

We have put substantial effort into the characterization of the HexNAcs constituting terminal motifs due to their importance as antigenic epitopes. We have, however, not confirmed the exact nature of all single HexNAcs within the GSL backbones. We assumed the backbone HexNAc to be GlcNAc, based on analogy with *S. mansoni* for which HexNAc stretches of the GSL backbones have been shown by monosaccharide analysis to be constituted predominantly of GlcNAc during previous studies (*refs #30, 32-34*). This is specified in the figure captions, and we have now added this information in the main text **L.106-114**. In addition, we have resolved the previously uncharacterized (white) hexose part of the *S. haematobium* GSL glycan core structure. Using UHPLC, we determined this trisaccharide sequence is Galα-Galβ-Glc-Cer. This new data has been incorporated into the manuscript in Figure S4, **L.90** (result description), **L.341(342), L.356(359)-362(365)** (discussion) and **L.634(648)-647(661)** (M&M section). Thus, the white hexoses were converted to Gal.

Lastly, we agree with the reviewer that the possibility of a methylated hexose and the rationale for its exclusion as a potential monosaccharide in the composition of the glycans characterized here are worth mentioning. We have revised the text **L.121-125** accordingly.

**NB: Please note that line numbers refer to the version of the manuscript without track changes, while numbers in brackets correspond to the version with track changes —except when they are identical in both versions.*

2. **Figure 3D - none of the higher Mr glycans above m/z 2300 are annotated, are they triantennary – although a composition is given in one of the Excel files. Inspection of the corresponding mzxml files on glycopost suggests the m/z window was not wide enough to detect the entire range as an obvious fucose series suddenly is cut of at m/z 3500. (In the Excel file cut off at m/z 3321.)**

   To tackle the complexity of the data presented in this study, we arbitrarily decided to solely annotate the “ions with signal-to-noise ratios > 4 and intensities above 1% of the total spectrum intensity” (specified in figure legends) which indeed resulted in ions with *m/z* > 2300 not being annotated in Figure 3.D, as they do not fulfill these criteria.

   Based on composition and digestion with β-HexNAcase and β-GlcNAcase (Figure S6.E) these structures are expected to be multiantennary and to harbor a variety of terminal motifs including LN, LeX and (fucosylated) LDN (Figure R2-A). Many of these masses are also observed in *S. mansoni* eggs,

although a poly-LacNAc series (*m/z* 3029, 3175, 3321…) present in *S. haematobium* was not detected in *S. mansoni* (Figure R2-B). As highlighted by the reviewer, it is very likely that this series encompasses larger structures with *m/z* > 3500 not measurable using this workflow. The amount of glycans released from *S. haematobium* eggs using PNGase A was limited, preventing further structural characterization of minor glycan subsets such as this one. For the same reason, these structures could not be included in the generated glycan array. However, we would not expect *N*-glycans containing terminal LN, which are widespread also in mammalian systems, to be antigenic.

We focused this study on the most divergent glycan structures between *S. mansoni* and *S. haematobium*, which centered the manuscript on the GSL glycans and the corresponding antibody responses from infected individuals. Thus, although interesting, we opted not to elaborate on minor *N*-glycan subsets.

3. **Figure 4 - it is a bit strange that there is a gap of some 1200 amu between the largest and second largest glycan (although there are traces apparently of intermediate peaks shown in the Excel file) – this assumes an almost 'quantitative' complete biosynthetic modification once the second 'core 2-like' arm is generated.**

   We thank the reviewer for their interesting observation that may give some insights regarding the *O*-glycan synthesis in schistosomes. Although less visible than in *S. haematobium*, a similar pattern can indeed be observed in the *O*-glycans of *S. mansoni* mature eggs (*ref #9 in manuscript*) where ions with m/z 1157.5 and 1780.8 also appear relatively more abundant than the intermediate structures.

