Embryonic development in the acoel *Hofstenia miamia*

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DOI: 10.1242/dev.188656

Editor: James Briscoe

Review timeline
Original submission: 18 January 2021
Editorial decision: 4 March 2021
First revision received: 28 April 2021
Accepted: 20 May 2021

Original submission

First decision letter

MS ID#: DEVELOP/2020/188656

MS TITLE: Embryonic development in the acoel *Hofstenia miamia*

AUTHORS: Julian O. Kimura, Lorenzo Ricci, and Mansi Srivastava

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication.All three referees suggest specific points that need clarification, addressing these will help make your work more accessible. Referee 1 suggests that, to avoid confusion, you should use standard embryological terminology that allows parallels to be drawn with other bilateria and where there are uncertainties about the equivalence this should be discussed. I also agree with Referee 3's that a discussion of the insight that the developmental data bring to the phylogenetic position of Xenacoelomorpha would be helpful. The seven questions raised by Referee 3 about methodological details are also important to address.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

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how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript "Embryonic development in the acoel Hofstenia miamia" by Kimura et al. provides a detailed morphological staging and transcriptomic profiling of embryogenesis in the acoel worm Hofstenia miamia.
Acoels are one of the most enigmatic bilaterian lineages, yet their position as sister group to Nephrozoa – or sister group to Ambulacraria – makes them fundamental to our understanding of bilaterally symmetrical animals. However, few acoel species are experimentally tractable, with the species Hofstenia miamia recently emerging as the reference acoel system thanks to Mansi Srivastava's lab efforts. However, most of the work done in this species is on whole-body adult regeneration, with Hofstenia's embryogenesis being still little explored. This manuscript sets the grounds to fill this knowledge gap by (i) describing the major events happening during Hofstenia development; (ii) setting up a basic set of techniques for future dev biol studies (e.g. in situ hybridisation, embryo injections/cell labelling); and (iii) providing a stage-specific transcriptomic dataset of Hofstenia embryogenesis. Based on morphology, Kimura and coworkers define 16 stages, from the fertilised oocyte to hatching. Morphological landmarks (e.g. cell number, an anterior notch, pigmentation overall shape, etc) define and help to identify each stage. Employing intracellular cell labelling, the authors describe two major ingression events, one after cleavage and another later on at the animal/anterior pole.
Transcriptomic profiling provides gene expression data supporting the major morphological events and stages. Finally, the authors characterise the gene expression patterns of tissue-specific markers (gut muscle, pharynx, epidermis and nervous system) during embryogenesis, which serves as an indication of the timing and sequence of events during organogenesis. As it is common to Srivastava's works, the data is of high quality, nicely presented, and available via public repositories. Overall, the manuscript is a nice read and a novel and valuable contribution/resource for the evo-devo and developmental community broadly.

Best,

Chema Martin

Comments for the author

My main concern resides in the use of common terms in developmental biology to describe Hofstenia development. I have the impression the authors tend to avoid applying generally accepted terms (e.g. "blastula", "gastrula", "elongation"), using instead layman's terms that refer to external landmarks of the Hofstenia embryo. While I see the value of this decision in helping to recognise those stages, I think in the long run it will make cross-lineage comparisons more difficult and thus I would encourage the authors to use those more common terms (or at least propose equivalences between their stages and those of other bilaterian embryos). For example, from previous work on Neochildia (Henry et al; doi:10.1006/dbio.2000.962) the engulfment of the 3rd duet macromeres (that form the endomesoderm) by the micromeres would be gastrulation (i.e. endomesoderm internalisation) in an acoel embryo. While the authors perhaps wanted to be cautious because no direct cell fate mapping is yet available for Hofstenia, I think it is reasonable to assume that giving the overall similarities in cleavage and development between acoels, the fate of those two macromeres will be similar between Neochildia and Hofstenia and thus propose this stage (the "Morula") as the "Gastrula" of Hofstenia. In fact, the term "Morula" is confusing because in most other embryos, a "morula" stage occurs early on, after cleavage and before blastula and gastrulation, when the embryo is not multilayered and when there are no germ layers yet. If what the authors refer to as morula is then gastrulation, this event happens via epiboly, as illustrated in Fig. 4C. (Actually, cleavage usually does not involve major cell movements, but the drawing in Fig4C suggests the movement of the animal micromeres occurs during early cleavage). Another observation supporting similarities between Neochildia and Hofstenia is the cell labelling of 1a/1b,

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which remains very clearly (Fig 4B, animal view) restricted to two longitudinal areas of the embryo (in Neochildia these are the ventral and dorsal sides).

Regarding the second ingression of cells at the animal pole, were the authors able to track the final location of the internalised cells in the hatchling? Did labelled embryos hatched or survived imaging? Clarifications on that would be helpful. Also, from Henry et al. (doi:10.1006/dbio.2000.962), 1a/1b forms ectoderm and neurons. Could thus this be the internalisation of the apical neuroectoderm to form the brain? Or is this internalisation related to the formation of the anterior pharynx? Could the authors comment on this?

Other comments:
- Line 100: brachyury is not per se a blastoporal marker, as it is lost in some lineages which otherwise form a blastopore during embryogenesis (e.g. flatworms and nematodes) and it is not expressed in the blastopore of many other invertebrates.
- Line 177-181: The first of these two sentences is redundant. To some extent, the same issue happens in Lines 200-202.
- Line 230: how does hatching happens? Is there a hatching cap in the eggshell (e.g. as in priapulids)? Do hatchlings tear the eggshell or is the eggshell resorbed?
- Line 511: why was a different criterion used to called differential expression between Pill and Prehatchling?
These stages do not separate that clearly in the PCA (Fig 5A). Is this a technical issue [sample collection] or is it true biological signal [the stages are probably very similar; Pill seems to be a short stage, based on Fig6A]? For Fig 5A, it would be good to indicate values in the axes and % of variance explained by PC1 and PC2.

