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The multi-scale architecture of mammalian sperm flagella and implications for ciliary motility

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Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this study, the authors use focused-ion beam (FIB) milling coupled with cryo-electron tomography and subtomogram averaging to uncover the structure of the elusive proximal and distal centrioles, as well as different regions of the axoneme in the sperm of 3 mammalian species: pig, horse, and mouse. The in-situ tomograms of the sperm neck region beautifully illustrate the morphology of both the proximal centriole, confirming the partial degeneration of mouse sperm, and intriguingly, asymmetry in the microtubule wall of pig sperm. In distal centrioles, the authors show that in all mammalian species, microtubule doublets of the centriole wall are organized around a pair of singlet microtubules. The presented segmentation of the connecting piece is beautiful and nicely shows the connecting piece forming a nine-fold, asymmetric, chamber the centrioles. The authors further use subtomogram averaging to provide the first maps of the mammalian central pair and identify sperm-specific radial spoke-bridging barrel structures. Lastly, the authors perform further subtomogram averaging to show to the connecting site of the outer dense fibers to the microtubule doublet of the proximal principal piece and confirm the presence of the TAILS microtubule inner protein complex (Zabeo et al, 2018) in the singlet microtubules occupying the tip of sperm tails.
The manuscript provides the clearest insight into flagellar base morphology to date, giving insight into the morphological difference between different mammalian cilia and centriole types. The manuscript is suitable for publication, once the following questions are addressed.

Major Points:
How many centrioles and axonemes were used in generating the averages presented in the paper? If too few samples were used, especially in centrioles undergoing dramatic remodeling or degeneration, the reality of MIPs and MAPs being present might be completely affected. For instance, In figure 1d, the authors present a cryoET map of the centriole microtubule triplet. However, centrioles are divided into several regions with different accessory elements. Here, the authors could show the presence of only part of the A-C linker. The A-C linker covers only 40% of the centriole, so does it mean that this

centriole is made only of the accessories that characterize the proximal side of the centriole? In the same line, what were the boundaries governing subtomogram extraction? For example, in the distal centriole, were microtubules extracted from just before the start of the transition zone, to the end of the microtubule vaulting, more pronounced at the end of the proximal region? There are known heterogeneities in centriole, as well as flagella, ultrastructure along the proximal distal axis. If no pre-classification was performed for subtomogram longitudinal position along with the centriole and axoneme, structural features may be averaged out, and or present and not reflecting their real longitudinal localization. The classification should be applied here if it was not the case.

Minor Points:
- In line 3, motile cilia are not only used to swim, they can move liquid or mucus for instance.
- In line 175, the authors stated " a prominent MIP associated with protofilament A9, was also reported in centrioles isolated from CHO cells (Greenan et al. 2018) and in basal bodies from bovine respiratory epithelia (Greenan et al 2020). Actually, this MIP has been seen in many other centrioles from other species, such as Trichonympha (https://doi.org/10.1016/j.cub.2013.06.061), Chlamydomonas, and Paramecium (DOI: 10.1126/sciadv.aaz4137). Citing these studies will reinforce the evolutionary conservation of this MIP and therefore its potential crucial role in the A microtubule.
- In Line178, the authors stated: "Protofilaments A9 and A10 are proposed to be the location of the seam (Ichikawa et 2017)". High-resolution cryoEM maps confirmed it: https://doi.org/10.1016/j.cell.2019.09.030 . This publication should be cited. Moreover, authors should also refer to this paper when discussing MIPs in the microtubule doublet.
- In Line 187-189 the authors stated, "We resolved density of the A-C linker (gold) which is associated with protofilaments C9 and C10." The A-C linker interconnects the triplets of the proximal centriole (Guichard et. al. 2013, Li et. al. 2019, Klena et. al. 2020) with distinct regions binding the C-tubule, as shown by the authors in gold, as well as an A-link, making contact with the A-tubule through various protofilaments in a species-specific manner, but always on protofilament A9. The authors may have identified the A-link, labeled in green, on the outside of protofilament A8/A9 in Figure 1d.
- In figure 1e, the authors provide a 9-fold representation of the centriole based on their map. How relevant is this model ? the distance between triplet is inconsistent here, which has not been observed before. Do they use true 3D coordinates to generate this model? The A-C linker, which is only partially reconstructed, does not contact the A microtubule. Is it really the case? did the authors see that the A-link density of the A-C linker has disappeared? If these points are not clearly specified, this representation might be misleading.
- The nomenclature regarding MIPs is sometimes confusing in this manuscript. For example, in lines 228-229 "We then determined the structure of DC doublets, revealing the presence of MIPs distinct from those in the PC." Does this include the gold and turquoise labeled structures in Figure 2j? These densities appear to correspond to the inner scaffold stem in the gold density presented in Figure 2j, and armA, presented in the turquoise density (Li et. al. 2011, Le Guennec et. al. 2020). The presence of this Stem here is important as it correlates with the presence of the molecular player making the inner scaffold (POC5, POC1B, CENTRIN): https://doi.org/10.1038/s41467-018-04678-

- The connecting piece is composed of column vaults emanating from the striated columns is compelling and beautiful segmentation data. However, it is important to note how many pig sperm proximal centrioles had immediate-short triplet side contact with the Y-shaped segmented column 9, as well as in how many mouse centrioles have the two electron-dense structures flanking the striated columns.

