A newly identified left–right asymmetry in larval sea urchins

Jason Hodin, Keegan Lutek and Andreas Heyland

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Original submission: 26 February 2016
1st revised submission: 20 May 2016
2nd revised submission: 26 July 2016
Final acceptance: 29 July 2016

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSOS-160139.R0 (Original submission)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
No

Are the interpretations and conclusions justified by the results?
No

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
Sorry I did not look into this

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
Yes
Recommendation?
Major revision is needed (please make suggestions in comments)

Comments to the Author(s)
This manuscript by Hodin et al is interesting – although it is an odd mix of developmental biology and ecology with applications for biomechanics. As a result the text appears a bit too broad for me. One is also left wondering what is the significance of the work especially as it appears to involve a very low sample size – with regard to the independent source of data – the parent or family. I would like to see the stats revisited. Because I am most concerned about the methods and how the data were handled I will focus on that aspect – with a few comments elsewhere.

1. Dendraster – crosses were set up with 2 females. Thus in my view here n=2 so that all they can do is a t-test. Larvae are not independent data sources – they are nested in female. For the other species n=4 so that is a bit better. Thus the conclusion on pg. 26 will need to be toned down – unless they can avail of data from the literature to extend their inferences on application of the phenomenon in disparate echinoids.

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Review form: Reviewer 2 (A. Richard Palmer)

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes
Is the language acceptable?
Yes

Is it clear how to access all supporting data?
The Supplementary Information file needs some serious work.

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
Yes

Recommendation?
Major revision is needed (please make suggestions in comments)

Comments to the Author(s)
See attached review file and annotated MS file. See Appendix A and B.

Decision letter (RSOS-160139)

19-Apr-2016

Dear Dr Heyland,

The editors assigned to your paper ("A newly-identified left-right asymmetry in larval sea urchins") has now received comments from reviewers. We would like you to revise your paper in accordance with the referee and Subject Editor suggestions which can be found below (not including confidential reports to the Editor). Please note this decision does not guarantee eventual acceptance.

Please submit a copy of your revised paper within three weeks (i.e. by the 12-May-2016). If we do not hear from you within this time then it will be assumed that the paper has been withdrawn. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office in advance. We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Editors, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers.

To revise your manuscript, log into http://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. Revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you must respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". Please use this to document how you have responded to the comments, and the adjustments you have made. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response.
In addition to addressing all of the reviewers’ and editor’s comments please also ensure that your revised manuscript contains the following sections as appropriate before the reference list:

- **Ethics statement (if applicable)**
  If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

- **Data accessibility**
  It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article’s supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data has been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that has been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link:

- **Competing interests**
  Please declare any financial or non-financial competing interests, or state that you have no competing interests.

- **Authors’ contributions**
  All submissions, other than those with a single author, must include an Authors’ Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

  All contributors who do not meet all of these criteria should be included in the acknowledgements.

  We suggest the following format:
  AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

- **Acknowledgements**
  Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

- **Funding statement**
  Please list the source of funding for each author.
Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Yours sincerely,
Matthew Allinson,
Editorial Coordinator, Royal Society Open Science

on behalf of Kevin Padian
Subject Editor, Royal Society Open Science
openscience@royalsociety.org

Associate Editor’s comments (Dr David Ferrier):
Associate Editor: 1
Comments to the Author:
The reviewers have made a number of helpful recommendations for improvement of the manuscript, mainly focused on the statistical analyses employed. Comments on improving aspects of the presentation, including points about the main text and figures as well as the supplementary information, should also be accommodated.

Associate Editor: 2
Comments to the Author:
(There are no comments.)

Comments to Author:

Reviewers' Comments to Author:
Reviewer: 1

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Reviewer: 2

Comments to the Author(s)
see attached review file and annotated MS file

Author's Response to Decision Letter for (RSOS-160139)

See Appendix C.

RSOS-160139.R1 (Revision)

Review form: Reviewer 2 (A. Richard Palmer)

Is the manuscript scientifically sound in its present form?
No

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
A link to Dryad (doi:10.5061/dryad.102tc) is provided, but I was not able to access this link and was therefore unable to judge it.

