nature portfolio

Peer Review File

Four camera eyes in the earliest vertebrates from the Cambrian

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Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Lei et al. present evidence that ancestral vertebrates contained two sets of camera eyes, one lateral and the other medial. Previous studies interpreted the medial sensory organs as nasal, possibly arising from paired olfactory placodes. Here, the authors reinterpret the fossil record to argue that they correspond to a second set of eyes.
The authors analyzed six specimens of Haikouichthys and four slabs of myllokunmingid fossils. They applied sophisticated imaging technologies (EDS, Raman spectroscopy, and XPS) to provide evidence that the two sets of pigmented sensory organs contain microbodies—melanin-containing melanosomes. ToF-Mass spec methods provide further support for the occurrence of melanin-containing melanosomes in both sets of sensory organs. This is a critical feature of camera eyes. The ovoid and spherical organizations of melanosomes are also consistent with this possibility.
The lens is another key property of camera eyes, and here the arguments are not quite as compelling as the case for melanosomes. Different indentations are interpreted as lenses, but the identification is not definitive. Any additional methods that could be used to strengthen these arguments would be a nice addition to the study.
The authors argue for a deep evolutionary and developmental connection between camera eyes and the pineal complex of fish and other vertebrates. Both organs arise from the same region of the anterior neural plate. There is quite a lot of published speculation regarding the relationship of these sensory cell types. In most scenarios, image-forming camera eyes arise from light-sensing pineal cells. Here, the authors envision a retrograde scenario, whereby their newly discovered medial eyes have degenerated into the pineal complex. Totally plausible, but many of their arguments are highly speculative and should be pruned and sharpened in a revised text.
Likewise, the arguments of whole-genome duplications and the subfunctionalization of different cell types in the eye and pineal complex are unconvincing and speculative. Cellular subfunctionalization does not depend on gene or genome duplication events.
In summary, the authors should attempt to strengthen the case for lenses and sharpen the discussion of retrograde evolution of medial eyes to form the pineal complex.

Referee #2

(Remarks to the Author)
This manuscript presents the first rigorously analysed evidence for the presence of paired, eye-like pineal/parapineal organs in the earliest vertebrates. The analysis is convincing (though I have some minor quibbles; see below) and falsifies the currently popular alternative interpretation of the structures in question as nasal sacs. This represents a major advance in our understanding of early vertebrate anatomy and evolution, even though the fundamental conclusion - that the pineal/parapineal originated from a pair of functional eyes - agrees with a lot of past theorising. It's one thing to theorise, another to find the hypothesised ancestral state actually preserved! The preservation of the fossils is sufficiently good to support the claims being made, and the phylogenetic position of myllokunmingiids (and Metaspriggina) as stem vertebrates is generally accepted. In other words, there are no serious problems with this manuscript, but as usual there are some lesser issues that need to be addressed:

Page 1, line 20: "facilitated" is the wrong word here; I suggest "supplemented".

P. 2, l. 23: "both of which are derived" suggests that "both" refers specifically to the paired lateral eyes; "both being derived" would be better, as "both" then refers unambiguously to both the eyes and the pineal complex.

P. 3, l. 57-58: Only three structures are listed but there are supposed to be four.

P. 5, l. 104: "after sliced" should be "after being sliced".

P. 5, l. 105: "central" should be "centre".

P. 6, l. 125-126 (and Fig. 2): Here's my most serious concern with the manuscript. Taking all the evidence together I am happy that the melanosomes have been correctly identified, but I don't quite understand how the authors can claim that the presence of melanin is verified by the PCA in Fig. 2a. In the first instance it is striking that NO fossil melanin sample overlaps with the range of modern melanins, either fresh or heated, so strictly speaking all we learn from the PCA is that the samples from Haikouichthys overlap with those from previously identified (presumably on the basis of location and appearance) fossil melanins. However, the samples from "myllokunmingiid sp." don't overlap with these at all! They form a discrete tight cluster, very far away, and only appear in the figure to belong to the 'fossil melanin cluster' because the (pale orange) background of that cluster has been extended to include them - essentially reflecting a prior judgement that they must also be melanin! This peculiar pattern needs to be properly discussed and explained. In particular, why are the two myllokunmingiids so different? Is this just taphonomy, or something more important reflecting real differences in the original molecules?

The question is made even more interesting by the observed differences in melanosome shape in the two taxa (pages 5-6). I would like to put an idea to the authors for their consideration in relation to these points: could it be that these "myllokunmingiids" are not in fact very closely related and show either 1) steps in the assembly of the vertebrate bauplan, or 2) unsuspected diversity? My point is that these are without a doubt very primitive vertebrates, and are thus likely to share large numbers of vertebrate plesiomorphies, which may make them look deceptively similar and mask important differences. To make an analogy, "myllokunmingiids" may be to vertebrates what "lobopods" are to arthropods. This is not a question that can be settled at present, but it would be worth thinking about and mentioning at least in passing in the text.

The legend of Fig. 2 also contains a small linguistic error: the position indicators "(up)" and "(below)" should be "(top)" and "(bottom)".

P. 9 and Fig. 3b: I agree that the paired dark structures are para/pineal eyes, not nasal sacs. However, in Fig. 3b (and elsewhere) we can see an obvious square midline notch cut into what appears to be a cartilaginous supporting rim of the upper lip, in front of the para/pinealS. Could this be a nasohypophyseal opening? Positionally it would make perfect sense. I think this possibility should be discussed briefly.

P. 9, l. 184-190: This claim is not supported by the fossil evidence. Sacabambaspis, the earliest vertebrate for which the nasal region is known, has widely separated left and right nostrils and thus cannot have had a nasohypophysial duct. Heterostracans (for which we only have impressions on the internal surface of the head shield as guides to their internal anatomy) do seem to have had an anterior midline opening, but the impressions of the left and right nasal sacs are widely separated and the condition of the hypophysis is unknown. In short, this story is nowhere near as clear-cut as the authors claim.