The resolution of the mammalian central pair is an important development brought by this work. The structural similarity between the central pair of pig and horse is convincing. However, with only 281 subtomograms being averaged for the murine central pair, corresponding to an estimated resolution of 49Å, the absence of the helical MIP of C1 with 8 nm periodicity suggests that there is simply not enough signal to capture it in the average. The same could be said for the smaller MIP displayed in Figure 4 c, panel ii. This point should be clearly stated.

Another piece of compelling data presented in this study is the attachment of the outer dense fibers to the axoneme of the midpiece and proximal and distal principal pieces. From the classification data presented along the flagellar length, it is clear that the only ODF contact made with the axoneme is at the proximal principle plate. However, this is far from obvious in the native top view images presented. Is it possible to include a zoomed inset of the connection between the A-tubule and ODF connection?

3. Significance:

Significance (Required)

This work is of good quality and provides crucial information on the structure of centriole and axoneme in 3 different species. This work complements well the previous works.
The audience for this type of study is large as it is of interest to researchers working on centrioles, cilium, and sperm cell architecture.

My expertise is cryo-tomography and centriole biology

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this study, Leung et al. used state-of-the-art EM imaging techniques, including FIB cryo-milling, Volta Phase plate, cryo-electron tomography and subtomogram averaging, to study the structure of sperm flagella from three mammalian species, pig, horse and mouse. First, they described two unique centrioles in the sperm, the PC and the DC. They found the PCs are composed of a mixture of triplet and doublet MTs. In contrast, the DCs are composed mainly of doublet and singlet MTs. By using subtomogram averaging, they identified a number of accessory proteins, including many MIPs bound to the MT wall. Many are unique to the mammalian sperm. They further described the connecting piece region of the sperm enclosing the centrioles and found an asymmetric arrangement. Furthermore, the authors presented the structure of sperm axonemes from all three species. These include the DMT and the CPA. Finally, they described the tail region of the sperm and described how the DMTs transitioned to the singlet MTs.

This is a beautiful piece of work! It is by far the most comprehensive structural study of mammalian sperm cells. These findings will serve as a valuable resource for structure and function analysis of the mammalian flagella in the future. Now the stage is set for identifying the molecular nature of the structures and densities described in this study.

The manuscript is clearly written. The data analysis is thorough. The conclusions are solid and not overstated. I don't have any major issues for its publication. A number of minor suggestions are listed below. Most are related to the figures and figure legends.

Figure 1d, the figure legend should mention this is the subtomogram average of PC triplet MTs from pig sperm, though this is mentioned in the text. Also, for convenience, the color codes for the MIPs should be mentioned in the figure legend.

Figure 2J, similarly, the figure legend should mention this is the subtomogram average of DC doublets. It also needs a description of the color codes of the identified MIPs. For the DMT, please indicate the A- and B-tubule, which are colored in light or dark blue.

Line 228, "We then determined the structure of DC doublet by subtomogram averaging"

For both Fig 2 and Fig 3. the DC doublets are colored in dark and light blue, please specify which is the A- or B-tubule in the figure legends.

Line 273, need space between "goldenrod"

Figure 4. need to expand the figure legend. Panels I, ii, iii, iv, are cut-through view of the lumen of CPA microtubules C1 and C2.

Line 338, Interestingly, the RS1 barrel is radially distributed asymmetrically around the axoneme

Figure 5, need color codes for the arrowheads (light pink, pink, magenta) in panels i~n,

Figure 7, (a-c) please use arrowheads to indicate the location of caps in the singlet MT.

3. Significance:

Significance (Required)

This is a beautiful and significant work - by far the most comprehensive analysis of mammalian sperm structure

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This is a very interesting study that explores the structural diversity of mammalian sperm flagella, in pig, mouse and horse, at high resolution using cryo-FIB milling and cryo-tomography. The study provides the first in situ cryo-EM structure of a mammalian centriole and describes a number of microtubule associated structures, such as MIPs and plugs at the plus-end of microtubules, that were not been reported so far. Additionally, the authors identify several asymmetries in the overall structure of the flagellum in the three species, which have implications for the understanding of the flagellar beat and waveform geometry in sperm, which are discussed by the authors. Although this study does not provide mechanistic novel information on the function of the described structures, it will undoubtedly serve as a reference for future theoretical and empirical work on the role of these structures in shaping the flagellar beat.
With the exception of a couple of "eclectic word choices" in the Introduction (see detailed feedback in Minor Comments), the manuscript is also well written. Image acquisition and analysis are sound.