Reviewer 2

Advance summary and potential significance to field

Kimura and collaborators present in the current manuscript a thorough investigation of the embryonic development of the acoel flatworm Hofstenia miamia. Using a combination of methodologies, from morphological observation to the analysis of transcriptomes and in situ hybridization, the authors provide a unique and detailed description of embryonic stages that they have defined. The result is an excellent study that nonetheless has some limitations (see below).

Comments for the author

While the study presents many different findings, I found the most relevant to be that acoels have a rather plastic system of embryogenesis. In addition to the so-called “duet spiral cleavage,” different clades seem to use variations on the timing and pattern of early cleavages. This finding is in line with others that have demonstrated that acoels’ tissues build in highly diverse configurations (e.g., in terms of neural architectures, the presence of a pharynx, and the position of the mouth).

The diversity of constructional modes has, somehow, an equivalent in early cleavage patterns. I find the second ingression of cells at the animal pole to be especially interesting, as this has not previously been reported for any acoel, and is a rare finding in bilaterians. This should be further investigated using more detailed lineage tracing and cell ablations, and perhaps by looking for specific molecular determinants at both poles, as have been described in Clytia.

My major criticism is that this work provides an initial description of the embryogenesis, and although the descriptions of the stages are appreciated, readers are left without the details of a fate map. There are also some useful experiments with the injection of micromeres, but these are still insufficient. Moreover, without ablation experiments, the specific contributions of blastomeres to cell lineages are very limited. The work of Henry et al. (2000) would have been a better guide to the understanding of early embryogenesis in Hofstenia. Furthermore, the extent to which the transcriptomic analysis was necessary is unclear, unless the selection of tissue markers derives from the analysis of these transcriptomes. I do not consider the inclusion of transcriptomic data to be a requirement to present the main message of the paper.

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Some of my minor comments on the manuscript – which I would like the authors to address – are listed below:

- The introduction contains some repetition. For instance, lines 88–92 and 104–110 are almost the same. Please revise this.
- In the description of the Morula stage, the detection of chromosomes is mentioned. How many were the authors able to detect and were all of similar size?
- The authors mention the use of tissue markers and that they are known but have not provided a reference for this. In addition, why did they select these markers? Was their selection based on previous studies or were the markers selected based on their profiles in the transcriptome data?
- The discussion is focused. However, when the authors report the variability of cleavage in acoels and indicate that Hofstenia probably represents the ancestral state, why have they not compared (or mentioned) the studies conducted on Meara (a nemertodermatid)? Borve and Hejnol's paper comes to mind.

Reviewer 3

Advance summary and potential significance to field

Kimura and colleagues unveil a comprehensive description of embryonic development for the acoel Hofstenia miamia, an emerging regenerative research organism informed by embryo morphology and transcriptomic data. This staging series was meticulously executed, clearly described, and aesthetically pleasing in its presentation. The prototypic imagery and movies are beautiful! This foundational work provides a strong basis for future fate mapping and functional studies to further elucidate key aspects of development in this species. As such, the work is appropriate for publication in the Resources section of Development. Points for clarification are addressed to the authors below.

Comments for the author

Introduction:
Given the contentious debate over the phylogenetic position of Xenacoelomorpha – are they basal bilaterians (Nephrozoa), an outgroup to the deuterostome lineage (Ambulacraria), or a member of the phylum Platyhelminthes – what are your opinions on how your embryonic staging data adds to the debate? Should the early embryonic cleavage pattern supersede claims made using sequencing data, or does it corroborate one interpretation on how to classify these animals? To me, it seems like prioritizing the sequencing data to make this call is problematic if the animals are highly divergent and we have a dearth of information (i.e., would the interpretation change if we had better coverage from more acoel species?). Is there good evidence to suggest that duet cleavage is not a derived form of Spiralian cleavage? This issue is brought up again in your discussion (lines 392-408), and given the uncanny similarities of acoel biology, as a whole, with that of freshwater and parasitic flatworms it seems like you should be open-minded to the possibility that duet cleavage could be a modified form of spiralian cleavage.

From my perspective, Hofstenia miamia's value as a research organism for regenerative and developmental biology is not diminished by the outcome of this debate, but I do see it as an area where your data may inform prevailing thoughts about this topic.

Materials and Methods; Figure 1; Figure S1:
To aid others establishing Hofstenia colonies, can you please comment on the following, or refer readers to other publications that would contain the following information:
1. Were the Hofstenia cultures used to report the staging series from an outbred population or an inbred strain?
2. From your description, it appears that mating and embryo laying are spontaneous events in your cultures. Do Hofstenia store sperm? I am wondering how the collections detailed here would be different than matings needed to perform directed crosses (e.g., between transgenic lines).
3. How do adult age, feed schedule, and culture density impact fertility rates (% viable embryos laid; number of viable embryos laid per adult per day)?

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4. How many embryos are laid by a mating pair post-lay (i.e., what is the size of a typical clutch)? Do you have any feel for the number of zygotes laid per animal per day, or the periodicity at which embryos are produced by mating pairs?
5. What is the reproductive lifespan of Hofstenia? (i.e., how long will sexually mature adults remain optimal for embryo production).
6. What was the rationale for culturing embryos at higher temperature than the laying adults post-collection?
7. What is the artificial seawater source/recipe for adult Hofstenia, and is this recipe modified at all for embryo culture?
Figure 2:
Please fix the grammatical error in Figure 2A, Zygote, description.

Lines 177-181: The sentences are partially redundant and start with the same phrase. The language can be cleaned up here.

Lines 204-207: You mention movement of embryos within the egg shell, without contraction/body wall movement. Do you have any evidence that the outer cell layer of the embryos is ciliated (e.g., acetylated tubulin antibody staining data?) This is not critical to address for this publication, but commercially available acetylated tubulin antibodies (Sigma T7451) that cross-react broadly across species may also work well for your samples.