Discussion, first paragraph: Why do the authors not mention that a "pineal" (or "parietal") foramen is very widely distributed among early osteichthyans - it is present more often than not - and persists throughout the tetrapod stem group right up into stem amphibians (temnospondyls), stem mammals (synapsids), and a variety of early reptiles? It is quite clear that lizards and tuataras retain this primitive condition, as opposed to having evolved it de novo. They are thus likely to be highly informative about the functional evolution of this anatomical complex. The way the text is written now, they come across as more of an evolutionary oddity, which does them a disservice.

P. 11, l. 235-238: This hypothesis about whole-genome duplications and eye-pineal divergence only works if you assume that both whole-genome duplications occurred in the vertebrate stem. However, the ever-expanding body of genomic evidence increasingly points to only one duplication having occurred in the vertebrate stem, with the second occurring in the gnathostome stem (see for example Hoffmann et al. 2021: https://academic.oup.com/gbe/article/13/10/evab205/6364188). This is incompatible with the hypothesis and pretty much falsifies it.

P. 12, l. 251: I know that the authors are simply following the published interpretation of Sacabambaspis here, but looking at Fig. 4e I'm struck by a sudden thought that I want to share with the authors. It is potentially important and they are welcome to incorporate it in their comparative analysis if they agree with it. I think the supposed "pineal / parapineal foramina" of Sacabambaspis have been misidentified. They are positioned far posterior to the eyes, in a manner that makes it difficult to understand how the nerve connections and brain morphology would work. However, they are in just the right place to be the openings of paired endolymphatic ducts - structures that are seen in many other early vertebrates.

Such a reinterpretation carries with it an interesting implication: there would be no known crown-group vertebrate in which the parapineal and pineal organs are symmetrical and equally eye-like. The myllokunmingiids and Metaspriggina would be unique in this regard, consonant with their position in the vertebrate stem group. The symmetrical and eye-like condition is presumably primitive, because from theoretical considerations it appears like a less modified version of an evolutionary (and developmental) 'ground state'.

In summary, I think this is an important manuscript, and while it does need some further work it is definitely on the right track. I will be happy to review a potential revised version in due course.

Per Erik Ahlberg

Referee #3

(Remarks to the Author)
The authors use new specimens to reevaluate the nature of the lateral dark spots in the earliest vertbrates from the Chengjioang biota. They show that these spots have melanosomes and a lens, suggesting that they are camera.-type eyes, with important implications for the evolution of vision. The fossils are amazing and the analyses are(mostly, see below) robust and convincing. The authors have done a thorough job of analyzing these samples and have made an important finding. Outside of this primary significance, I also found the potential discovery of “hollow” melanosomes to be worthy of further discussion (see below), and have some doubts about some of the chemical data. I also have some wording suggestions. But overall this is an excellent manuscript.
L41:”comprise of”->is comprised of
L42: “the hemispherical retina”, “the optical nerve..”
L70: “of”->”that”
L77:”did have”->had
L77: Delete “with their four camera eyes”
L92: Why exactly is this “high resolution”? The equipment seems to be a standard SEM
L104:”after slicing”
L105: “small hole occurring at the central”->small hole in the center
L117: Authors should emphasize this point more. Hollow melanosomes are only found in birds, but melanosomes with eumelanin shells surrounding pheomelanin cores have been identified in human neuromelanin (https://doi.org/10.1073/pnas.060401010). Perhaps these melanosomes had pheomelanin cores that were not preserved? This could have exiting implications for the evolution of pheomelanin (thought to have evolved after eumelanin),a nd perhaps even for melanin functon(could it serve a simialr role to neuromelanin)?
Fig. 1: There must be a typo in the figure legends pertaining to the size of the scale bars, as the melanosomes would be orders of magnitude larger than the fossils themselves.
L126 and fig. 2: this is not very convincing. The tof-sims data from fossils in this paper are well outside those of extant melanin, and even on the edge of other fossil melanins. Furthermore, it is unclear exactly what data are shown here (what is the source of the “fossil melanin” data?). related, how was the fossil melanin identified as fossil melanin? By chemical similarity to extant melanin (I would guess not, considering that much of it is far away extant melanin)? This therefore runs the risk of being circular. It is thus probably best to say that the chemical data did not show what you expected (similarity to extant melanin), but that there could be several explanations for this, including taphonomy, evolution of the chemistry of melanin. . In any case the morphological data are completely convincing and the chemical data are of secondary importance.
Please add the source of the fossil melanin tof-sims data, as well as the data themselves.
L248:”of the presence”
Extended data fig. 2: Not sure what is the point of this figure? It should include data from extant melanin, as well as from the matrix for comparison.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
I am satisfied with the revised manuscript. The authors have thoughtfully incorporated my key concerns and I think the paper provides an exciting advancement in the field.

Referee #2

(Remarks to the Author)
This revised version of the manuscript is substantially improved; the authors have addressed the criticisms of the reviewers in a constructive and collegial spirit. I have only a few comments this time (the one dealing with lines 209-214 being the most serious), and if these are also addressed I don't think the manuscript will need another round of review.

Lines 45, 199, 219: "Metaspriginna" should be "Metaspriggina".

Lines 121-122: "Extended Data Fig. 6k, l)" do not exist! Something has gone wrong here.

Line 146: "show no sign of overlapping" is excessively dramatic and sounds like the authors are trying to exaggerate the importance of this observation; "do not overlap" would be better.

Line 195: "which" is undefined here. Better to end the sentence with "(Fig. 3c-j)." and then begin a new one "It is similar..."