However, I have some suggestions that should help the authors to strengthen their claims and present their results. The study is in principle suitable to be published, after the following points will be addressed:

Major comments:

- A major concern is that it is not clear how many animals, sperms and lamellae the authors used to acquire the data presented in the manuscript. This information needs to be provided, because it not uncommon to encounter aberrant flagella, even in a wildtype animal. The authors should state how many animals, and how many flagella per each animal were analyzed, in order to allow the reader to have an opinion on the reliability of their observations.
- The figures are esthetically pleasing; however, the figures legends should be carefully revised to include necessary information about color codes, image annotations.

Minor comments:
- Line 26. I do not think that the word "menagerie" is properly used in this context.
- Line 29. The same is true for the word "Bewildering" in this sentence.
- Line 286 "Our structures of the CPA are the first from any mammalian system, and our structures of the doublets are the first from any mammalian sperm, thus filling crucial gaps in the gallery of axoneme structures." Sentences like this one would fit much better in the Conclusions or at least in the Discussion.
- Line 377 "Large B-tubule MIPs have so far only been seen in human respiratory cilia (Fig. 5j) and in Trypanosoma (the ponticulus, Fig. 5n), but the morphometry of these MIPs differs from the helical MIPs in mammalian sperm." Please insert the citations for the studies about respiratory cilia and Trypanosoma flagella.
- In Figure 1. What do the stars shown in panel a and a' indicate?
Given the complexity of the structures that compose the flagellar system of sperms, it would be helpful to add an illustration of the sperm with careful annotation of the centriole structures and the various segments of the flagellum.
- Figure 2. Explanation of the used color codes is missing. Additionally, the authors should include an explanation for the black and white arrows and for the 2 insets in i.
- In "(j) In situ structure of the pig sperm DC with the tubulin backbone in grey and microtubule inner protein densities colored individually" ...it should be written "...sperm DC microtubule doublet..."
- In this figure, but also in every other figure that shows centriole, axoneme, or even microtubule averages it is important to indicate the microtubule polarity. Please add the symbol + and - to indicate microtubule polarity in the figures.
- Figure 3. Additional to the images in a,b, and c, the original tomographic slices (without segmentation) should be shown here, to allow the reader to visualize the structure.
- Figure 7. Scale bars are missing in d-f.
- Scale bars are missing in most Supplementary figures.
- Table S1. The Information about horse and mouse centriole data is missing.

3. Significance:

Significance (Required)

This study provides several novel structural insights in to the sperm flagellum structure that have implications for the understanding of the flagellar beat and waveform geometry in sperm. Although this study does not provide mechanistic novel information on the function of the described structures, it will undoubtedly serve as a reference for future theoretical and empirical work on the role of these structures in shaping the flagellar beat.

Overview of changes

We thank the ReviewCommons editorial team for facilitating a fair review process. We thank all the reviewers for taking the time to carefully evaluate our manuscript. We feel that all reviewers genuinely appreciated the work, and also provided concrete suggestions for improving this paper. In particular, all reviewers agree that our work is 'beautiful and significant work - by far the most comprehensive analysis of mammalian sperm structure'. Providing the 'clearest insight into flagellar base morphology to date'. All reviewers appreciated that our manuscript is packed with novel observations on mammalian centrioles and flagella that 'have implications for the understanding of the flagellar beat and waveform geometry in sperm'. Furthermore, the reviewers agree that our data 'will undoubtedly serve as a reference for future theoretical and empirical work on the role of these structures in shaping the flagellar beat'.

All reviewers agree that the manuscript is well written and (almost) ready for publication and is relevant for broad audience: 'the audience for this type of study is large as it is of interest to researchers working on centrioles, cilium, and sperm cell architecture'.

To address reviewers 1 and 3 request for more information about the number of animals, cells, and tomograms used to generate the averages. We added this information for each average in Table S1. For more qualitative observations, we include the numbers in the material and methods (see detailed response to Reviewer 1).

Reviewer 1 requested clarification on the boundaries for subtomogram extraction, given known variations along the proximodistal axes of ciliary assemblies. We now clearly state precisely which regions of the centriole/axoneme each average comes from, both in the main text and in the figure legends.