4. **Page 15 - the authors say "once validated" without briefly stating at this point what they mean (except when the reader digs into the supplement).**

   To clarify this, we have added a brief statement in the main text (**L.276-278**) and a short paragraph in the Materials and Methods section (**L.673(691)-675(693)**), both pointing to Figure S9 (formerly Figure S8), which provides the detailed version of the experimental validation procedure. We hope that these additions sufficiently clarify how the microarray validation was conducted while keeping the corresponding results and data in the supplementary materials for the sake of restricting the main text length.

5. **Methods - replace MQ by water - other manufacturers also have machines capable of generating pure water.**

   We appreciate the reviewer's point, however, for the sake of methodological reproducibility, we prefer to specify that ultrapure water was used. Therefore, we retained the abbreviation 'MQ' but changed its meaning to ultrapure water (see **abbreviation list**).

6. **Ref 84 - check author names, perhaps for McShaneb and Doddb, b is a superscripted indication of affiliation. Ref 90 is the same as Ref 29**

   We apologize for the oversight which has now been rectified, and we thank the reviewer for their attention to detail.

7. **Figure S2 - what is meant by "purified glycans", "purified fraction" ... if it corresponds to a certain HPLC fraction, then this should be stated in terms of fraction number or time. Three decimal places for standard MALDI is over-exact - also in the Excel files. Are all the annotated m/z in the supplementary figures actually glycans or glycan fragments or just what the software annotates without human interpretation? What is the blank spectrum for the glucuronidase? The enzyme seems particularly full of contaminants.**

   "purified glycans" and "purified fractions" in Figure S2, indeed referred to UHPLC fractions generated during the glycan array construction. They have been replaced by their respective fraction ID numbers as listed in Table S5.

   We have standardized the decimal number to two decimal places, for uniformity between the various MS-based techniques employed in this study. This also aligns with the resolution and mass accuracy typically achievable in reflector mode on Bruker MALDI-TOF instruments (Autoflex, Ultraflex, Rapiflex) and is commonly seen in publications presenting MALDI-TOF-MS spectra of glycans.

   All the glycan and glycan fragment annotations both in main and supplementary figures were interpreted and assigned manually by the authors with the help of GlycoWorkBench. Currently available tools for annotation of MALDI-TOF-MS data are designed for mammalian *N*-glycans and are not able to handle non-mammalian structures such as those found in schistosomes. Therefore, manual annotation remains necessary.

   The β-glucuronidase appears to indeed contain a few contaminant peaks (Figure R3). The ion with *m/z* 1418 for instance can be distinguished in β-glucuronidase digestion spectra in Figure S2.B. The MS spectrum shown in Figure S2.B (panel 2) appears particularly noisy due to the low amount of glycan material available from the pool of acidic worm GSL glycans used as starting material. Unfortunately, this sample was exhausted, preventing us from generating a cleaner, more concentrated replicate. However, when more abundant material was available, such as in the case of GSL glycans derived from eggs (Figure S2.C), a life stage yielding significantly higher quantities of acidic GSL glycans, the contaminant peaks became negligible and were no longer detectable.

8. **Figure S5 - not all the glycosidic bonds are easily seen. Figure S8 - refers to a previous study for the O-glycans - however, the basic characteristics of these samples should be stated here (e.g., also AA labelled?). Which entire O-glycan pools are meant in the methods? Were the concentrations defined on the basis of comparison to a standard?**

   The glycosidic bonds represented in Figure S6 (former Figure S5) appear clearly visible to us, and we are uncertain about the issue the reviewer is experiencing.
   Glycan amounts were indeed estimated by comparing UHPLC peak heights to those of RNase B standards. Height values were converted to pmol amount based on calibration curves generated using glycans released from known quantities of RNase B. This information has been added to the caption of now Figure S9 (previously Figure S8) for the reader's reference. We have also edited the M&M section "Glycan array construction and validation" (**L.659(673)-671(689)**) to clarify the points brought to our attention by the reviewer.