Lines 209-214: the argumentation is strange in this section. It is neither here nor there whether galeaspids have been "surmised" to be the first vertebrates with well-separated nasal sacs. The case for the dark spots of myllokunmingiids being para/pineal eyes rather than nasal sacs rests entirely on the POSITIVE evidence that they had melanosome-containing

retinae as well as lenses. That evidence is sufficient on its own, because such structures are never present in nasal sacs but are known to occur in the para/pineal organs of living vertebrates, and the position of the dark spots in myllokunmingiids is compatible with their identification as para/pineal eyes. This identification is not dependent on any argumentation about the position or number of nasal sacs in other early vertebrates. I would recommend cutting out those arguments altogether.

Line 224: "indicating that" or "suggesting that" would be better than "prompting".

Line 257: "posed" is wrong here; the correct word is "posited".

Lines 300-303: an obvious thought, given their position and orientation, is that the para/pineal eyes would detect threats coming from above. The fact that they are smaller than the lateral eyes suggests that they had a different and perhaps simpler visual function; this is speculative, but you could for example imagine them serving to trigger a flight reaction in response to a dark shape passing above, whereas the later eyes would be 'seeing' in a conventional sense and allow the animal to navigate its environment.

Line 418: "continually collected"? Given that the collecting of any particular specimen is a brief and discrete action - starting when you find it and ending when you pack it for transport home - I don't understand what "continually collected" means. This needs to be reworded.

Figure 3: why are the lenses in the reconstruction (3b) not shown in the same position as in the specimen (3a)? There's no reason to believe that the lenses in the fossil have been displaced. The difference matters, because it has functional implications: in the reconstruction it looks like the animal is focused on what's going on in front of it, in a rather human-like way, but the specimen suggests that the para/pineal eyes looked straight upwards and the lateral eyes to the sides. The reconstruction should be modified.

There's another point as well: looking at the Metaspriggina eyes shown in Fig. 3s-v, it looks like the para/pineal eyes in this animal are much smaller relative to the lateral eyes than in myllokunmingiids. This is an interesting difference, perhaps suggesting that Metaspriggina shows a more derived condition, but I don't think it's mentioned anywhere in the text.

Figure 4: the lens positions should be adjusted in some of these reconstructions as well. Lampreys do NOT have stereoscopic vision! Even in Metaspriggina, where the eyes are anterior in position, it is not clear from the preserved lens position that the eyes looked forwards in the way shown here. In Sacabambaspis, on the other hand, it is clear that they did.

Referee #3

(Remarks to the Author)
The authors use new specimens to reevaluate the nature of the lateral dark spots in the earliest vertbrates from the Chengjioang biota. They show that these spots have melanosomes and a lens, suggesting that they are camera-type eyes, with important implications for the evolution of vision. The authors have done a nice job responding to my comments and the those of the other reviewers. I still, however, am not convinced by the ToF-SIMS data. It does show that the fossil melanin is distinct from the sediment, but it is also distinct from other melanins, including other fossil melanin. Of course, there may be explanations for this pattern, but it should not be considered strong evidence of presence of melanin. Indeed, if this was the only evidence of the presence of melanin (which it is not), it would not be acceptable. I recommend straightforwardly acknowledging the uncertainty of these results, but pointing out that it is secondary to the morphological evidence.
other small point:
L36: Darwins'->Darwin's

Referee #1 (Remarks to the Author):

Lei et al. present evidence that ancestral vertebrates contained two sets of camera eyes, one lateral and the other medial. Previous studies interpreted the medial sensory organs as nasal, possibly arising from paired olfactory placodes. Here, the authors reinterpret the fossil record to argue that they correspond to a second set of eyes.

The authors analyzed six specimens of *Haikouichthys* and four slabs of myllokunmingid fossils. They applied sophisticated imaging technologies (EDS, Raman spectroscopy, and XPS) to provide evidence that the two sets of pigmented sensory organs contain microbodies—melanin-containing melanosomes. ToF-Mass spec methods provide further support for the occurrence of melanin-containing melanosomes in both sets of sensory organs. This is a critical feature of camera eyes. The ovoid and spherical organizations of melanosomes are also consistent with this possibility.

Response: We thank the referee's positive comments and are pleased that the referee agrees with our interpretations for the RPE in the fossil specimens and the two median structures (also with melanosomes) as two additional eyes.

The lens is another key property of camera eyes, and here the arguments are not quite as compelling as the case for melanosomes. Different indentations are interpreted as lenses, but the identification is not definitive. Any additional methods that could be used to strengthen these arguments would be a nice addition to the study.

Response: Following the referee's helpful suggestions, we have now reworded the related text to clarify the process in identifying the lens in myllokunmingids and other vertebrate fossils (see line 182-194).

It is true that identification of a lens in fossils is somewhat challenging due to the lack of explicit criteria to follow. Indeed, in the context of fossils they are rarely mentioned, even when visible in fossils illustrated. To take on board the referee's helpful suggestion we have now explicitly stated the criteria we have used to make an interpretation of 'lens' in these fossil specimens. The primary distinguishing feature is the consistently regular circular morphology of the structure, accompanied by relief; its dimensions, shape, and mode of preservation remain

uniform across all examined Chengjiang specimens. The shape and position of the structure is consistent with lens interpretation. Importantly, the mode of preservation – as a structure with relief – is also critical. In the eye it is only the lens that has sufficient recalcitrance to retain three-dimensional relief during decay and produce an impression in the sediment (now seen as relief) even though the biological material of the lens is now absent. Such relief has been used in the identification of eye lenses in other putative vertebrate fossils e.g. *Metaspriggina walcotti* from the Burgess Shale (*Conway Morris & Caron, 2014, Nature 512, 419-422*) and *Mayomyzon* in the Mazon Creek (*Gabbott et al. 2016, Proc. R. Soc. B 283, 20161151*).