Lines 140-237: As you describe morphology typical of each defined stage of embryogenesis, it would also be helpful to delineate when the adult body axes are specified and when adult organogenesis gets underway, to the extent that this is possible. (My guess is that the axes are specified by the start of the Pill stage prior to the shape changes that are observed, and that organogenesis is underway during or after the dimple stage).

Lines 259-261 and Movie S4:
“The internalizing cells appeared to be less bright, retaining less of the fluorescent label relative to their neighboring cells that don’t become internalized. This suggests that the internalizing cells had undergone more cell divisions.”

Are these cells truly retaining less label? Differences in intensity look like they could correlate with state/shape of the cells, differences in intensity could be due to depth relative to the focal plane once the cells are internalized.

Without substantive evidence to support this claim I would temper or remove speculative comments.

RNA-Seq time course analysis (Figure 5):
Figure 5B: Cluster 7’s behavior is interesting, but the GO annotation appears to be a misnomer – these aren’t likely to be neural biomarkers per se, but rather genes that function in a biological process typically associated witn neurons (cell adhesion? Pathfinding? Cell migration?)

What is known about when the MZT occurs in acoel embryos, and whether this is a phased, multi-step process? I realize that, given the pooling of the earliest cleavages and lack of an oocyte sample, that this can’t be stated definitively from your data?

Based on adult Hofstenia transcriptome data, and homology-based comparison with other species (e.g., planarians), can you infer more about the timeline for organogenesis by looking at genes that may be specific to early lineage-specific progenitors, and not just terminally differentiated cell types?

Figure 6B: Please include the orientation of the embryos, relative to the Animal-Vegetal axis, prior to the shape changes. Are these lateral views? It would be helpful for non-expert readers if each series of WISH panels was labeled with the tissue type (e.g., gut, muscle, etc) – there’s plenty of room to do that to the left of the panels.

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Figure 7: It would be nice to include the chronological timeline, along with labels for the developmental milestones you've noted at other points in the paper in the summary model figure (e.g., the two cell internalization events, the start of organogenesis).

Discussion:
Given the eccentricities of neoblast-driven development, I would steer clear of too heavy-handed a discussion about the necessity for germ layers to generate a triploblastic animal. It will be interesting in the future to see whether the epiboly and invagination events that you see correlate with cell fate specification, what cell cycle behavior looks like during the course of embryogenesis, and to investigate how the construction of the adult anatomy relates to neoblast-driven postnatal maintenance of the body plan.

First revision

Author response to reviewers' comments

Response to Reviewer Comments

We thank the reviewers for their careful reading of the paper and for recognizing the value of this work. Their comments have helped us improve the manuscript. We explain below in detail how we have addressed most of the comments of the reviewers.

Reviewer 1

The manuscript "Embryonic development in the acoel Hofstenia miamia" by Kimura et al. provides a detailed morphological staging and transcriptomic profiling of embryogenesis in the acoel worm Hofstenia miamia. Acoels are one of the most enigmatic bilaterian lineages, yet their position as sister group to Nephrozoa – or sister group to Ambulacraria – makes them fundamental to our understanding of bilaterally symmetrical animals. However, few acoel species are experimentally tractable, with the species Hofstenia miamia recently emerging as the reference acoel system thanks to Mansi Srivastava's lab efforts. However,most of the work done in this species is on whole- body adult regeneration, with Hofstenia's embryogenesis being still little explored. This manuscript sets the grounds to fill this knowledge gap by (i) describing the major events happening during Hofstenia development; (ii) setting up a basic set of techniques for future dev biol studies (e.g. in situ hybridisation, embryo injections/cell labelling); and (iii) providing a stage-specific transcriptomic dataset of Hofstenia embryogenesis. Based on morphology, Kimura and coworkers define 16 stages, from the fertilised oocyte to hatching. Morphological landmarks (e.g. cell number, an anterior notch, pigmentation, overall shape, etc) define and help to identify each stage. Employing intracellular cell labelling, the authors describe two major ingression events, one after cleavage and another later on at the animal/anterior pole. Transcriptomic profiling provides gene expression data supporting the major morphological events and stages. Finally, the authors characterise the gene expression patterns of tissue-specific markers (gut, muscle, pharynx, epidermis and nervous system) during embryogenesis, which serves as an indication of the timing and sequence of events during organogenesis. As it is common to Srivastava's works, the data is of high quality, nicely presented, and available via public repositories. Overall, the manuscript is a nice read and a novel and valuable contribution/resource for the evo-devo and developmental community broadly.

Best,

Chema Martin

We thank Dr. Martin for the in-depth reading of the manuscript, for the constructive comments, and for the generous comments about the quality of our work.

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My main concern resides in the use of common terms in developmental biology to describe Hofstenia development. I have the impression the authors tend to avoid applying generally accepted terms (e.g. "blastula", "gastrula", "elongation"), using instead layman's terms that refer to external landmarks of the Hofstenia embryo. While I see the value of this decision in helping to recognise those stages, I think in the long run it will make cross-lineage comparisons more difficult and thus I would encourage the authors to use those more common terms (or at least propose equivalences between their stages and those of other bilaterian embryos). For example, from previous work on Neochildia (Henry et al; doi:10.1006/dbio.2000.962) the engulfment of the 3rd duet macromeres (that form the endomesoderm) by the micromeres would be gastrulation (i.e. endomesoderm internalisation) in an acoel embryo. While the authors perhaps wanted to be cautious because no direct cell fate mapping is yet available for Hofstenia, I think it is reasonable to assume that giving the overall similarities in cleavage and development between acoels, the fate of those two macromeres will be similar between Neochildia and Hofstenia and thus propose this stage (the "Morula") as the "Gastrula" of Hofstenia. In fact, the term "Morula" is confusing because in most other embryos, a "morula" stage occurs early on, after cleavage and before blastula and gastrulation, when the embryo is not multilayered and when there are no germ layers yet. If what the authors refer to as morula is then gastrulation, this event happens via epiboly, as illustrated in Fig. 4C. (Actually, cleavage usually does not involve major cell movements, but the drawing in Fig4C suggests the movement of the animal micromeres occurs during early cleavage). Another observation supporting similarities between Neochildia and Hofstenia is the cell labelling of 1a/1b, which remains very clearly (Fig 4B, animal view) restricted to two longitudinal areas of the embryo (in Neochildia these are the ventral and dorsal sides).