9. **Excel file "631032_0_data_set_11107602_sz8r9r" - the "accuracy" varies between the N-and O-glycans - was the MALDI calibrated with an external standard on a daily basis? Are the "null" masses 'junk'? Perhaps there are limitations to what the rather old Glycoworkbench program can find. For the Oglycans, there is a mix of H and Na forms - some have only an H form, which is suspicious – considering the Na-rich release method, finding only Na or having both H and Na seems logical, rather than having some glycans solely as protonated forms.**

   External MALDI calibration was performed using the Bruker Peptide Calibration Standard II (#8206195, Bruker Daltonics) as specified in the M&M (**L.586(601)**) prior to each measurement. Measurements were recorded over several months and years using different MALDI instruments, which likely account for some of the observed variation. This had been omitted in the M&M section and has now been added **L.579(594)-581(596)**. Additionally, while GSL and *N*-glycans were measured in negative-ion mode, *O*-glycans were measured in positive-ion mode.
   The null masses fall into two categories: known non-glycan peaks (DHB matrix or hexose polymer contaminants) and masses to which a plausible glycan composition could not be assigned. GlycoWorkbench, while reliable and widely used, has inherent limitations, in particular the fact that its annotation capabilities are constrained by various user-defined settings including the predefined pool of allowed monosaccharides and ppm threshold. It is unclear whether “null” masses are not annotated because they truly are non-glycan peaks or because the proper monosaccharide were not provided as an input for the software. Of note, peaks with “null” masses largely correspond to low-intensity ions and capturing all relevant glycan signals while avoiding the inclusion of noise is inherently challenging. This is part of our rationale for excluding masses with signal-to-noise ratio below 4 from our MALDI-TOF-MS analysis.
   We thank the reviewer for pointing out the inconsistencies in the *O*-glycan assignments in Table S1. As correctly noted, ions with protonated ($[M+H]^+$) adducts should not have been included in the analysis, since our workflow exclusively yielded sodiated ions ($[M+Na]^+$). We have carefully reexamined Table S1 and revised the annotations of the raw MALDI data accordingly.

10. **Excel file "631032_0_data_set_11107609_sz8r9r" - although the glycan fractions were printed in triplicate, I guess only mean values are shown. No individual data points are shown in the relevant supplementary figures S8-S10. What are the corresponding wet or dry weights of the numbers of eggs, worms, etc (methods and legend to Figure S8)?**

    The reviewer is correct, only mean values of triplicates are shown in Figures S9-S11 (previously Figures S8-S10), as averaging fluorescence intensities is one of the initial data processing steps alongside background correction using the average fluorescence intensity of buffer spots (**see M&M L.697(715)**). Raw data for individual triplicates are available in the GitHub repository (see Data Availability section).

    In Figures S9 (previously S8) and S11 (previously S10), we chose to display averaged MFI (median fluorescence intensity) values to simplify visualization and facilitate interpretation. These figures are intended to provide a clear overview of mAb binding (Figure S9) and Ig binding to specific array

fractions (Figure S11). In Figure S10, however, the heatmap allows visualization of the variation in IgM responses to schistosome glycan antigens from the different individuals.

We did not record the weight of the starting biological material used for glycan microarray construction. However, we believe that the information provided in the manuscript should support experimental reproducibility, as quantification by counting eggs, worms, or cercariae is a standard and widely accepted method in the field for estimating and handling *Schistosoma* material.

11. **The Excel files should be headed in the file with the same Table number as in the text, considering the renumbering by the editorial management system.**

    We regret the confusion caused by the editorial management system's handling of the file names. We appreciate the reviewer's suggestion and have added headers indicating the corresponding table numbers as referenced in the main text in the resubmitted manuscript, in hopes of making the review process more straightforward.

POINT-BY-POINT REPLY TO REVIEWER COMMENTS

Revision of NCOMMS-25-53181A (Major human schistosome species express different glycans with immunological and diagnostic implications.)