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No
Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)

GENERAL COMMENT

I) Change in arm length with age/size. Something seems decidedly odd about the graphs in figures 2 - 5. Why does arm length not increase with increasing larval age/stage beyond day 25/stage C? Shouldn't all of these arms get longer as the larvae grow and increase in size? If these numbers are correct, the authors should comment on this near absence of any increase in arm length with age/size in the discussion.

SPECIFIC COMMENTS (line numbers as assigned by MS Word, NOT assigned by the pdf)

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Fig. 3- The labeling of the lines for R and L, and the font colors, aren't consistent in these panels. Presumably R is grey and dashed throughout but this is not so in Panels B and D.

Tables 1 & 2 legends- Explain to what "regression analysis" refers.

A.R. Palmer

See Appendix D

Decision letter (RSOS-160139.R1)

15-Jul-2016

Dear Dr Heyland:

On behalf of the Editors, I am pleased to inform you that your Manuscript RSOS-160139.R1 entitled "A newly-identified left-right asymmetry in larval sea urchins" has been accepted for publication in Royal Society Open Science subject to minor revision in accordance with the referee suggestions. Please find the referees' comments at the end of this email.

The reviewers and Subject Editor have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the comments and revise your manuscript.

• Ethics statement
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.
• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article’s supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data has been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that has been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link: http://datadryad.org/submit?journalID=RSOS&manu=RSOS-160139.R1

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors’ contributions
All submissions, other than those with a single author, must include an Authors’ Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

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• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days (i.e. by the 24-Jul-2016). If you do not think you will be able to meet this date please let me know immediately.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under “Manuscripts with Decisions”. Under “Actions,” click on “Create a Revision.” You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the
processing of the revised manuscript, please be as specific as possible in your response to the referees.

When uploading your revised files please make sure that you have:

1) A text file of the manuscript (tex, txt, rtf, docx or doc), references, tables (including captions) and figure captions. Do not upload a PDF as your "Main Document".
2) A separate electronic file of each figure (EPS or print-quality PDF preferred (either format should be produced directly from original creation package), or original software format)
3) Included a 100 word media summary of your paper when requested at submission. Please ensure you have entered correct contact details (email, institution and telephone) in your user account
4) Included the raw data to support the claims made in your paper. You can either include your data as electronic supplementary material or upload to a repository and include the relevant doi within your manuscript
5) Included your supplementary files in a format you are happy with (no line numbers, vancouver referencing, track changes removed etc) as these files will NOT be edited in production

Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes

Andrew Dunn
Senior Publishing Editor, Royal Society Open Science

on behalf of Kevin Padian
Subject Editor, Royal Society Open Science

openscience@royalsociety.org

Comments to Author:
Reviewer: 2

Comments to the Author(s)
GENERAL COMMENT

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A.R. Palmer

Author's Response to Decision Letter for (RSOS-160139.R1)

See Appendix E.
### A newly-identified left-right asymmetry in larval sea urchins

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| Complete List of Authors: | Hodin, Jason; Stanford University, Hopkins Marine Station  
Lutek, Keegan; University of Guelph, Integrative Biology  
Heyland, Andreas; University of Florida, Integrative Biology |
| Subject: | developmental biology < BIOLOGY, evolution < BIOLOGY, biomechanics < BIOLOGY |
| Keywords: | heterochrony, development, plasticity, feeding, fluctuating asymmetry, morphometrics |
| Subject Category: | Biology (whole organism) |
A newly-identified left-right asymmetry in larval sea urchins

Jason Hodin¹, Keegan Lutek² and Andreas Heyland²,*

¹ University of Washington, Friday Harbor Laboratories, Friday Harbor, WA USA
² University of Guelph, Department of Integrative Biology, Guelph, ON Canada