Thank you for your suggestions regarding the naming convention of our embryonic stages. We agree that having a common staging terminology with other embryos would greatly help with cross-lineage comparisons of development. As you mentioned, there are many parallels that could be drawn between *Hofstenia* and *Neochildia* development. *Hofstenia* differs in that they undergo a second, previously undescribed internalization event at the Dimple stage, making it difficult to ascertain when gastrulation truly occurs in this system. Given this uncertainty, we had chosen alternative nomenclature, to minimize making assumptions about the embryo. However, you are absolutely right in that the name “morula” is rather confusing, as it is defined as a ball of cells that result from the division of a single celled zygote. Whereas in *Hofstenia*, this stage is reached through the internalization of macromeres. Given that this process is conserved among acoels, and has been shown in *Neochildia* to be gastrulation, we have renamed this stage as the Gastrula.

Regarding the second ingression of cells at the animal pole, were the authors able to track the final location of the internalized cells in the hatchling? Did labelled embryos hatched or survived imaging? Clarifications on that would be helpful. Also, from Henry et al. (doi:10.1006/dbio.2000.962), 1a/1b forms ectoderm and neurons. Could thus this be the internalization of the apical neuroectoderm to form the brain? Or is this internalization related to the formation of the anterior pharynx? Could the authors comment on this?

This is an excellent point. We were also curious about what the cells at the Dimple stage gave rise to. We found that when we injected the 1a and 1b cells with fluorescent dextran, the embryos hatched, but we had some difficulty detecting bright signal deriving from the fluorescent dye. What’s more, given that not all of the 1a and 1b progeny internalize at the Dimple, more rigorous approaches are needed to specifically target the internalized cells. This is an important next step for us, and we are developing several approaches, including a transgenic line where we would utilize photoconversion to trace the cells.

Other comments:
- Line 100: brachyury is not per se a blastoporal marker, as it is lost in some lineages which otherwise form a blastopore during embryogenesis (e.g. flatworms and nematodes) and it is not expressed in the blastopore of many other invertebrates.

Thank you for your insightful comment. We agree that with the current wording, it suggests that *brachyury* is a highly conserved blastopore marker, when there are several instances

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where it does not mark the blastopore. We have re-worded both line 100 and line 242 to highlight the fact that the presence of *brachyury* is simply one line of evidence that suggests the internalization of macromeres in *Neochildia* is gastrulation.

- Line 177-181: The first of these two sentences is redundant. To some extent, the same issue happens in Lines 200-202.

Thank you for catching the awkward wording and redundancy in the paper. We have made the necessary edits to these lines in the paper.

- Line 230: how does hatching happens? Is there a hatching cap in the eggshell (e.g. as in priapulids)? Do hatchlings tear the eggshell or is the eggshell resorbed?

We have found that the worm appears to tear out of the eggshell during hatching. However, we have not been able to identify a "weak point" in the shell like a hatching cap where the embryo could escape from. This hatching event could be seen in two different individuals at the end of Movie S1 at 2:02 and 2:18.

- Line 511: why was a different criterion used to called differential expression between Pill and Prehatchling? These stages do not separate that clearly in the PCA (Fig 5A). Is this a technical issue [sample collection] or is it true biological signal [the stages are probably very similar; Pill seems to be a short stage, based on Fig6A]? For Fig 5A, it would be good to indicate values in the axes and % of variance explained by PC1 and PC2.

The different criterion was used because differential expression between the Pill and Prehatchling stages resulted in no genes that retained statistical significance after multiple test correction (False Discovery Rate Adjusted p- value). Regardless, the non-corrected p-values derived from the Likelihood Ratio Test provided an ordered list of genes, so we relied on them for our analyses. We believe that the PCA plot and differential expression analysis both reflect true biological signal, with these stages possessing similar transcriptomic profiles. We have now indicated the values for the percent of variance explained by PC1 and PC2 on the axes Figure 5.

Reviewer 2

Kimura and collaborators present in the current manuscript a thorough investigation of the embryonic development of the acoel flatworm Hofstenia miamia. Using a combination of methodologies, from morphological observation to the analysis of transcriptomes and in situ hybridization, the authors provide a unique and detailed description of embryonic stages that they have defined. The result is an excellent study that nonetheless has some limitations (see below).

We thank the reviewer for their thorough reading of the manuscript and for their constructive comments.

While the study presents many different findings, I found the most relevant to be that acoels have a rather plastic system of embryogenesis. In addition to the so-called "duet spiral cleavage," different clades seem to use variations on the timing and pattern of early cleavages. This finding is in line with others that have demonstrated that acoels' tissues build in highly diverse configurations (e.g., in terms of neural architectures, the presence of a pharynx, and the position of the mouth). The diversity of constructional modes has, somehow, an equivalent in early cleavage patterns. I find the second ingression of cells at the animal pole to be especially interesting, as this has not previously been reported for any acoel, and is a rare finding in bilaterians. This should be further investigated using more detailed lineage tracing and cell ablations, and perhaps by looking for specific molecular determinants at both poles, as have been described in Clytia.