In addition, the feature we interpret as the lens is located within the eyes and is thus logically compressed between two layers of carbonaceous films (from retinal pigment epithelium) in the exceptionally preserved fossils. When splitting the rock block, the carbonaceous films superimposed with the lens can present either in the part or the counterpart slab, leaving the lens region rich or absent in carbon films. In practice, the absence of the carbonaceous film in the eyes is easier to discern and can be used to facilitate the identification of a lens in some cases. At least two of our specimens show that the relief of eye lens is identical with the absence of carbonaceous films, both of which show regularly ovoid shape (Extended Data Fig. 1d, h, i). We also follow this rule when identifying the lens in both lateral eyes and median eyes in Chengjiang myllokunmingiids and *Metaspriggiga waloctti* (Fig 3s-v.).

Our identification of eye lens is facilitated with multiple techniques, including low-angle light, fluorescent light, SEM tomography and Element mapping. It is worthy to note though that technique such as RTI (Reflectance Transformation Imaging) also potentially works in showing the relief of eye lens. However, the prerequisites of spheres near the object makes it impossible to photograph specimens of extremely small size (such as lens here), thus limiting the use of this technique in our practise (the spere would impose shadow on the project when the light source is at extremely low angels).

The authors argue for a deep evolutionary and developmental connection between camera eyes and the pineal complex of fish and other vertebrates. Both organs arise from the same region of the anterior neural plate. There is quite a lot of published speculation regarding the relationship of these sensory cell types. In most scenarios, image-forming camera eyes arise from light-sensing pineal cells. Here, the authors envision a retrograde scenario, whereby their newly discovered medial eyes have degenerated into the pineal complex. Totally plausible, but many of their arguments are highly speculative and should be pruned and sharpened in a revised text. Likewise, the arguments of whole-genome duplications and the subfunctionalization of different cell types in the eye and pineal complex are unconvincing and speculative. Cellular subfunctionalization does not depend on gene or genome duplication events.

Response: Following the referee's suggestion, we have now heavily revised our discussion section (See line 227-272), thoroughly summarizing the distribution of morphology and

function of pineal complex in vertebrates. In addition, we have removed the discussion of whole-genome duplication. The referee is correct that this is speculative, as also noted by referee 2. We have now revised our discussion to focus the readers attention to the retrograde of the median eyes in early vertebrates to the pineal complex in extant vertebrates, which is more robustly evidenced by our work.

In summary, the authors should attempt to strengthen the case for lenses and sharpen the discussion of retrograde evolution of medial eyes to form the pineal complex.

Response: We now revised our manuscript following these insightful suggestions (see above for detailed response). See line 182-192 for lens arguments and lines 226-277 for pertaining to the discussion of the retrograde nature of the median eyes.

Referee #2 (Remarks to the Author):

This manuscript presents the first rigorously analysed evidence for the presence of paired, eye-like pineal/parapineal organs in the earliest vertebrates. The analysis is convincing (though I have some minor quibbles; see below) and falsifies the currently popular alternative interpretation of the structures in question as nasal sacs. This represents a major advance in our understanding of early vertebrate anatomy and evolution, even though the fundamental conclusion - that the pineal/parapineal originated from a pair of functional eyes - agrees with a lot of past theorising. It's one thing to theorise, another to find the hypothesised ancestral state actually preserved! The preservation of the fossils is sufficiently good to support the claims being made, and the phylogenetic position of myllokunmingiids (and Metaspriggina) as stem vertebrates is generally accepted. In other words, there are no serious problems with this manuscript, but as usual there are some lesser issues that need to be addressed:

Response: We thank the referee for all these positive comments, which highlight the importance of our work in understanding the anatomy and evolution of early vertebrates.

Page 1, line 20: "facilitated" is the wrong word here; I suggest "supplemented".

Response: We now replaced the word "facilitated" with "supplemented", see line 18.

P. 2, l. 23: "both of which are derived" suggests that "both" refers specifically to the paired lateral eyes; "both being derived" would be better, as "both" then refers unambiguously to both the eyes and the pineal complex.

Response: We now rephrase as ‘both being derived’, see line 21.

P. 3, l. 57-58: Only three structures are listed but there are supposed to be four.

Response: We have reworded the sentence, listing the four structures as "the frontal ‘eye’, the

lamellar body (ciliary), the Joseph cells and the dorsal ocelli", see line 55-57.

P. 5, l. 104: "after sliced" should be "after being sliced".

Response: We have now replaced " after sliced " with " after being sliced ", see line 102.

P. 5, l. 105: "central" should be "centre".

Response: We have replaced " central " with " centre ", see line 103.

P. 6, l. 125-126 (and Fig. 2): Here's my most serious concern with the manuscript. Taking all the evidence together I am happy that the melanosomes have been correctly identified, but I don't quite understand how the authors can claim that the presence of melanin is verified by the PCA in Fig. 2a. In the first instance it is striking that NO fossil melanin sample overlaps with the range of modern melanins, either fresh or heated, so strictly speaking all we learn from the PCA is that the samples from *Haikouichthys* overlap with those from previously identified (presumably on the basis of location and appearance) fossil melanins. However, the samples from "myllokunmingiid sp." don't overlap with these at all! They form a discrete tight cluster, very far away, and only appear in the figure to belong to the 'fossil melanin cluster' because the (pale orange) background of that cluster has been extended to include them - essentially reflecting a prior judgement that they must also be melanin! This peculiar pattern needs to be properly discussed and explained.

Response: We sincerely appreciate that the referee agrees on our identification of melanosomes, which are mainly supported by the morphology (Fig. 1g-j). We have now updated Fig. 2 (and Extended Data Fig. 7) and revised the manuscript to explicitly explain the identification of melanin based on the ToF-SIMS data and PCA results (see line 124-159).