* Corresponding author: aheyland@uoguelph.ca

Running title: Left-right asymmetry in larval sea urchins
ABSTRACT

Directional asymmetry in body form is a widespread phenomenon in animals and plants alike, and a functional understanding of such asymmetries can offer profound insights into the ways in which ecology and development interface to drive evolution. Echinoids (sea urchins, sand dollars and their kin) with planktotrophic development have a bilaterally-symmetrical feeding pluteus larva that undergoes a dramatic metamorphosis into a pentameral juvenile that enters the benthos at settlement. The earliest stage of this transformation involves a directional asymmetry: the echinus rudiment will form the oral field of the juvenile. Here we show for the first time in two echinoid species that the larval body itself subsequently exhibits a corresponding directional asymmetry in the length of the bilateral pairs of larval arms, with shorter arms on the rudiment (left) side. We then demonstrate a mechanistic connection between the rudiment and arm length asymmetries by examining rare, aberrant purple urchin larvae that have rudiments on both the left and the right side. Our data suggest that this asymmetry is likely a broadly-shared feature characterizing ontogeny in the class Echinoidea. We propose several functional hypotheses—including developmental constraints and water column stability—to account for this newly-identified asymmetry.

Keywords: development, plasticity, fluctuating asymmetry, morphometrics, larval ecology, evolution
BACKGROUND

Many species of benthic invertebrates have a planktonic larval phase, which may allow these taxa to exploit alternative resources across life history stages, increase their dispersal ability and maintain connectivity among populations [1-3]. The echinoderms including sea urchins, sea stars and sea cucumbers exhibit a wide variety of such planktonic larval forms, both feeding and non-feeding [4-8]. These forms are the result of evolutionary pressures which appear to shape larval morphology within the confines of opposing functional constraints, in particular on feeding ability versus stability in the water column [9, 10]. Specifically, feeding structures generally require large surface area for particle capture, whereas stability, especially in turbulent waters, relies upon minimal surface area [8].

Consistent with these proposed trade-offs are the derived, non-feeding larval forms that have evolved independently and repeatedly across echinoderm taxa. Such larvae avoid the aforementioned functional constraints on feeding versus stability, and thus tend to be relatively simple in overall structure, with multiple ciliated bands circling their spheroid bodies to facilitate movement [11]. By contrast, feeding larvae exhibit more complex morphologies, and two classes of echinoderms—the ophiuroids (brittle stars and basket stars) and the echinoids (sea urchins, sand dollars and kin)—have independently evolved similar-looking pluteus larvae [12, 13], with 2-8 or more larval arms supported by internal skeletal rods. These arms are used for feeding and swimming, provide structural support, and might assist in passively orienting the larvae and offering protection from predation [14-17]).
The diverse echinoderm larvae described above share one key developmental feature: at a certain point in larval development, a directional asymmetry appears when juvenile structures begin to form on the left side in the otherwise bilaterally-symmetrical larva. In most echinoids with feeding larvae, this asymmetry is first visible as an invagination of ectoderm on the left side called the "echinus rudiment", which ultimately contacts a coelomic pouch, and they jointly transform into the oral portion of the pentamerally-symmetrical juvenile [18-22]. The juvenile structures will continue to grow and differentiate until the larva reaches competence, at which point if it subsequently encounters suitable substrate, the larva will settle irreversibly on the benthos. The relationship between the juvenile and the larva is in a sense parasitic as the juvenile structures develop at the expense of larval growth [reviewed in 23].

The directionally-asymmetrical rudiment invagination is particularly well studied in the purple sea urchin, *Strongylocentrotus purpuratus*. Aihara and Amemiya [21] provided strong experimental evidence that the right side of the larva is largely responsible for differentiating the L-R axis: laterally-bisected larvae (before rudiment invagination) all regenerate and develop to competence, but the larvae developing from the left halves rarely exhibited normal L-R patterning, whereas those from the right side almost always developed normally. More targeted removals of portions of the right side also resulted in larvae with abnormal L-R patterning.

Recent molecular evidence has further supported this scenario of right side control of the L-R asymmetry. The identified genes that appear to regulate L-R asymmetry in urchins encode two secreted growth factor-like proteins – Nodal and Lefty- and the Pitx2 paired-class homeodomain protein, all three of which are
expressed primarily on the right side of the larva; their proper expression restricts rudiment formation to the left side [24]. BMP (Bone Morphogenetic Protein) signaling is asymmetrically activated and is required for the development of left sided structures and marker genes [25]. Additionally, a H,K-ATPase-like protein also appears to be important, most likely via either H$^+$ or K$^+$ gradients that occur upstream of the asymmetric gene expression of nodal, lefty and pitx2 [26].