All reviewers had suggestions to improve the figures and text, which we addressed as thoroughly as we could. The exact changes are detailed below in the responses to individual reviewers.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this study, the authors use focused-ion beam (FIB) milling coupled with cryo-electron tomography and subtomogram averaging to uncover the structure of the elusive proximal and distal centrioles, as well as different regions of the axoneme in the sperm of 3 mammalian species: pig, horse, and mouse. The in-situ tomograms of the sperm neck region beautifully illustrate the morphology of both the proximal centriole, confirming the partial degeneration of mouse sperm, and intriguingly, asymmetry in the microtubule wall of pig sperm. In distal centrioles, the authors show that in all mammalian species, microtubule doublets of the centriole wall are organized around a pair of singlet microtubules. The presented segmentation of the connecting piece is beautiful and nicely shows the connecting piece forming a nine-fold, asymmetric, chamber the centrioles. The authors further use subtomogram averaging to provide the first maps of the mammalian central pair and identify sperm-specific radial spoke-bridging barrel structures. Lastly, the authors perform further subtomogram averaging to show to the connecting site of the outer dense fibers to the microtubule doublet of the proximal principal piece and confirm the presence of the TAILS microtubule inner protein complex (Zabeo et al, 2018) in the singlet microtubules occupying the tip of sperm tails.

The manuscript provides the clearest insight into flagellar base morphology to date, giving insight into the morphological difference between different mammalian cilia and centriole types. The manuscript is suitable for publication, once the following questions are addressed.

We are ecstatic that the reviewer shares our enthusiasm for this work. We are particularly grateful that the reviewer appreciates the significance of the unique, and hitherto under-explored biology of the sperm centrioles and the flagellar base.

Major Points:

How many centrioles and axonemes were used in generating the averages presented in the paper? If too few samples were used, especially in centrioles undergoing dramatic remodeling or degeneration, the reality of MIPs and MAPs being present might be completely affected. For instance, In figure 1d, the authors present a cryoET map of the centriole microtubule triplet. However, centrioles are divided into several regions with different accessory elements. Here, the authors could show the presence of only part of the A-C linker. The A-C linker covers only 40% of the centriole, so does it mean that this centriole is made only of the accessories that characterize the proximal side of the centriole? In the same line, what were the boundaries governing subtomogram extraction? For example, in the distal centriole, were microtubules extracted from just before the start of the transition zone, to the end of the microtubule vaulting, more pronounced at the end of the proximal region? There are known heterogeneities in centriole, as well as flagella, ultrastructure along the proximal distal axis. If no pre-classification was performed for subtomogram longitudinal position along with the centriole and axoneme, structural features may be averaged out, and or present and not reflecting their real longitudinal localization. The classification should be applied here if it was not the case.

These are all valid points. Because there is no easy way to target the PC/DC when cryo-FIB milling, and because there is only one of each structure in every cell, the chances of catching them in ~150-nm-thin lamellae are slim (not to mention the number of things that can and do go wrong when doing cryo-ET on lamellae). As such, the averages of the PC were generated from 3 tomograms (3 cells) and those of the DC from 2 tomograms (2 cells).

We do have more tomograms with the PC/DC, but these were used for segmentation/visual inspection since we only used the best tomograms for averaging. These numbers are not entirely atypical for cryo-FIB datasets; the only other *in situ* centriole structures are from 5-6 centrioles (from *Chlamydomonas*, from Le Guennec et al 2020 doi: 10.1126/sciadv.aaz4137 and Klena et al 2020 doi: 10.15252/embj.2020106246).

To allow readers to adjust their interpretations according to the small number of cells analysed, we explicitly stated the number of animals/cells/tomograms used to generate averages in Table S1. Furthermore, we amended the text to clarify which regions of the centrioles our averages represent. These changes are detailed below:

(1) proximal centriole

The lamellae used for averaging PC triplets caught mostly the proximal end of the centriole, and essentially all of the particles come from the most proximal ~ 400 nm. In a sense, this was a form of pre-classification. We now state explicitly that our structure represents only the proximal region and that proximal/distal differences may be identified in the future (see section on distal centriole below). Despite the limited particle number, we are confident in the presence of the MIPs as these are also visible in the raw data (the striations in Fig. 1a, now Fig. 1d, for instance). Page 7, Line 165 was edited accordingly as well as the legend to Fig. 1.

(2) distal centriole

The subtomograms used for the DC average were extracted from the region of the distal centriole closest to the base of the axoneme (i.e; the region marked “distal centriole” in Fig. 2h-i). Because the DC doublet average in Fig. 2j was generated from very few particles, we tried to be very conservative when interpreting it. Page 9, Line 216 was edited accordingly likewise the legend to Fig. 2.