Reviewer #1 (Remarks to the Author):

The revised manuscript has addressed several key concerns, namely characterization of the core structure of the GSLs, clarified the limitations of their studies in regard to linkages of sugars, expanded on their insights into the differential antibody responses to glycans previously observed versus their current observations, addressed the limited nature of the study's insights regarding linkages to the genome, clarified the nature of glycosylation in worms isolated from hamsters compared to human hosts, addressed the limitation of the study in regard to lack of glycoproteomic information, provided additional information in Figure S9 regarding structure of glycans recognized by antibodies, revised their discussion regarding the presence of glucuronic acid in glycans, the lack of quantitation of glycan expression, which is a common limitation of current technologies, and the individualistic nature of immune responses to glycan antigens.

Overall, the revised manuscript provides excellent information about glycan structures in these parasites, as well as developmental and species-specific difference in glycosylation.

We are pleased to have been able to provide satisfactory responses to the reviewer's comments, and we would like to thank the reviewer once again for the careful evaluation of our work and for the many constructive suggestions that have substantially improved the manuscript.

Reviewer #2 (Remarks to the Author):

Overall, the manuscript is of interest due to the variations in glycan structures between the two Schistosoma species as well as due to the glycan array data. The data is new, the insights into S. haematobium are novel. The methodology is typical of that from the authors' laboratory. With such a large dataset, it is not possible to assess all files, but spot checks still result in some questions. The authors have revised their manuscript, adding further data and explanations as well as making corrections. My line numbers refer to the 'tracked changes' version which follows the revised manuscript in the single "manuscript" pdf.

We thank the reviewer for confirming the relevance of our work and the continued detailed evaluation of our manuscript in order to achieve further improvements and clarity

Heading - line 77 - "chains" or "oligomers" rather than "stretches"

The section title has been changed from "*S. haematobium* GSL glycans are built on a trihexosyl core extended with β-linked N-acetylhexosamine (HexNAc) stretches" to "*S. haematobium* GSL glycans are built on a trihexosyl core extended with oligomeric chains of β-linked N-acetylhexosamine (HexNAc) residues" (see L.76-77).

Line 93 - if both a general and a specific b-galactosidase worked, then the linkage is Galb1,4Glc and if the a-Gal is not a1,4, then it can be hypothesised that it is Gala1,3Galb1,4Glc (Gala1,6 in an animal would probably be unusual). Possibly in vitro a1,3-galactosylation of lactose with a commercially available enzyme to generate an alternative standard would resolve this in the absence of enough material for linkage analysis - alternatively Elictyl actually sell Isoglobotriose.

We fully concur with the reviewer that the isoglobo series Galα1-3Galβ1-4Glcβ1-Cer is the most likely composition of the core of *S. haematobium* GSL glycans. Thus, we have modified the text accordingly in the results (L.97-102) and discussion (L.361-371).

Line 108 - specify S. pneumoniae in the text, not just the table.

The organism has been specified (L.110-111).

Line 370 - certainly one alpha and one beta galactosyltransferase

"While *S. mansoni* uses a putative β1-4-N-acetylgalactosaminyltransferase to extend the glucosylceramide with GalNAc, one or more galactosyltransferase(s) must be involved to synthesize the trihexosyl core in *S. haematobium*" was changed to "While *S. mansoni* uses a putative β1-4-N-acetylgalactosaminyltransferase to extend the glucosylceramide with GalNAc, *S. haematobium* employs both an α- and a β-galactosyltransferase to synthesize the trihexosyl core." (L. 376-378).

Comment 2: The S/N ratio of 4 and a 1% intensity as a cut-off for defining glycan structures seems arbitrary. Can these larger glycan structures be annotated by careful manual interpretation or are the MS2 spectra uninterpretable? Also, it should be possible to detect and fragment glycans above m/z 3500 using at least the Rapiflex mentioned in the methods. The result of these omissions is an underestimation of the glycomic potential of the organism. Testing interactions of the long LN repeats with either mammalian lectins or antibodies would be of interest; again, it is the authors' assumption that they will be immunologically invisible, which if true is also of interest in terms of 'glycan mimickry'.