Here we show that concomitant with this L-R asymmetry in rudiment formation is an asymmetry of the larval arms in advanced echinoid pluteus larvae that has not previously been described. We first document the asymmetry in two disparate echinoids separated by 250 million years, suggesting that this asymmetry may be a common feature among echinoids with feeding larvae. We further explore the phenomenon in S. purpuratus to evaluate the possible connection between the rudiment and larval arm asymmetries, and by examining aberrant larvae with rudiments on both the left and right sides. We discuss our results in the context of several hypotheses concerning the function of this newly-identified asymmetry in sea urchin plutei. In so doing, we highlight the ways in which directional asymmetries offer a unique window into how ecology and development work together to drive organismal evolution.

METHODS

Source populations, maintenance of adults and larval cultures

For the characterization of the arm-length asymmetries in Dendraster excentricus (Escholtz) sand dollar larvae, we used adults collected at low tide (-0.25 meters) from
a large, intertidal population in East Sound (Orcas Island, WA, USA) on July 17, 2015. The adults were maintained at Friday Harbor Labs (FHL; Friday Harbor, WA, USA) in flowing sea water in sand bins until spawning. On August 26, 2015, we spawned several adults by intra-coelomic injection with 0.5M KCl. We set up crosses from two females: one by standard methods [27] using sperm collected dry that same day from a single spawning male, the second by aspirating off the eggs from the aboral surface of a second female who began spawning after we returned her to an aquarium. This second female’s eggs were already fertilized, therefore we are unsure of the paternity in this second cross (several males were also spawning in the aquarium at that time, including the male from the first cross). Thus, the two crosses were either half sibs from different mothers, non-sibs, or a mixture. We conducted fertilizations and all subsequent rearing steps in 0.45 µm Millipore-filtered natural sea water (MFSW) at room temperature, which varied between 19 and 22°C.

Sand dollar embryos had hatched by 1 day after fertilization, at which point we set up one jar from each cross at approximately 1 larva/ml of MFSW, and fed them a combination of *Rhodomonas* spp. (2500 cells/ml) and *Dunaliella tertiolecta* (3000 cells/ml), and kept them gently stirred using a gyratory shaker table. We changed their water every 2 days by gentle reverse filtration of >95% of the water volume and gave the larvae fresh MFSW and food. On day 3 (comparable to soft tissue stage iii from [22]), we reduced the larval density to 1 larva/5 ml MFSW, and maintained them at that density until day 9 (more or less equivalent to skeletogenic stage 10 from [22]) when we conducted all larval arm measurements.
For the characterization of the corresponding arm-length asymmetries in purple urchins as well as the ontogenetic characterizations and feeding trials, we used adult *Strongylocentrotus purpuratus* (Stimpson), collected at Slip Point (Clallam Bay, WA, USA) and maintained in subtidal cages suspended off the floating docks at FHL, fed throughout the year *ad libitum* with drift kelp (mainly blades of *Nereocystis leutkeana*). We spawned 2 males and 2 females on March 27, 2015 at FHL, by intra-coelomic injection with 0.5M KCl. We then set up the four pairwise fertilizations in MFSW using standard methods [27] at 11°C. We transported embryos the next day to the University of Washington (Seattle, WA, USA) and continued to maintain the cultures at 11°C. On day 5, when the embryos had reached the late prism/early 4-arm larval stage, we set up a single gallon jar in MFSW at approximately 1 larva/ml from equal proportions of the 4 fertilizations, fed them a combination of *Dunaliella tertiolecta* and *Rhodomonas spp.* as described above, and over about an hour, warmed the culture to 15°C in a shaking water bath, where we maintained all cultures for the remainder of the experiment. Every two days, we cleaned the cultures and fed them as described above.

On day 15, most of the larvae had reached the rudiment invagination stage (soft tissue stage i from [22]), at which point we reduced the larval density to approximately 1 larva/1.3 ml MFSW, and then to 1 larva/2 ml MFSW on day 17, with food at the same concentration as previously. On day 20 (approximately soft tissue stage iv from [22]), we individually selected 1000 of the optimally developing larvae, reduced the density to 1 larva/6 ml, and fed them as before. This stepwise reduction in density was an attempt to limit bouts of asexual budding, which can be induced by sudden shifts in density.
(unpublished observations), and would be expected to increase variability in arm length within cultures [28].