Comparison of ToF-SIMS spectra indicates that the characteristic peaks of melanin collected from the eyes of the Chengjiang myllokunmingids are strikingly resemble those of fresh melanin in the eyes of living frog but differ from the data collected from the Chengjiang sediment (as negative control) (Fig. 2b; Extended Data Fig. 7b), which strongly indicates there are melanin remains in Chengjiang melanosomes. Further PCA analyses, conducted by integrating our ToF-SIMS data with those previously published, show that the Chengjiang vertebrate eye samples do not overlap with the non-melanin negative controls, including two Chengjiang rock samples (sample 26, 27 in the updated Extended Data Fig. 7). We should note that the negative control samples also contain fossils (leaves) and modern organic samples, not just sediment (See updated Fig. 2a and Extended Data Fig. 7a, d). We have now adjusted the PCA plot to highlight the original control samples and our samples as different groups (see response to the next point for further details), to clarify the potential misleading when reading our results. The results support there are true chemical signals of melanin in the Chengjiang vertebrate eye samples. We have now rewritten this section in the main text (see lines 124-142).

As noted by the referee, our melanin samples, like other previously identified fossil melanin samples, don't overlap with modern melanin samples: a phenomenon recovered by an extensive maturation experiment (*Colleary et al., 2015, PNAS, 112(41): 12592-12597*). The reason for the separation of fossil melanin from modern samples was discussed by Colleary et al. (2015), which is attributed to diagenetic alteration of melanin after burial. Possible reasons for this might include a much more complicated degradation history of fossil melanin than the simple experimental temperature/pressure maturation and the impact of other fossilized organic remain in fossil samples, such as secondary introduction of kerogen or sulfurisation. We now highlighted this explanation in the main text (see lines 143-150).

We also would like to highlight a more recent study (*Roy et al., 2023, Paleobiology, 49(4), pp.712-732*) that found more wide spread of experimentally matured samples when matured within sediment, which allow for volatiles to dissipate, as opposed to within sealed gold tubes as conducted by Colleary et al. (2015). Unfortunately, Roy et al. didn't include negative control samples, which would affect the loadings on the axes and making their analyses not directly comparable to ours.

In particular, why are the two myllokunmingiids so different? Is this just taphonomy, or something more important reflecting real differences in the original molecules? The question is made even more interesting by the observed differences in melanosome shape in the two taxa (pages 5-6). I would like to put an idea to the authors for their consideration in relation to these points: could it be that these "myllokunmingiids" are not in fact very closely related and show either 1) steps in the assembly of the vertebrate bauplan, or 2) unsuspected diversity? My point is that these are without a doubt very primitive vertebrates and are thus likely to share large numbers of vertebrate plesiomorphies, which may make them look deceptively similar and mask important differences. To make an analogy, "myllokunmingiids" may be to vertebrates what "lobopods" are to arthropods. This is not a question that can be settled at present, but it would be worth thinking about and mentioning at least in passing in the text.

Response: We thank the referee for these inspiring comments and agree that both taphonomy and original molecular components might together account for the disperse distribution of data points from the Chengjiang vertebrate samples in our PCA result. In addition, there is a difference in depositional environment, geochemical conditions and diagenetic processes experienced across fossil sites from which we extracted melanin samples (updated Extended Data Fig. 7), and these differences also probably account for the dispersed pattern of the data points distribution. However, the difference between the two Chengjiang myllokunmingids plotted is somewhat unexpected because the preservation of Chengjiang fossils is consistent across localities (*Gabbott et al., 2004, Geology, 32: 901-904; Zhu et al., 2005, Palaoe3, 220: 31-46*) and they are supposed to have experienced the same preservation process. We have now provided further explanations for this (line 143-159).

We further divided the negative control samples, based on their nature, to sediment, modern carbon and fossil leaves subgroups, to testing whether the Chengjiang vertebrate samples are overprinted by later weathering or contamination. The PCA result shows that these factors can be ruled out based on the separation among our samples from all negative controls (Fig. 2a; Extended Data Fig. 7a). We now clarify this (see line 143-150) and address that the observed difference in distribution pattern between the two myllokunmingid species is likely due to their possessing a different melanin composition – this is consistent with their different melanosome morphologies (see line 156-159). The correlation between melanosome morphology and melanin compositions has been previously noted by *Colleary et al., 2015, PNAS, 112(41): 12592-12597; D'Alba & Shawkey, 2019, Physiol. Rev. 99:1-19)*. And we do concur with the referee that this is an aspect that would need further scrutiny to be fully resolved.

Interestingly, we noticed that this variation in the morphology of fossilized melanosomes also present in the eyes of vertebrates from Mazon Creek. We have addressed a desperate need to investigate this phenomenon in living taxa to understand the reason for this variation (line 166-171).

In terms of the monophyly of myllokunmingids, we agree with the referee that there is a high possibility that myllokunmingids were a diverse group, or an evolutionary grade, which can be further inferred from the size variation of the two species, and the unique anterior dorsal plate (or the upper lip) only present in *Haikouichthys*. However, we are unsure that the difference in melanosome morphology necessarily has evolutionary/phylogenetic significance, given that this has also been observed in the Carboniferous *Bandringa*. It may be an ecological adaptation in terms of how melanosomes become distributed amongst the rods and cones, in which the elliptical melanosomes are usually situated in between the rods and cones, while the ovoid melanosomes form the underlying layer beneath the elongate melanosomes and the photoreceptor cells (rods and cones). Hence, if this pattern persists in a taxon with only ovoid melanosomes, it will allow for light to scatter between photoreceptors but not be reflected from the base. Again, it would be most beneficial to establish if any recent vertebrate exhibits such a RPE melanosome distribution. As such, we only leave these observations in terms of their significance. And, for now (and as said by the reviewer) we have desisted from adding this more speculative discussion to our manuscript.

The legend of Fig. 2 also contains a small linguistic error: the position indicators "(up)" and "(below)" should be "(top)" and "(bottom)".

Response: Sorry, these are now corrected. See line 518-519.