(3) axoneme

We did attempt to average the axoneme from different regions of flagella (midpiece, proximal principal piece, distal principal piece). This is shown in Fig. 6d-l. The major difference we found was at the doublet-ODF connection. We did not find any striking differences in MIP densities, or in radial spoke densities along the proximodistal axis. As such, the averages in Fig. 5 are from the entire principal piece (but not the midpiece), which we state in the figure legend.

Because mammalian sperm flagella are very long, it *is* possible that we missed more subtle differences. We now state this in the Discussion (page 20, line 491):

Minor Points:

- In line 3, motile cilia are not only used to swim, they can move liquid or mucus for instance.

Done. Page 3, line 64

- In line 175, the authors stated " a prominent MIP associated with protofilament A9, was also reported in centrioles isolated from CHO cells (Greenan et al. 2018) and in basal bodies from bovine respiratory epithelia (Greenan et al 2020). Actually, this MIP has been seen in many other centrioles from other species, such as Trichonympha (https://doi.org/10.1016/j.cub.2013.06.061), Chlamydomonas, and Paramecium (DOI: 10.1126/sciadv.aaz4137). Citing these studies will reinforce the evolutionary conservation of this MIP and therefore its potential crucial role in the A microtubule.

We thank the reviewer for pointing out these very important papers, we added them to the manuscript (page 7, lines 175-176).

- In Line178, the authors stated: "Protofilaments A9 and A10 are proposed to be the location of the seam (Ichikawa et 2017)". High-resolution cryoEM maps confirmed it: https://doi.org/10.1016/j.cell.2019.09.030 . This publication should be cited. Moreover, authors should also refer to this paper when discussing MIPs in the microtubule doublet.

Done (page 7, lines 178-179 and page 13, line 329).

We also now cite Ma et al (along with Ichikawa et al 2019 doi: 10.1073/pnas.1911119116 and Khalifa et al 2020 doi: 10.7554/eLife.52760) in the Discussion when alluding to high-resolution structures as a possible means of identifying MIPs (page 19, lines 479).

- In Line 187-189 the authors stated, "We resolved density of the A-C linker (gold) which is associated with protofilaments C9 and C10." The A-C linker interconnects the triplets of the proximal centriole (Guichard et. al. 2013, Li et. al. 2019, Klena et. al. 2020) with distinct regions binding the C-tubule, as shown by the authors in gold, as well as an A-link, making contact with the A-tubule through various protofilaments in a species-specific manner, but always on protofilament A9. The authors may have identified the A-link, labeled in green, on the outside of protofilament A8/A9 in Figure 1d.

We thank the reviewer for pointing this out. The position of the olive green density associated with A8/A9 is indeed consistent with the A-link, and this is also now illustrated more clearly in the new version of Fig. 1e (now Fig. 1h, see below). We accordingly edited page 8, lines 187-188.

- In figure 1e, the authors provide a 9-fold representation of the centriole based on their map. How relevant is this model ? the distance between triplet is inconsistent here, which has not been observed before. Do they use true 3D coordinates to generate this model? The A-C linker, which is only partially reconstructed, does not contact the A microtubule. Is it really the case? did the authors see that the A-link density of the A-C linker has disappeared? If these points are not clearly specified, this representation might be misleading.

In order to avoid misleading readers, we replaced this panel with a model generated directly by plotting back the averages into their original positions and orientations in the tomogram (new Fig. 1h). This model now shows that the olive green density on A8/A9 is in the right position to form part of the A-C linker (as Reviewer 1 correctly pointed out in their previous point). We have amended the figure legend accordingly. We also described how the plotback was generated in the Materials and Methods section (page 26, line 648).

As the reviewer points out, the distance between triplets does indeed seem inconsistent in the plotback. This is an interesting observation, but we feel it is a bit too preliminary to discuss in detail here. This can be explored in a follow-up study more focused on sperm centriole geometry.

- The nomenclature regarding MIPs is sometimes confusing in this manuscript. For example, in lines 228-229 "We then determined the structure of DC doublets, revealing the presence of MIPs distinct from those in the PC." Does this include the gold and turquoise labeled structures in Figure 2j? These densities appear to correspond to the inner scaffold stem in the gold density presented in Figure 2j, and armA, presented in the turquoise density (Li et. al. 2011, Le Guennec et. al. 2020). The presence of this Stem here is important as it correlates with the presence of the molecular player making the inner scaffold (POC5, POC1B, CENTRIN): https://doi.org/10.1038/s41467-018-04678-8

While we were initially very conservative with interpreting the DC doublet average (as stated above it comes from very few particles), we agree with the reviewer's assessment that the gold and turquoise densities in Fig. 2j are consistent with the Stem and armA respectively of the inner scaffold. Because the inner scaffold contributes to centriole rigidity, it will be

interesting to determine if and how it changes during remodelling of the atypical DC in mammalian sperm. Intriguingly, at least some inner scaffold components (including POC5, POC1B) reorganise into two rods in the mammalian sperm DC (Fishman et al 2018 doi: 10.1038/s41467-018-04678-8). We expanded the section on the DC average (page 9, lines 218-220):

- The connecting piece is composed of column vaults emanating from the striated columns is compelling and beautiful segmentation data. However, it is important to note how many pig sperm proximal centrioles had immediate-short triplet side contact with the Y-shaped segmented column 9, as well as in how many mouse centrioles have the two electron-dense structures flanking the striated columns.