On day 25 (approximately skeletogenic stage 1 from [22]), we selected out 83 larvae into each of 6 jars with 500 ml MFSW (so still at 1 larva/6 ml) and randomly assigned each jar to one of two treatments: 3 replicate jars of high food (full ration of Dunaliella:Rhodomonas at 1:4 cells/µl) and 3 replicate jars of low food (25% ration at 0.25:1 cells/µl) for the remainder of the experiment. Approximately 50% of the larvae in the high food treatment had reached metamorphic competence by day 42; the low food larvae had not quite reached metamorphic competence by the time we concluded the experiment on day 45.

For the "double rudiment" experiment we used purple urchins originally obtained from The Cultured Abalone Ltd. (Goleta, California, USA) and that we have maintained at the Hagen Aqualab at the University of Guelph (Guelph, Ontario) in an artificial seawater system on an 8:16 hr light:dark photoperiod at approximately 12ºC and 34 ppt salinity, fed ad libitum with rehydrated Kombu kelp (Laminaria spp.). Over the last several years, we have repeatedly noted an unusually high proportion (approximately 2-5%; data not shown) of offspring of these urchins that exhibit rudiments on both their right and the left sides – so-called "double rudiments"– a phenomenon typically seen in fewer than 1% of larvae [29]; our unpublished observations). In September 2013, we obtained gametes from one male and one female adult S. purpuratus by intracoelomic injection following protocols described above. After fertilization and hatching, we set up cultures at an approximate density of 1 larva/2 ml of 0.45 µm Millipore filtered artificial sea water (MFASW), agitated to prevent the larvae from settling out of the water.
column. We transferred larvae three times per week to clean beakers with new water and fed them with either *Dunaliella tertiolecta* or *Rhodomonas spp.* at 12 cells/ml or 6 cells/ml respectively. We collected twenty to thirty individuals for measurement at 21 days post-fertilization. We then stage-matched equal numbers of these individuals to randomly selected siblings with single rudiments, and assessed asymmetry as in the previous experiment.

**Staging and Measurements**

In FHL, we measured ten live 9-day old sand dollar larvae from each of the two crosses, gently immobilized on slides under raised cover glass, using an Olympus BH-2 microscope. In Seattle, we staged and measured live purple urchin larvae, immobilized as above, using a Leitz Wetzlar Ortholux microscope. For the purple urchins, we employed the staging scheme as outlined in [22]. Note that we used stage bins (as defined in the legend to Fig. 2) for the analyses of the urchin data in an attempt to equalize the numbers of individuals within each bin for this dataset. In both FHL and Seattle, we measured skeletal rod lengths on haphazardly-chosen larvae using a calibrated ocular micrometer, and calculated the z-axis offset of each measured skeletal rod using the gradated focus knob, which we had calibrated using a slide and cover glass of known thickness (measured with a micron caliper).

To account for possible measurement error and/or bias in our measurements, we used larvae fertilized and reared in Guelph as described above, but from an August 3, 2015 fertilization. On day 21, we packed and shipped overnight approximately 200 of
these live larvae to Seattle, in order to conduct the error measurements on the Leitz microscope set-up used for the majority of our data (see above). The larvae arrived in good condition on day 22 at approximately 13°C, and we conducted the error measurements on that day as follows. We haphazardly chose 20 larvae and placed them on individual microscope slides with raised cover glass as described above, and then staged and measured each one as above. Then, a colleague uninvolved in the study re-labeled all 20 slides and we re-measured each of the 20 larvae a second time; thus, the second measurement on these same larvae was done “blind.” We calculated measurement error using the difference between each of the paired measurements, and used this error calculation to ensure that any reported differences in L-R asymmetry fell outside of the experimentation error range (Supplementary material).

For the double rudiment experiment, we staged larvae under a Nikon Eclipse Ti microscope, according to our published staging scheme (see Tables 1 and 2 in [22]), and we made three-dimensional "z-stacks" (pictures taken at 10 µm steps through the larvae) with a Nikon Digital sight DS-Fi1 camera. We then conducted measurements on these z-stacks using a 3D measurement macro (calibrated to account for both the x-y and z-y plane distances covered) using ImageJ software Fiji. We measured postoral (PO), posterodorsal (PD) and anterolateral (ALA) arm lengths on the rudiment and non-rudiment sides of the larvae.