We agree with Reviewer #2 that there are indeed differences in the cleavage pattern and body/tissue configurations among acoels. However, the differences in cleavage order are minor with a few exceptions, and the core of the duet cleavage program as a whole appears

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to be highly conserved. This shows interesting parallels to the Spiralia, which is a group of morphologically diverse lineages whose embryos undergo stereotyped spiral cleavage. The spiral cleavage program, which we know to be highly conserved, shows variations in cleavage and gives rise to an even wider diversity of body plans (including neural architectures) depending on the species (ranging from flatworms, to annelids, to molluscs etc.).

We also completely agree that given the conserved nature of the duet program, the second cell internalization event, which is unique, becomes all the more interesting. We are very excited about what these cells do.
However, straightforward dye labeling approaches are not sufficient here, as we would need to specifically label all cells that are internalizing at the Dimple event. As you can see from Movie S4, a large number of cells are internalized and it is unclear whether the cells internalized are all derived from the same early blastomeres. Only a subset of the progeny of labeled 1a/1b micromeres are internalized, for example. We have attempted an approach at labeling large swaths of cells using a technique called the “buzz box”, which uses an electric current to transfer dil to the cells. We found that this technique was harsh on our embryos, resulting in low viability after labeling. What’s more, the dye transfer did not occur very efficiently. We are currently developing more robust approaches to tackle the question of cell lineage contributions, such as making a transgenic line where cells can be photoconverted for lineage tracing. However, this new work would be beyond the scope of this manuscript. We are also currently working on identifying molecular determinants of the animal-vegetal axis, but this is also a substantial project in its own right.

My major criticism is that this work provides an initial description of the embryogenesis, and although the descriptions of the stages are appreciated, readers are left without the details of a fate map. There are also some useful experiments with the injection of micromeres, but these are still insufficient. Moreover, without ablation experiments, the specific contributions of blastomeres to cell lineages are very limited. The work of Henry et al. (2000) would have been a better guide to the understanding of early embryogenesis in Hofstenia. Furthermore, the extent to which the transcriptomic analysis was necessary is unclear, unless the selection of tissue markers derives from the analysis of these transcriptomes. I do not consider the inclusion of transcriptomic data to be a requirement to present the main message of the paper.

Once again, the reviewer is right. A fate map is crucial data in understanding the early development of any system, and is something that will be needed to gain further insight into *Hofstenia* development. The characterization of embryonic development as presented in this paper was a much needed first step to make sense of a fate map in a future study. As stated in our response to Reviewer #1, we did attempt to perform tracings, but traditional dye-based tracing approaches will not be sufficient for obtaining a fate map for *Hofstenia*. We look forward to having more insights on this as part of our next project.

Some of my minor comments on the manuscript – which I would like the authors to address – are listed below:

• The introduction contains some repetition. For instance, lines 88–92 and 104–110 are almost the same. Please revise this.

We apologize for the awkwardness of the introduction. We have made the necessary revisions.

• In the description of the Morula stage, the detection of chromosomes is mentioned. How many were the authors able to detect and were all of similar size?

Although individual chromosomes were detectable, the exact number of individual chromosomes was difficult to ascertain. However, we did find that all of the chromosomes appeared to be of similar size.

• The authors mention the use of tissue markers and that they are known but have not provided a reference for this. In addition, why did they select these markers? Was their

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selection based on previous studies or were the markers selected based on their profiles in the transcriptome data?

The tissue markers we included in the manuscript were chosen based on different screens done in the lab. Over the years we have tested candidate genes and generated different datasets which resulted in the identification of genes that mark specific tissues in the hatched worm. We have added this information to the methods section of the manuscript.

• The discussion is focused. However, when the authors report the variability of cleavage in acoels and indicate that Hofstenia probably represents the ancestral state, why have they not compared (or mentioned) the studies conducted on Meara (a nemertodermatid)? Borve and Hejnol's paper comes to mind.

This is an excellent point, and we have incorporated a comparison of the cleavage patterns between *Meara* and
Hofstenia in our discussion.

Reviewer 3

Kimura and colleagues unveil a comprehensive description of embryonic development for the acoel Hofstenia miamia, an emerging regenerative research organism, informed by embryo morphology and transcriptomic data. This staging series was meticulously executed, clearly described, and aesthetically pleasing in its presentation. The prototypic imagery and movies are beautiful! This foundational work provides a strong basis for future fate mapping and functional studies to further elucidate key aspects of development in this species. As such, the work is appropriate for publication in the Resources section of Development. Points for clarification are addressed to the authors below.

We thank the reviewer for the extremely generous comments on our manuscript!

Introduction:
Given the contentious debate over the phylogenetic position of Xenacoelomorpha – are they basal bilaterians (Nephrozoa), an outgroup to the deuterostome lineage (Ambulacraria), or a member of the phylum Platyhelminthes – what are your opinions on how your embryonic staging data adds to the debate? Should the early embryonic cleavage pattern supersede claims made using sequencing data, or does it corroborate one interpretation on how to classify these animals? To me, it seems like prioritizing the sequencing data to make this call is problematic if the animals are highly divergent and we have a dearth of information (i.e., would the interpretation change if we had better coverage from more acoel species?). Is there good evidence to suggest that duet cleavage is not a derived form of Spiralian cleavage? This issue is brought up again in your discussion (lines 392-408), and given the uncanny similarities of acoel biology, as a whole, with that of freshwater and parasitic flatworms it seems like you should be open-minded to the possibility that duet cleavage could be a modified form of spiralian cleavage. From my perspective, Hofstenia miamia's value as a research organism for regenerative and developmental biology is not diminished by the outcome of this debate, but I do see it as an area where your data may inform prevailing thoughts about this topic.