Indeed, we applied a criteria of a S/N ratio of 4 and a minimum intensity of 1% to define the cut-off in our MALDI MS analysis. However, these thresholds are not arbitrary but rather were selected because they provide a conservative and robust cut-off for reliably distinguishing true signal from noise in our MALDI MS workflow. We aimed to apply consistent and fair criteria across all samples, and, in our opinion altering this cut-off for a specific sample or glycan class would be difficult to justify without introducing bias and fundamentally affecting the rationale of our study.

We however fully agree with the reviewer that the upper limit of the mass range (normally set at *m/z* 3500) should be extended when the presence of structures with higher molecular weight meeting the aforementioned criteria may be suspected, such as in the case highlighted by the reviewer, in order not to overlook any important glycosylation feature.

To address this, we acquired new mass spectrum with a mass range extended to 5000 Da. Unfortunately, the original sample containing the *N*-glycans released using PNGase A from the *S.*

haematobium mature eggs was exhausted. A fifth of this sample was concentrated using C18 ziptip (as detailed in the M&M section) and subjected to MALDI analysis to obtain the spectrum presented in Figure 3 while the remaining amount was used for exoglycosidase digestions presented in Figure S6-D. Applying a newly generated sample containing *S. haematobium* eggs at different stages of maturity, we detected the structures of composition $H_{5-7}N_{6-8}F_{2-3}X_1$ (*m/z* 2591.1, 2737.1, 2794.2, 2940.2, 3321.1, 3467.0,) previously pointed out by the reviewer, originating from the mature eggs. The resulting spectrum is presented in Figure R. This *N*-glycan series is continued by ions with *m/z* 3623.9, 3670.8, 3920.5, 4082.3 ($H_{7-9}N_8F_{2-3}X_1$) and several high mass structures of composition $H_9N_8F_{2-5}$ (*m/z* 3512.8, 3658.7, 3804.6, 3950.5) were also visible. However, the intensities of these ions only decrease further with increasing molecular weight, indicating that no significant glycans with *m/z* values beyond the mass range applied in this study were overlooked.

We were unable to obtain interpretable MS2 spectra for ions detected in the 2500–5000 m/z range of the *S. haematobium* egg *N*-glycans released using PNGase A, due to their low intensities. Indeed, these ions exhibit low intensities in the range of 600-2000 arbitrary intensity units (a.u.). For comparison, the MS/MS data shown in Figure S6-E were obtained by fragmenting parent ions with intensities between $4x10^4$ and $5x10^4$ a.u.. Rather than being instrumentation-related, this limitation is due to the scarce amount of material available. Mature *S. haematobium* eggs are obtained through a specific separation procedure (detailed in M&M) from mixed-age eggs isolated from infected hamsters. Their quantity is therefore very limited. For this reason, it is also not possible to perform functional experiments to investigate the potential interactions of these particular structures with lectins or (infected) sera, although we fully agree with the reviewer that such studies could be valuable to confirm our suggestion that LN repeats are not relevant from an antigenic point of view. The minimum amount needed of these native isolated glycans of interest for microarray printing (low pmol) is far higher than the obtainable amount.

Although we are unable to further address the structural and antigenic features of this low abundance mature egg-specific glycan subset, we agree with the reviewer that its uniqueness, being exclusively found in mature eggs and released with PNGase A, deserves acknowledgment as it constitutes an additional feature of the parasite glycosylation potential. We hope that the newly added Figure S6-G and the reference in the main text (L.241-243) address this point by showing the specific expression of these structures of higher molecular mass, even though they do not meet our criteria nor the minimally required amount for further quantitative or qualitative study.

Comment 8: Importing from Glycoworkbench does have the tendency to make slightly blurry pictures and one has to really zoom in to distinguish the rather small alphas and betas, which could be added rather with a drawing program for legibility.