As indicated in Figures 1A and 2C, the PO and PD rods are relatively straight, so our calculation of these skeletal rod lengths were straightforward. By contrast, the preoral (PRO) and ALA rods are somewhat curved in both species. Our reported PRO and ALA rod lengths represent the linear distance between the landmarks indicated in
Fig 1C, and are thus are our best approximations of these rod lengths using the methods we employed.

**Statistical Analysis**

We conducted all statistical tests and graphs using SPSS v23. We analyzed all morphological comparisons using SPSS MANOVA commands with sub-commands further specified in the Results section and Supplementary material. We considered results “significant” if α was < 0.05. We report all results as ± one standard error unless stated otherwise. We conducted our principle component (PC) analyses using SPSS factor commands using regression analysis. PCs with eigenvectors >1 were extracted.

We tested for fluctuating asymmetry using Levene’s Test of Equality with SPSS commands.

**RESULTS**

We here present our experiments that test whether echinopluteus larval arms are directionally asymmetrical during mid- to late-larval development, and whether any detected asymmetry changes as a function of developmental age and stage.
Larvae of the sand dollar *Dendraster excentricus* show directional asymmetry in larval arm growth

We measured all eight larval arms of *D. excentricus* 9 days post-fertilization at ~20°C; we analyzed differences in arm lengths between the left and right side of the larva (Fig. 1). We found that the postoral (PO) arms of *D. excentricus* larvae were significantly shorter on the side of the juvenile rudiment (left side) compared to the right side ($F_{1,19}=7.60, p<0.01$). We did not find differences in arm length between the left and right side of *D. excentricus* larvae for the other three pairs of arms [posterodorsal (PD: $F_{1,19}=2.38, p=0.13$); anterolateral (ALA: $F_{1,19}=0.06, p=0.81$); and preoral (PRO: $F_{1,19}=0.10, p=0.76$); see Supplementary material for complete ANOVA results]. A principle component (PC) analysis using all arm lengths extracted one PC that explained 69% of the total variance in arm lengths (Supplementary material) but did not show any difference between the left and right side of the larva ($F_{1,20}=1.51; p=0.23$).

Note also that we setup two separate fertilizations (crosses) for this experiment and we did not find a significant interaction between cross and side for any arm pairs (PO: $F_{1,1,19}=0.29, p=0.59$; ALA: $F_{1,1,19}=0.01, p=0.92$; PD: $F_{1,1,19}=0.31, p=0.58$; PRO: $F_{1,1,19}=0.01, p=0.93$). This indicates that asymmetry patterns are not different between the two crosses.
Larvae of the purple sea urchin, *Strongylocentrotus purpuratus*, show directional asymmetry in larval arm growth that changes over ontogeny.

To determine if the late-stage asymmetry we observed in sand dollar larvae is a more widespread feature among echinoids, and to examine how any effect progresses during ontogeny, we examined arm asymmetry in the purple sea urchin *S. purpuratus* at four time points post-fertilization. We then analyzed arm length morphometrics as a function of age (dPF) and binned stage (see Fig. 2 legend for details).

Based upon the sand dollar data and our preliminary observations on *S. purpuratus* larvae, we expected asymmetry to first become manifest in later development, after the juvenile rudiment begins to form. Therefore, our earliest measurement date was on day 15, right around the time when we first observed rudiment invagination (Fig. 2D). We made additional measurements during the growth of the juvenile rudiment on days 25, 33 and finally on day 39 (Fig. 2E), at which point the larvae were at or near competence to transform to the juvenile stage (not shown).