The reviewer's point is important and duly noted. Indeed, the debate in terms of the placement of Xenacoelomorpha on the metazoan phylogeny is a very important one. Ever since (Ruiz-Trillo et al., 1999) first applied molecular data to study the question of the placement of acoels, no analysis has placed acoels as part of the phylum Platyhelminthes. The current debate is about whether acoels belong to the earliest-diverging bilaterian lineage or placed closer to vertebrates within ambulacraria. These analyses have applied genome-scale data from several acoel species (Hejnol et al., 2009; Jondelius et al., 2011; Kapli and Telford, 2020; Marlétaz et al., 2019; Mwinyi et al., 2010; Philippe et al., 2007, 2011, 2019; Ruiz-Trillo et al., 1999, 2002, 2004; Ruiz-Trillo and Paps, 2016; Sempere et al., 2007; Telford et al., 2003).
While morphological and developmental traits were used to build phylogenies prior to the availability of molecular data, the state-of-the-art approach to inferring relationships of organisms is to utilize phylogenomics, where statistical frameworks and models of sequence

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evolution are applied to large-scale transcriptome and genome data. Phylogenies built with molecular data can then serve as the backbone for making inferences about trait evolution. For example, regardless of the current debate, all phylogenomicists agree that acoels and platyhelminthes are distantly-related. This tells us that duet cleavage is unlikely to be a simplified version of spiral cleavage, unless spiral cleavage was the ancestral cleavage program for all bilaterians. We agree with the reviewer, the similarity is uncanny, but a major emerging theme in evolutionary biology has been that convergent evolution of traits is a lot more common than we had previously assumed. We think the similarity is an exciting opportunity to study and compare molecular mechanisms between duet-cleavers and spiral-cleavers because that can tell us something about how developmental mechanisms evolve.

Materials and Methods; Figure 1; Figure S1:
To aid others establishing Hofstenia colonies, can you please comment on the following, or refer readers to other publications that would contain the following information:

1. Were the Hofstenia cultures used to report the staging series from an outbred population or an inbred strain?

The *Hofstenia* cultures represent many generations of worms derived from a group of 120 worms collected from Bermuda in 2010. The embryos used were the progeny generated through the random matings of these worms, making them a polymorphic, lab-bred population. Thus these animals are indeed inbred, but the number of generations has not been counted. We have added this information to the methods section.

2. From your description, it appears that mating and embryo laying are spontaneous events in your cultures. Do Hofstenia store sperm? I am wondering how the collections detailed here would be different than matings needed to perform directed crosses (e.g., between transgenic lines).

You are correct, mating and embryo laying are spontaneous events in our cultures, and it is unknown whether *Hofstenia* store sperm. The embryos here were collected daily from plastic boxes that have about 20-30 sexually mature animals. Thus, we did not keep track of which individual was mating with which. If, however, we needed to perform directed crosses, we would simply place two individuals together in a single box, and allow them to mate. To account for the possibility that *Hofstenia* could store sperm from previous mating partners, we would place two individuals who have yet to reach sexual maturity together, and wait until they start mating. We have added this information to the methods section.

3. How do adult age, feed schedule, and culture density impact fertility rates (% viable embryos laid; number of viable embryos laid per adult per day)?

Typically, adults produce about four embryos per week, totaling to 100's of embryos per day in laboratory culture as shown in (Srivastava et al., 2014). We have found that the optimal density appears to be about 20-30 worms in about 1L of water, as higher density beyond this resulted in cannibalism. In terms of the proportion of viable embryos laid, we have not observed any variations in viability. However, this observation has not been quantified. We have added this information to the methods section.

4. How many embryos are laid by a mating pair post-lay (i.e., what is the size of a typical clutch)? Do you have any feel for the number of zygotes laid per animal per day, or the periodicity at which embryos are produced by mating pairs?

We have found that a typical clutch consists of about 4-7 embryos, but can be as small as 1, and large as 20-30 embryos. We have found that a natural periodicity occurs, where the animals seem to lay more embryos during the night. However, this could be simply due to the fact that these cultures are not disturbed by lab members opening incubator doors, or collecting embryos. We have added this information to the methods section.

5. What is the reproductive lifespan of Hofstenia? (i.e., how long will sexually mature adults

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remain optimal for embryo production).

Typically, upon reaching sexual maturity, adults will retain a high embryo production rate for about 6-8 months. However, given proper care, we have found that adults over a year old will still produce embryos at a rate large enough to support experiments. We have added this information to the methods section.

6. What was the rationale for culturing embryos at higher temperature than the laying adults post-collection?

Time-lapse imaging was done at room temperature (the room we performed the imaging was kept at a constant 23˚C). To maintain consistency in the timing of different morphological milestones being detected in the time- lapse, the embryos were also cultured at 23˚C. We have added this information to the methods section.

7. What is the artificial seawater source/recipe for adult Hofstenia, and is this recipe modified at all for embryo culture?

The artificial seawater is made using instant ocean sea salt. Instant ocean sea salt was mixed with deionized water to create a solution with a salinity of 37ppt, and with a pH that falls within the range of 7.8-8.0. This recipe is not modified for embryo culture. We have added this information to the methods section.

Figure 2:

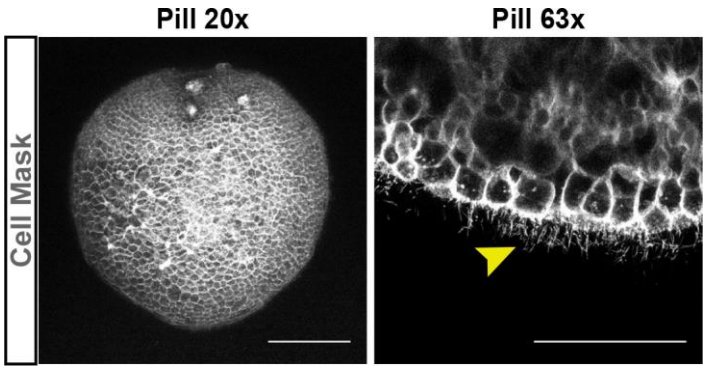
Please fix the grammatical error in Figure 2A, Zygote, description. We have made the necessary corrections.