Done. Material and Methods Page 25, lines 615-619.

The resolution of the mammalian central pair is an important development brought by this work. The structural similarity between the central pair of pig and horse is convincing. However, with only 281 subtomograms being averaged for the murine central pair, corresponding to an estimated resolution of 49Å, the absence of the helical MIP of C1 with 8 nm periodicity suggests that there is simply not enough signal to capture it in the average. The same could be said for the smaller MIP displayed in Figure 4 c, panel ii. This point should be clearly stated.

We agree with the reviewer that the quality of the mouse CPA structure is not on par with the pig and horse CPA structures. We now explicitly state this caveat in the text (pages 11, lines 276-277):

Another piece of compelling data presented in this study is the attachment of the outer dense fibers to the axoneme of the midpiece and proximal and distal principal pieces. From the classification data presented along the flagellar length, it is clear that the only ODF contact made with the axoneme is at the proximal principle plate. However, this is far from obvious in the native top view images presented. Is it possible to include a zoomed inset of the connection between the A-tubule and ODF connection?

We are very happy that the reviewer finds this data exciting. As Fig. 6 is quite cluttered as is, we instead tried to better annotate the cross-section views of the axoneme by tracing one doublet-ODF pair in each image (or only a doublet in the case of the distal principal piece). This shows that there is a gap between the doublet and the ODF in the midpiece, and that there is no such gap in the principal piece. We also hope that annotating one doublet-ODF pair helps the reader see that the same pattern holds true for the other doublets/ODFs. The legend to Fig. 6 was changed accordingly.

Reviewer #1 (Significance (Required)):

This work is of good quality and provides crucial information on the structure of centriole and axoneme in 3 different species. This work complements well the previous works.

The audience for this type of study is large as it is of interest to researchers working on centrioles, cilium, and sperm cell architecture.

We are pleased the reviewer appreciate the quality of our work and see the interest for broad audience.

My expertise is cryo-tomography and centriole biology

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this study, Leung et al. used state-of-the-art EM imaging techniques, including FIB cryo-milling, Volta Phase plate, cryo-electron tomography and subtomogram averaging, to study the structure of sperm flagella from three mammalian species, pig, horse and mouse. First, they described two unique centrioles in the sperm, the PC and the DC. They found the PCs are composed of a mixture of triplet and doublet MTs. In contrast, the DCs are composed mainly of doublet and singlet MTs. By using subtomogram averaging, they identified a number of accessory proteins, including many MIPs bound to the MT wall. Many are unique to the mammalian sperm. They further described the connecting piece region of the sperm enclosing the centrioles and found an asymmetric arrangement. Furthermore, the authors presented the structure of sperm axonemes from all three species. These include the DMT and the CPA. Finally, they described the tail region of the sperm and described how the DMTs transitioned to the singlet MTs.

This is a beautiful piece of work! It is by far the most comprehensive structural study of mammalian sperm cells. These findings will serve as a valuable resource for structure and function analysis of the mammalian flagella in the future. Now the stage is set for identifying the molecular nature of the structures and densities described in this study.

We thank the reviewer for their positive evaluation! We are very happy that they share our excitement for the work, and that they also see it as “setting the stage” for future studies at the molecular level.

The manuscript is clearly written. The data analysis is thorough. The conclusions are solid and not overstated. I don't have any major issues for its publication. A number of minor suggestions are listed below. Most are related to the figures and figure legends.

Figure 1d, the figure legend should mention this is the subtomogram average of PC triplet MTs from pig sperm, though this is mentioned in the text. Also, for convenience, the color codes for the MIPs should be mentioned in the figure legend.

Done.

Figure 2J, similarly, the figure legend should mention this is the subtomogram average of DC doublets. It also needs a description of the color codes of the identified MIPs. For the DMT, please indicate the A- and B-tubule, which are colored in light or dark blue.

Done, except we would prefer not to enumerate the MIPs as we did not name them nor discuss them extensively in the main text as we do not want to over-interpret the MIPs at this point as the average is from relatively small number of particles. However, we did specify that the gold and turquoise densities on the luminal surface are consistent with the inner scaffold. The figure legend was edited accordingly.