P. 9 and Fig. 3b: I agree that the paired dark structures are para/pineal eyes, not nasal sacs. However, in Fig. 3b (and elsewhere) we can see an obvious square midline notch cut into what appears to be a cartilaginous supporting rim of the upper lip, in front of the para/pineals. Could this be a nasohypophyseal opening? Positionally it would make perfect sense. I think this

possibility should be discussed briefly.

Response: We agree with the referee on the potential presence of a nasohypophyseal opening in *Haikouichthys*. Evidence supporting this identification only comes from a single specimen in our study (Figs 1a,b; 3a,b), but with several previously illustrated specimens (fig 1a-d in *Shu et al. 2003, Nature, 421: 526-529*) potentially showing similar morphology. Following the referee's suggestion, we have now edited the text to reflect this fact (line 215-218). We hope to find more specimens with upper lips to underpin this possibility in the future.

P. 9, l. 184-190: This claim is not supported by the fossil evidence. *Sacabambaspis*, the earliest vertebrate for which the nasal region is known, has widely separated left and right nostrils and thus cannot have had a nasohypophysial duct. Heterostracans (for which we only have impressions on the internal surface of the head shield as guides to their internal anatomy) do seem to have had an anterior midline opening, but the impressions of the left and right nasal sacs are widely separated, and the condition of the hypophysis is unknown. In short, this story is nowhere near as clear-cut as the authors claim.

Response: We thank the referee for this comment. It is true that there are some early vertebrates that have been inferred to bear paired nostrils/nasal sacs. However, nasal sacs in almost all these early vertebrates are not preserved as carbonaceous films. We have now revised the manuscript to clarify current evidence concerning the preservation of olfactory organ in early vertebrates, including *Sacabambaspis*, see line 205-209.

Discussion, first paragraph: Why do the authors not mention that a "pineal" (or "parietal") foramen is very widely distributed among early osteichthyans - it is present more often than not - and persists throughout the tetrapod stem group right up into stem amphibians (temnospondyls), stem mammals (synapsids), and a variety of early reptiles? It is quite clear that lizards and tuataras retain this primitive condition, as opposed to having evolved it de novo. They are thus likely to be highly informative about the functional evolution of this anatomical complex. The way the text is written now, they come across as more of an evolutionary oddity, which does them a disservice.

Response: Following the referee's suggestion, we have now reorganised the discussion section, sorted the text structure and clarified the distribution of morphology and function of pineal complex across major clades of crown vertebrates, including the distribution of pineal or parietal foramen throughout crown gnathostomes (see line 227-277).

P. 11, l. 235-238: This hypothesis about whole-genome duplications and eye-pineal divergence only works if you assume that both whole-genome duplications occurred in the vertebrate stem. However, the ever-expanding body of genomic evidence increasingly points to only one duplication having occurred in the vertebrate stem, with the second occurring in the gnathostome stem (see for example Hoffmann et al.

2021: https://academic.oup.com/gbe/article/13/10/evab205/6364188). This is incompatible with the hypothesis and pretty much falsifies it.

Response: We have now removed the discussion about whole-genome duplications (WGD). See response to Referee 1 for the same concern.

P. 12, l. 251: I know that the authors are simply following the published interpretation of *Sacabambaspis* here, but looking at Fig. 4e I'm struck by a sudden thought that I want to share with the authors. It is potentially important and they are welcome to incorporate it in their comparative analysis if they agree with it. I think the supposed "pineal / parapineal foramina" of *Sacabambaspis* have been misidentified. They are positioned far posterior to the eyes, in a manner that makes it difficult to understand how the nerve connections and brain morphology would work. However, they are in just the right place to be the openings of paired endolymphatic ducts - structures that are seen in many other early vertebrates.

Such a reinterpretation carries with it an interesting implication: there would be no known crown-group vertebrate in which the parapineal and pineal organs are symmetrical and equally eye-like. The myllokunmingiids and *Metaspriggina* would be unique in this regard, consonant with their position in the vertebrate stem group. The symmetrical and eye-like condition is presumably primitive, because from theoretical considerations it appears like a less modified version of an evolutionary (and developmental) 'ground state'.

Response: We thank the referee for sharing this inspiring thinking and agree that the 'pineal / parapineal foramina' of *Sacabambaspis* is too far from the eyes. However, if the presence of two pineal eyes being an ancestral state were accepted, there should be, in theory, more early vertebrates bearing two pineal eyes/organs. In this sense, the interpretation of the anterior paired openings as nasal sacs in some early vertebrates deserves a re-thinking as potential paired pineal eyes/foramina. Accordingly, it would be a plausible alternative that the previously interpreted nasal openings of *Sacabambaspis* are indeed paired pineal foramina given their position relative to the eyes. In this sense, the presence of potential pineal organs might have been mis-interpreted in several early vertebrates (such as those listed in the table below), such as the well-defined circular carbonaceous films in the Early Carboniferous conodont animal (*Briggs et al., 1983, Lethaia, 16: 1-14*).

We do not expand our discussion to this extent due to the length limitation. We have according retained the citations to the current interpretation of the pineal / parapineal foramina of *Sacabambaspis*. However, we do agree with the referee, and we pointed out their unusual relative position to the eyes and denoted a question mark in Fig. 4. Furthermore, following the referee's suggestion, we also have highlighted the necessary to think about the gap between the state of two pineal / parapineal eyes (or foramina) and the state of one pineal foramen in crown vertebrates. See line 263-267.

Taxa	Geological Time	Original interpretation of potential pineal organ	References and referred figures therein
Sacabambaspis janvieri	Ordovician	nasal sacs	Gagnier, 1993 (fig. 2b)
Eriptychius americanus	Ordovician	olfactory bulbs	Dearden et al, 2023 (fig. 2f)
Euphanerops longaevus	Devonian	olfactory organs	Gabbott, 2021 (fig. 4a)
Clvdugnathus? cf. *cuvusformis*	Carboniferous	two dark spots	Briggs, 1983 (fig. 2c)

In summary, I think this is an important manuscript, and while it does need some further work it is definitely on the right track. I will be happy to review a potential revised version in due course.