Lines 177-181: The sentences are partially redundant and start with the same phrase. The language can be cleaned up here.

We apologize for the awkward phrasing and have made edits to this section.

Lines 204-207: You mention movement of embryos within the egg shell, without contraction/body wall movement. Do you have any evidence that the outer cell layer of the embryos is ciliated (e.g., acetylated tubulin antibody staining data?) This is not critical to address for this publication, but commercially available acetylated tubulin antibodies (Sigma T7451) that cross-react broadly across species may also work well for your samples.

We found that cell mask (Invitrogen C37608) works well in staining the cilia in our animals. 63x imaging at the Pill stage revealed the outer cell layer of the embryo is indeed ciliated. This panel is now Figure S4.



Lines 140-237: As you describe morphology typical of each defined stage of embryogenesis, it would also be helpful to delineate when the adult body axes are specified and when adult organogenesis gets underway, to the extent that this is possible. (My guess is that the axes are

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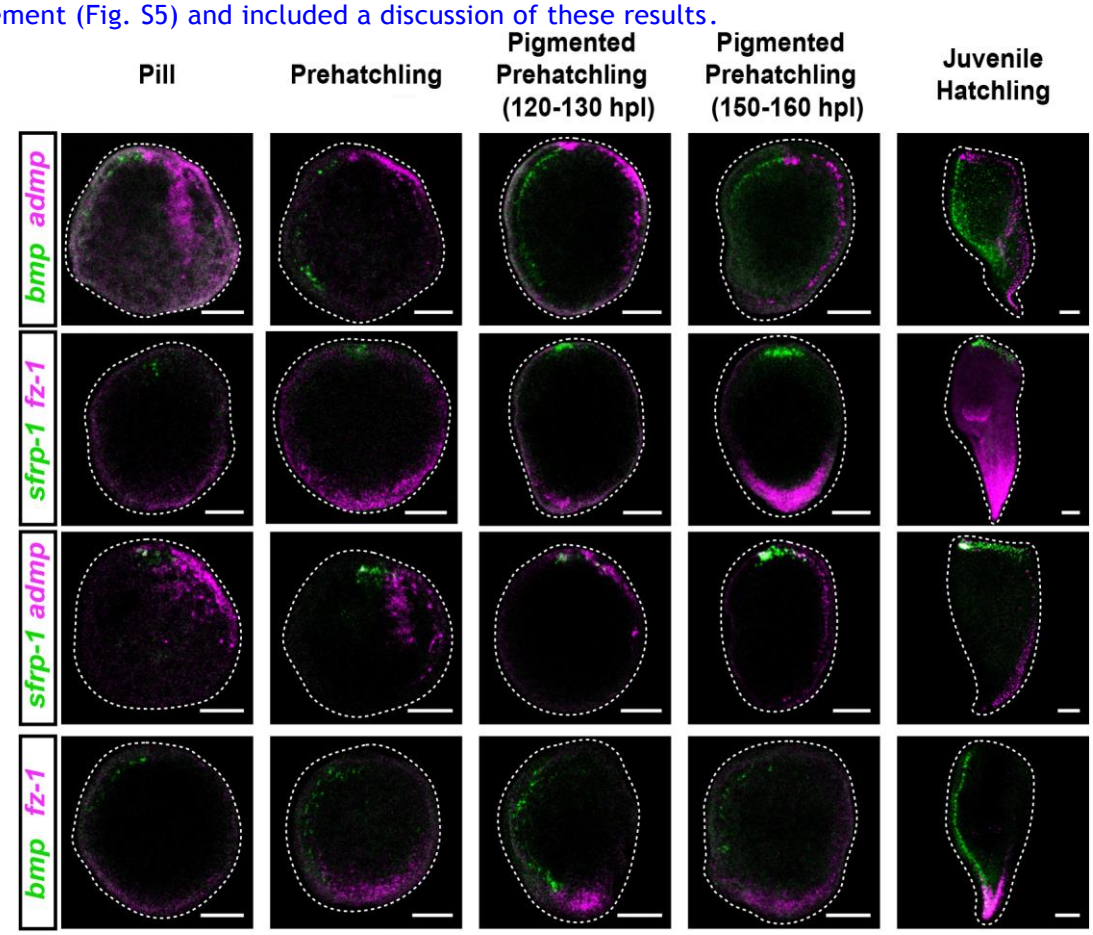
specified by the start of the Pill stage, prior to the shape changes that are observed, and that organogenesis is underway during or after the dimple stage).

You are correct! Axis markers are situated on opposite poles to one another at the Pill stage. The genes *bmp* and *admp* mark the dorsal and ventral axes respectively, while *sfrp-1* and *fz-1* mark the anterior and posterior axes respectively. We have added this figure to the supplement (Fig. S5) and included a discussion of these results.



Lines 259-261 and Movie S4:
"The internalizing cells appeared to be less bright, retaining less of the fluorescent label relative to their neighboring cells that don't become internalized. This suggests that the internalizing cells had undergone more cell divisions."Are these cells truly retaining less label? Differences in intensity look like they could correlate with state/shape of the cells, differences in intensity could be due to depth relative to the focal plane once the cells are internalized.
Without substantive evidence to support this claim I would temper or remove speculative comments.

The Reviewer provides an excellent point, and we have removed speculative comments.

RNA-Seq time course analysis (Figure 5):

Figure 5B: Cluster 7's behavior is interesting, but the GO annotation appears to be a misnomer – these aren't likely to be neural biomarkers per se, but rather genes that function in a biological process typically associated with neurons (cell adhesion? Pathfinding? Cell migration?)

We agree with the reviewer, it is indeed unlikely that neural biomarkers are present this early in development. We have clarified this.