Line 228, "We then determined the structure of DC doublet by subtomogram averaging"

Done.

For both Fig 2 and Fig 3. the DC doublets are colored in dark and light blue, please specify which is the A- or B-tubule in the figure legends.

Done.

Line 273, need space between "goldenrod"

We would prefer to keep “goldenrod” spelled as is since this is how the color is referred to in Chimera and ChimeraX.

Figure 4. need to expand the figure legend. Panels I, ii, iii, iv, are cut-through view of the lumen of CPA microtubules C1 and C2.
Done.

Line 338, Interestingly, the RS1 barrel is radially distributed asymmetrically around the axoneme

Done.

Figure 5, need color codes for the arrowheads (light pink, pink, magenta) in panels i~n,

Done.

Figure 7, (a-c) please use arrowheads to indicate the location of caps in the singlet MT.

Done.

Reviewer #2 (Significance (Required)):

This is a beautiful and significant work - by far the most comprehensive analysis of mammalian sperm structure

We are thrilled the reviewer appreciate the novelty of our work.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

This is a very interesting study that explores the structural diversity of mammalian sperm flagella, in pig, mouse and horse, at high resolution using cryo-FIB milling and cryo-tomography. The study provides the first in situ cryo-EM structure of a mammalian centriole and describes a number of microtubule associated structures, such as MIPs and plugs at the plus-end of microtubules, that were not been reported so far. Additionally, the authors identify several asymmetries in the overall structure of the flagellum in the three species, which have implications for the understanding of the flagellar beat and waveform geometry in sperm, which are discussed by the authors. Although this study does not provide mechanistic novel information on the function of the described structures, it will undoubtedly serve as a reference for future theoretical and empirical work on the role of these structures in shaping the flagellar beat.

With the exception of a couple of "eclectic word choices" in the Introduction (see detailed feedback in Minor Comments), the manuscript is also well written. Image acquisition and analysis are sound.

We thank the reviewer for positively evaluating our work. We are glad that they feel our study will “serve as a reference” to inform future studies.

However, I have some suggestions that should help the authors to strengthen their claims and present their results. The study is in principle suitable to be published, after the following points will be addressed:

Major comments:

- A major concern is that it is not clear how many animals, sperms and lamellae the authors used to acquire the data presented in the manuscript. This information needs to be provided, because it not uncommon to encounter aberrant flagella, even in a wildtype animal. The authors should state how many animals, and how many flagella per each animal were analyzed, in order to allow the reader to have an opinion on the reliability of their observations.

- The figures are esthetically pleasing; however, the figures legends should be carefully revised to include necessary information about color codes, image annotations.

We thank the reviewer for raising these points. We completely agree that the numbers of animals and cells are important pieces of information. As such, we now explicitly state the number of animals/cells/tomograms used for each average in Table S1. For more qualitative observations (such as the relationship between the asymmetry of the pig sperm PC and the Y-shaped segmented columns), we now state in the number of cells and animals in which we see each feature (see detailed response to Reviewer 1).

Minor comments:

- Line 26. I do not think that the word "menagerie" is properly used in this context.

- Line 29. The same is true for the word "Bewildering" in this sentence.

We apologise for our somewhat eclectic word choice. We see the reviewer's point that unconventional word choice may distract readers, so we replaced these two words with 'diverse' and 'an extensive', respectively.

- Line 286 "Our structures of the CPA are the first from any mammalian system, and our structures of the doublets are the first from any mammalian sperm, thus filling crucial gaps in the gallery of axoneme structures." Sentences like this one would fit much better in the Conclusions or at least in the Discussion.

We thank the reviewer for this suggestion, but we would prefer to keep this sentence where it is, if possible. We think it is useful to tell the audience upfront why these structures are significant, especially since readers who aren't deep in the field may be bogged down by all the details.

- Line 377 "Large B-tubule MIPs have so far only been seen in human respiratory cilia (Fig. 5j) and in Trypanosoma (the ponticulus, Fig. 5n), but the morphometry of these MIPs differs from the helical MIPs in mammalian sperm." Please insert the citations for the studies about respiratory cilia and Trypanosoma flagella.

Done.

- In Figure 1. What do the stars shown in panel a and a' indicate?

We indeed failed to specify what the asterisks/stars indicate. They are meant to emphasise that the electron-dense material in the lumen of the PC is continuous with the CP. We have now specified this in the text (page 10, lines 245).

Given the complexity of the structures that compose the flagellar system of sperms, it would be helpful to add an illustration of the sperm with careful annotation of the centriole structures and the various segments of the flagellum.