We appreciate the reviewer's suggestion to improve the manuscript quality and have added the linkage information using a different program to Glycoworkbench in the following tables and figures: Table 2, Figure S3, Figure S5, Figure S8.

Comment 9: It is indeed good that the authors found the mistakes in the table due to misassignments as protonated ions. The m/z 738 glycan shown in Tab S4 is not found in both replicates, but m/z 779, 1402, 1432, 1475 found in one replicate are not in Tab S4, while m/z 1636 is in both replicates, but is not in Tab S4. Is Tab S4 meant to be a consensus set - if so, then m/z 738 should not feature if it was only found in one experiment, while m/z 1636 should be included. Without me checking: has the Table S1 - Raw MALDI-TOF-MS data been cross-checked with the Tabs S2-S4?

Indeed, data included in Table S4 (and Figure 4) were selected based on the following criteria: ions with S/N > 4, intensities above 1% of the total spectrum intensity, and presence in both technical replicates. The reviewer is correct in noting that the ion at *m/z* 738 does not meet these criteria and should therefore not have been included in Table S4 or Figure 4. Both have now been corrected. Ion with *m/z* 1636, however, although present in both duplicates is less than 1% of total intensity, and thus does not fulfill the cut-off criteria.

Tables S1 and S2–S4 have been thoroughly cross-checked. Table S1 contains the complete dataset, which serves as the basis for the subsets presented in Tables S2–S4 (ions with S/N > 4, intensities above 1% of the total spectrum intensity, and presence in both technical replicates).

POINT-BY-POINT REPLY TO REVIEWER COMMENTS

Revision of NCOMMS-25-53181B (Major human schistosome species express different glycans with immunological and diagnostic implications.)

Reviewer #2:

The authors have again revised their manuscript, adding some 'zoomed' spectra for the higher molecular weight range. Unfortunately, their attempt at another preparation did not work well as the 'only for referees' supplement shows a spectrum full of polyhexose contaminants. Nevertheless a statement could be included in the legend to Supp 6 that: "In experiments with total eggs, there were indications of higher molecular weight N-glycans of approximately 4000 Da, whereby no interpretable MS2 could be obtained to verify whether these had either a fourth antenna or extra LacNAc repeats".

We typically observe polyhexose contaminants when pushing towards the detection limits in MALDI-TOF MS. Despite the presence of these unwanted peaks in the spectrum, it can be still concluded that high molecular weight *N*-glycans are not present in a significant amount that would fall within the study criteria set. To convey the point raised by the reviewer that [minor amounts of] *N*-glycans with *m/z* up to 4000 Da may still be present we included the sentence below in the caption of Figure S6.

"Of note, in experiments in which PNGase A was applied to a mixture of eggs at various stages of maturity, minor ions corresponding to N-glycan structures up to ~4000 Da were observed (data not shown). The lack of interpretable MS2 spectra prevented verification of whether these carried four antennae or polyLacNAc repeats."

As previously indicated, a perfect "corroboration" for the GSL core would be to do PGC-nano-LC-MS with isoglobotriose from Elictyl.

We proceeded with the recommended experiment consisting of running an isoglobotriose standard alongside our biological sample, indeed yielding additional confirmation of the core structure of the *S. haematobium* glycosphingolipid glycans. We have edited Figure S5-A to incorporate the newly acquired data and referd to the GSL glycan standards in the Methods section (lines 637-639). The main text was also slightly modified accordingly in the Results (lines 102-105) and Discussion (lines 354-360) sections.

Line 585 - does the Rapiflex® have a 1 kHz Smartbeam II laser or a 10 kHz one?

We appreciate the reviewer's attention to this discrepancy. Unlike the UltrafleXtreme® and Autoflex® instruments, the Rapiflex® is indeed equipped with a 10 kHz Smartbeam laser. We have updated the Methods section to provide the correct value (lines 590-592).

We thank the reviewer for their valuable suggestions and hope that our revisions satisfactorily address them.