What is known about when the MZT occurs in acoel embryos, and whether this is a phased,

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multi-step process? I realize that, given the pooling of the earliest cleavages and lack of an oocyte sample, that this can't be stated definitively
from your data?

It is currently unknown about when/how MZT occurs in acoel embryos, and unfortunately, given that we do not have an oocyte sample, we cannot definitively know from our data. We do find that the shift in transcriptional profile at the Dimple stage is quite interesting, but we cannot directly tie this to MZT. During the review period, we tried using a Click-iT RNA AlexaFluor 594 Imaging Kit (C10330) to detect newly synthesized RNA using 5-ethynyl uridine (EU), but this experiment was not successful.

Based on adult Hofstenia transcriptome data, and homology-based comparison with other species (e.g., planarians), can you infer more about the timeline for organogenesis by looking at genes that may be specific to early lineage-specific progenitors, and not just terminally differentiated cell types?

We thank the reviewer for bringing up this point. Unfortunately, very few markers chosen based on homology are guaranteed to be associated with certain organ/tissue types in a particular metazoan species. For example, although *pax6* is known to be required for eye formation in many species such as mice and flies, it is not involved in this process in planarians. The gene *myoD,* which is associated with muscle formation in many systems including planarians, is not encoded in the *Hofstenia* transcriptome. An even more direct example would be how *foxA*, which marks the pharynx in planarians, does not mark the pharynx in *Hofstenia*. Instead, we find that *foxA* is expressed among cells that are in a distribution similar to that of neoblasts (see image below). We think that the question of figuring out when organogenesis begins is an important one, but it will require us to first identify *Hofstenia*-specific specification programs for these organs/tissues.

Figure 6B: Please include the orientation of the embryos, relative to the Animal- Vegetal axis, prior to the shape changes. Are these lateral views? It would be helpful for non-expert readers if each series of WISH panels was labeled with the tissue type (e.g., gut, muscle, etc) – there's plenty of room to do that to the
left of the panels.

We thank the Reviewer for the insightful comment on this particular panel. We agree that the orientation of the embryos and labeling of the tissue types would greatly help with the reader's ability to interpret the data. We have made the necessary changes.

Figure 7: It would be nice to include the chronological timeline, along with
labels for the developmental milestones you've noted at other points in the paper, in the summary model figure (e.g., the two cell internalization events, the start of organogenesis).

We agree, and have added a chronological timeline to our summary figure.

Discussion:
Given the eccentricities of neoblast-driven development, I would steer clear of too heavy-handed a discussion about the necessity for germ layers to generate a triploblastic animal. It will be interesting in the future to see whether the epiboly and invagination events that you see correlate with cell fate specification, what cell cycle behavior looks like during the course of embryogenesis, and to investigate how the construction of the adult anatomy relates to neoblast-driven postnatal maintenance of the body plan.

This is a fantastic point. *Hofstenia* is unique in that they possess pluripotent stem cells that are utilized for continuous tissue turnover in the adult. However, embryonic development in this species appears to occur in a very stereotyped way, reminiscent of spiralian embryos where there are defined fates to particular blastomeres. This brings about the question of whether *Hofstenia* embryos undergo a classical developmental model where cells differentiate into specific germ layers first, and then later switch to a neoblast-driven mode of development. We agree that this is worth delving into in the discussion and have expanded on this topic.

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We also agree that it would be interesting to see whether the two internalization events result in fate specification, and whether cell cycle behavior could shed light on whether *Hofstenia* embryos undergo a “switch” where the neoblasts start to contribute to mature cell types.

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Second decision letter

MS ID#: DEVELOP/2020/188656

MS TITLE: Embryonic development in the acoel <em fr-original-style=3D"font-style: italic; margin: 0px; padding: 0px; border: 0px; outline: 0px; font-size: 100%; vertical-align: baseline; background: transparent;" style=3D"font-style: italic; margin: 0px; padding: 0px; border: 0px; outline: 0px; font-size: 100%; vertical-align: baseline; background: transparent;">Hofstenia miamia

AUTHORS: Julian O. Kimura, Lorenzo Ricci, and Mansi Srivastava
ARTICLE TYPE: Techniques and Resources Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. I'd also encourage you to follow Referee 3's advice and ensure instance of "Morula" are replaced in the text. The referee reports on this version are appended below.

Reviewer 1

Advance summary and potential significance to field

The authors have incorporated and/or addressed all my previous comments and I think the manuscript is now improved. The work by Kimura et al is a nice piece of work that will definitely serve as basis for exciting future work in acoels!

Comments for the author

I don't have further comments.

Reviewer 2

Advance summary and potential significance to field

The authors describe here the embryogenesis of the acoel flatworm Hofstenia miamia. To do this, they rely, mostly, on morphological features of the different developmental stages. It is true that I would have liked to see a lineage map her but I agree with the authors that this is a primary (and necessary) step for understanding Hofstenia's embryogenesis.

Comments for the author

The authors have addressed all my questions/doubts. It is true that I would have liked to see a lineage map her but I agree with the authors that this is a primary (and necessary) step for understanding Hofstenia's embryogenesis. I recommend publication in the current form. Congratulations.

Reviewer 3

Advance summary and potential significance to field

The revised manuscript by Kimura and colleagues reads well and it provides a wealth of detailed, practical descriptive knowledge that will surely aid newcomers to the field. It is an invaluable educational resource and is a strong foundational work for future studies. All reviewer questions that were feasible to address for this study were handled appropriately and in full. Kudos to the authors for assembling an impressive body of work during an especially challenging time.

Comments for the author

I would urge the authors to comb through the text, figure legends, and supplementary material to be sure that all instances of the word "Morula" are replaced with the new term, "Gastrula:" e.g., Movie S1; Figure S3 legend, Figure 6A.

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