This is an excellent suggestion. To help orient readers, we added three panels to Fig. 1 (Fig. 1a-c) showing low-magnification images of whole sperm cells. We annotated different parts of the flagellum (neck, midpiece, principal piece, endpiece) so that readers can refer back to these panels in case they want to know which part of the cell the averages are from.

- Figure 2. Explanation of the used color codes is missing. Additionally, the authors should include an explanation for the black and white arrows and for the 2 insets in i.

Done. For the color code, please see response to Reviewer 2. For the black and white arrows, we edited the figure legend.

- In "(j) In situ structure of the pig sperm DC with the tubulin backbone in grey and microtubule inner protein densities colored individually" ...it should be written "...sperm DC microtubule doublet..."

Done.

- In this figure, but also in every other figure that shows centriole, axoneme, or even microtubule averages it is important to indicate the microtubule polarity. Please add the symbol + and - to indicate microtubule polarity in the figures.

Done. In order to avoid overcrowding, we only labelled the pig structures as the horse and the mouse structures are always shown in the same orientations as the pig.

- Figure 3. Additional to the images in a,b, and c, the original tomographic slices (without segmentation) should be shown here, to allow the reader to visualize the structure.

We now include three additional supplementary movies slicing through the respective tomograms.

- Figure 7. Scale bars are missing in d-f.

Done.

- Scale bars are missing in most Supplementary figures.

Done.

- Table S1. The Information about horse and mouse centriole data is missing.

The reviewer is correct, but this information is missing because we did not average from the horse and the mouse. For the mouse, the triplets were in various stages of degeneration, resulting in heterogeneity that precluded us from averaging. For the horse, we simply did not catch enough centrioles to generate a meaningful structure.

Reviewer #3 (Significance (Required)):

This study provides several novel structural insights in to the sperm flagellum structure that have implications for the understanding of the flagellar beat and waveform geometry in sperm. Although this study does not provide mechanistic novel information on the function of the described structures, it will undoubtedly serve as a reference for future theoretical and empirical work on the role of these structures in shaping the flagellar beat.

Great to see the reviewer appreciate the novelty of our work.

1st Editorial Decision 30th Nov 2020

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have now read your manuscript, the reviewer comments and your response to them. Based on our editorial assessment and the referees' positive evaluations, I would like to invite you to submit a revised version of the manuscript.

I understand that the manuscript you have submitted to us has already been revised according to the reviewer instructions, but the invitation to revise is a formal and technical requirement from our side to be able to resume the review process. Moreover, we routinely perform an initial quality control on all revised manuscripts before re-review, for which we require the files to be in a specific format. Please revise your manuscript according to the instructions that follow below.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

The authors performed the requested changes.

1st Revision - Editorial Decision 26th Jan 2021

Thank you for submitting a revised version of your manuscript. I apologise for the delay in the processing of your manuscript due to the unusually high number of submissions to our office at the moment. Your revised study has now been seen by one of the original referees, who finds that their main concerns have been addressed and recommend publication of the manuscript. There now remain only a few minor editorial issues that have to be clarified before I can extend the official acceptance of the manuscript.

Referee #1:

I took pleasure in rereading this article with the changes/corrections made. The additional panels and extended figures added to the manuscript add clarity to the microtubule-associated structures of the basal body/flagellar tails and MIPs, and are aesthetically quite beautiful. The inclusion of the number of animals, cells, and tomograms imaged and analyzed also facilitates interpretation of the data, and is appreciated. Furthermore, the display of outer dense fiber connection to the axoneme from raw tomograms, presented in figure 6d-l, is very elegant and clear. This manuscript represents a large advancement in the knowledge of mammalian sperm centrioles and flagella. I recommend this manuscript for publication in EMBO Journal.

2nd Authors' Response to Reviewers 10th Feb 2021

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 12th Feb 2021

Editor accepted the manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: TZVIYA ZEEV-BEN-MORDEHAI
Journal Submitted to: THE EMBO JOURNAL
Manuscript Number: EMBOJ-2020-107410

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	N/A
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	N/A
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
Is there an estimate of variation within each group of data?	No, we are reporting qualitative observations and make only qualitative comparisons between structures.

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	We did not perform any statistical comparisons between groups.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	N/A
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Pigs (Sus scrofa) were commercial artificial insemination boars from the company Varkens KI Nederland. Horses were Warmblood stallions attending the Faculty of Veterinary Medicine at Utrecht University for breeding soundness examination. Mice were adult male C57BL/6 housed under standard conditions at the Utrecht University animal facility.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Experiments were not performed directly on animals.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Subtomogram averages have been deposited to the EMDB and have the following accession codes: EMD-12067, 12068, 12069, 12070, 12071, 12072, 12076, 12077, 12078, 12079, 12131, 12132, 12133, 12134, 12135, 12136.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A