Per Erik Ahlberg

Response: We thank Prof. Ahlberg again for all his positive and insightful comments.

Referee #3 (Remarks to the Author):

The authors use new specimens to reevaluate the nature of the lateral dark spots in the earliest vertbrates from the Chengjioang biota. They show that these spots have melanosomes and a lens, suggesting that they are camera-type eyes, with important implications for the evolution of vision. The fossils are amazing and the analyses are (mostly, see below) robust and convincing. The authors have done a thorough job of analyzing these samples and have made an important finding. Outside of this primary significance, I also found the potential discovery of "hollow" melanosomes to be worthy of further discussion (see below), and have some doubts about some of the chemical data. I also have some wording suggestions. But overall this is an excellent manuscript.

Response: We thank the referees for the positive comments.

L41:"comprise of"->is comprised of

Response: Corrected, see line 38-39.

L42: "the hemispherical retina", "the optical nerve.."

Response: 'the' is now added in each of these two phrases, see line 40 and 41.

L70: "of"->"that"

Response: We have reworded this sentence, see line 68-71.

L77:"did have"->had

Response: We have reworded this sentence, see line72-75.

L77: Delete "with their four camera eyes"

Response: We have deleted "with their four camera eyes".

L92: Why exactly is this "high resolution"? The equipment seems to be a standard SEM

Response: This phrase is removed now.

L104:"after slicing"

Response: This is now rephrased as 'after been sliced'", see line 102.

L105: "small hole occurring at the central"->small hole in the center

Response: This is now replaced, see line 103.

L117: Authors should emphasize this point more. Hollow melanosomes are only found in birds, but melanosomes with eumelanin shells surrounding pheomelanin cores have been identified in human neuromelanin (https://doi.org/10.1073/pnas.060401010). Perhaps these melanosomes had pheomelanin cores that were not preserved? This could have exiting implications for the evolution of pheomelanin (thought to have evolved after eumelanin), and perhaps even for melanin function (could it serve a similar role to neuromelanin)?

Response: We thank the referee for this insightful suggestion. Following the referee's suggestions, we have updated the discussion concerning the interpretation of the observed central hole in the melanosomes of Chengjiang myllokunmingid sp., highlighting that the degradation of pheomelanin cores is most likely the only reason accounting for the central hole, as the hollow melanosomes in the avian feathers have also been suggested to be a result of the loss of pheomelanin cores (*Simon & Peles, 2010, Acc. Chem. Res. 43: 1452-1460; D'Alba & Shawkey, 2019, Physiol. Rev. 99:1-19*). See lines 113-123.

To support this update in our manuscript, we conducted more TEM scanning and can now convincingly demonstrate that the 'hollow' central hole is quite common in all examined melanosomes (Fig. 1 k-n), and that the size of the hole is positively correlated with the overall size of the melanosomes bearing them. More intriguingly, we observed that the central hole is not really hollow, instead it bears a similar scaffold frame of material that show higher electron density, although the scaffolds are much less compacted than those in the outer shell region (Fig.1 m, n; Extended Data Fig. 6k, l). To reflect these changes, we have expanded the discussion in the manuscript (see line 113-123)

We agree with the referee that this interpretation is an exciting finding. However, the difficulty in identify pheomelanin (*D'Alba & Shawkey, 2019, Physiol. Rev. 99:1-19*) limits us to further discuss its potential importance. We look forward to future discoveries that may

provide feasible method to discern pheomelanin from eumelanin and welcome the opportunity to work with the referee in this project.

Fig. 1: There must be a typo in the figure legends pertaining to the size of the scale bars, as the melanosomes would be orders of magnitude larger than the fossils themselves.

Response: Sorry, the scale is corrected now.

L126 and fig. 2: this is not very convincing. The tof-sims data from fossils in this paper are well outside those of extant melanin, and even on the edge of other fossil melanins. Furthermore, it is unclear exactly what data are shown here (what is the source of the "fossil melanin" data?). related, how was the fossil melanin identified as fossil melanin? By chemical similarity to extant melanin (I would guess not, considering that much of it is far away extant melanin)? This therefore runs the risk of being circular. It is thus probably best to say that the chemical data did not show what you expected (similarity to extant melanin), but that there could be several explanations for this, including taphonomy, evolution of the chemistry of melanin. In any case the morphological data are completely convincing and the chemical data are of secondary importance. Please add the source of the fossil melanin tof-sims data, as well as the data themselves.

Response: We thank the referee for this comment, which is also raised by Referee 2. We have now implicitly clarified the identification of melanin and the implication of PCA analyses (see reply to Referee 2 above). In addition, we have added the source of all the data in the updated Fig. 2 and Extended Data Fig. 7.

248:"of the presence"

Response: This has been corrected now.

Extended data fig. 2: Not sure what is the point of this figure? It should include data from extant melanin, as well as from the matrix for comparison.

Response: This Raman spectra figure, together with Extended Data Fig. 3, is designed to show that the black film in the eyes of Chengjiang myllokunmingid are organic remains, as denoted by the D and G bands in all samples except to the Chengjiang sediment. Furthermore, the plotting of multiple specimens in this figure aims to show the consistency and robustness of Raman data across multiple samples. The figure and its caption are now updated.

Referee #1 (Remarks to the Author):
I am satisfied with the revised manuscript. The authors have thoughtfully incorporated my key concerns and I think the paper provides an exciting advancement in the field.
Response: We thank the referee for this supportive comment.

Referee #2 (Remarks to the Author):
This revised version of the manuscript is substantially improved; the authors have addressed the criticisms of the reviewers in a constructive and collegial spirit. I have only a few comments this time (the one dealing with lines 209-214 being the most serious), and if these are also addressed I don't think the manuscript will need another round of review.
Response: We appreciate that the referee is happy with our revision.

Lines 45, 199, 219: "Metaspriginna" should be "Metaspriggina".
Response: Sorry, this has now been corrected in the text.

Lines 121-122: "Extended Data Fig. 6k, l)" do not exist! Something has gone wrong here.
Response: This is now corrected.

Line 146: "show no sign of overlapping" is excessively dramatic and sounds like the authors are trying to exaggerate the importance of this observation; "do not overlap" would be better.
Response: This has been rephrased per the suggestion.

Line 195: "which" is undefined here. Better to end the sentence with "(Fig. 3c-j)." and then begin a new one "It is similar..."
Response: This has been rephrased per the suggestion.

Lines 209-214: the argumentation is strange in this section. It is neither here nor there whether galeaspids have been "surmised" to be the first vertebrates with well-separated nasal sacs. The case for the dark spots of myllokunmingiids being para/pineal eyes rather than nasal sacs rests entirely on the POSITIVE evidence that they had melanosome-containing retinae as well as lenses. That evidence is sufficient on its own, because such structures are never present in nasal sacs but are known to occur in the para/pineal organs of living vertebrates, and the position of the dark spots in myllokunmingiids is compatible with their identification as para/pineal eyes. This identification is not dependent on any argumentation about the position or number of nasal sacs in other early vertebrates. I would recommend cutting out those arguments

altogether.
Response: We have now removed the discussion of nasal sacs of galeaspids following the referee's suggestion.

Line 224: "indicating that" or "suggesting that" would be better than "prompting".
Response: This is now reworded following the suggestion.

Line 257: "posed" is wrong here; the correct word is "posited".
Response: This is now corrected.

Lines 300-303: an obvious thought, given their position and orientation, is that the para/pineal eyes would detect threats coming from above. The fact that they are smaller than the lateral eyes suggests that they had a different and perhaps simpler visual function; this is speculative, but you could for example imagine them serving to trigger a flight reaction in response to a dark shape passing above, whereas the later eyes would be 'seeing' in a conventional sense and allow the animal to navigate its environment.
Response: We thank the referee for sharing this idea. We have rephrased the relevant sentence to highlight the potential functional differentiation between the para/pineal and lateral eyes. (see line 299)

Line 418: "continually collected"? Given that the collecting of any particular specimen is a brief and discrete action - starting when you find it and ending when you pack it for transport home - I don't understand what "continually collected" means. This needs to be reworded.
Response: We have now reworded this sentence to clarify this misunderstanding. The specimens in this study, together with thousands of other Chengjiang specimens, were collected during fieldwork between 2019 and 2024. (see line 452-455)

Figure 3: why are the lenses in the reconstruction (3b) not shown in the same position as in the specimen (3a)? There's no reason to believe that the lenses in the fossil have been displaced. The difference matters, because it has functional implications: in the reconstruction it looks like the animal is focused on what's going on in front of it, in a rather human-like way, but the specimen suggests that the para/pineal eyes looked straight upwards and the lateral eyes to the sides. The reconstruction should be modified. There's another point as well: looking at the Metaspriggina eyes shown in Fig. 3s-v, it looks like the para/pineal eyes in this animal are much smaller relative to the lateral eyes than in myllokunmingiids. This is an interesting difference, perhaps suggesting that Metaspriggina shows a more derived condition, but I don't think it's mentioned anywhere in the text.
Response: We thank the referee for these suggestions. We have now updated the reconstruction of Fig. 3b, showing the orientation of lens toward more laterally, although it deserves to point out that the orientation of eye lens is not necessarily fixed in a lateral direction. In addition, Fig. 3b is a schematic reconstruction designed to depict the four camera eyes in *Haikouichthys*. The authentic, specimen-based

drawing of the lenses is shown in Fig. 3a. We have now clarified the differences of these two reconstructions in the figure legend.

We thank the referee for pointing out the relatively small size of the median dark spots in *Metaspriggina*; we have now stated this in the text. (see line 218)

Figure 4: the lens positions should be adjusted in some of these reconstructions as well. Lampreys do NOT have stereoscopic vision! Even in *Metaspriggina*, where the eyes are anterior in position, it is not clear from the preserved lens position that the eyes looked forwards in the way shown here. In Sacabambaspis, on the other hand, it is clear that they did.

Response: We thank the referee for these suggestions. We have now updated this figure following the referee's suggestions. The schematic reconstructions in this figure are designed to highlight differences of para/pineal complex among various clades of vertebrates. They are drawn neither to scale nor in strict accordance with original positions. We now also clarify this in the figure legend.

Referee #3 (Remarks to the Author):

The authors use new specimens to reevaluate the nature of the lateral dark spots in the earliest vertbrates from the Chengjioang biota. They show that these spots have melanosomes and a lens, suggesting that they are camera-type eyes, with important implications for the evolution of vision. The authors have done a nice job responding to my comments and the those of the other reviewers.

Response: We appreciate that the referee is satisfied with our revision.

I still, however, am not convinced by the ToF-SIMS data. It does show that the fossil melanin is distinct from the sediment, but it is also distinct from other melanins, including other fossil melanin. Of course, there may be explanations for this pattern, but it should not be considered strong evidence of presence of melanin. Indeed, if this was the only evidence of the presence of melanin (which it is not), it would not be acceptable. I recommend straightforwardly acknowledging the uncertainty of these results, but pointing out that it is secondary to the morphological evidence.

Response: We agree with the referee that the ToF-SIMS data should not be used as the sole evidence to verify the presence of melanin. Following the referee's suggestions, we have now edited the relevant text in the manuscript to clarify that the ToF-SIMS results are supportive of our morphological evidence (see line 123-142).

other small point:

L36: Darwins'->Darwin's

Response: Sorry, this is now corrected.