Three of the four arm pairs in *S. purpuratus* larvae showed directional asymmetry across developmental ages (Fig. 2A; PO: $F_{1,54}=6.35$, $p=0.01$; PD: $F_{1,54}=4.43$, $p=0.04$; ALA: $F_{1,54}=9.48$, $p<0.01$), with shorter arms on the left (juvenile rudiment) side. Furthermore, we found that asymmetry increases as a function of age for PO and PD, in that these arm pairs show directional asymmetry starting on day 39 (Fig. 2A; PO: $F_{1,14}=6.35$, $p=0.02$; PD: $F_{1,14}=12.12$, $p<0.01$). By contrast, ALA arms only showed directional asymmetry on day 33 (Fig. 2A; $F_{1,36}=16.21$, $p<0.01$).
When we grouped the larvae into binned stages (regardless of age; Fig. 2B), we found evidence for arm pair asymmetry across stages in PO ($F_{1,54}=5.43$, $p=0.02$); PD ($F_{1,54}=3.94$, $p=0.05$) and ALA arms ($F_{1,54}=9.84$, $p<0.01$). Specifically, we detected arm asymmetries for ALA only at stage bin D (Fig. 2B; $F_{1,13}=7.56$, $p=0.01$). and for PO and PD only in stage bin F (Fig. 2B; PO: $F_{1,14}=4.70$, $p=0.04$; PD: $F_{1,14}=10.45$, $p<0.01$). We summarize the ANOVA results in Supplementary material, where we also present the results for a principle component (PC) analysis of arm lengths across age and stage for *S. purpuratus*. As a whole, the results of the PC analysis are consistent with the analysis of individual arms lengths described above.

Although it is not the focus of the current study, we undertook an analysis of these data for the presence of fluctuating asymmetry (FA) as well. We see no convincing evidence for FA in any of the stages that we examined (see Supplementary material for details).

In sum, arm length asymmetry in *S. purpuratus* larvae increases with age and stage in PO and PD arms, but the pattern is more complex with ALA arms.

**Food does not affect larval arm asymmetry in *S. purpuratus***

One hypothesis that could explain the previously noted directional asymmetries is that there is competition for limited resources or materials between the rudiment and the nearby left arms. If so, we might expect to observe a more dramatic asymmetry in larvae raised in food limited conditions, where such materials/resources would be in reduced supply.
To test this hypothesis, we reared larvae under a high food ration for 25 days, and then shifted a subset of the larvae into a reduced food ration (25% of the high food ration) for the remainder of larval development. We then analyzed changes in arm size as a function of age and stage for the low food- and high food-reared larvae.

Plasticity in larval arm growth overall as a function of food level has been well demonstrated in *S. purpuratus* and numerous other echinoids [reviewed in 23]: in general, larvae under low food conditions have longer arms relative to growth of juvenile structures. In our dataset, we did not observe arm length plasticity (i.e., an effect of food level) for any arms as a function of age (Fig. 3; PO: F$_{1,89}$=3.52, p=0.06; ALA: F$_{1,89}$=0.14, p=0.71; PD: F$_{1,89}$=2.86, p=0.09; PR: F$_{1,89}$=0.01, p=0.92). We did however observe arm length plasticity for all arm pairs as a function of binned stage (Fig. 4; PO: F$_{1,89}$=21.19, p<0.01; ALA: F$_{1,89}$=3.85, p=0.05; PD: F$_{1,89}$=11.97, p<0.01; PR: F$_{1,89}$=5.62, p=0.02). We summarize the ANOVA results from the food experiment in Supplementary material.

We then tested whether food environment would impact the extent of arm asymmetry in *S. purpuratus* larvae. Statistically, such a food effect on arm length asymmetry would manifest as a significant interaction between food treatment (high or low) and side (L or R). We did not find evidence for a statistically significant interaction between food treatment and directional asymmetry for any larval arms (i.e., a difference in length between the left and right side) either by age or by stage (Figs. 3 and 4; Supplementary material).
S. purpuratus larvae with double rudiments have symmetrical arms

We analyzed aberrant larvae with naturally occurring double rudiments (i.e., larvae with “twin” juvenile rudiments developing simultaneously on the right and left sides) to test the hypothesis that larval arm asymmetry is functionally linked to the formation of the juvenile rudiment (Fig. 5A-E). In our double rudiment larvae, none of the arm pairs showed signs of directional asymmetry (PO: $F_{1,7}=0.28$, $p=0.60$; PD: $F_{1,7}=1.17$, $p=0.30$; ALA: $F_{1,7}=0.00$, $p=0.96$), whereas their full siblings with single rudiments showed directional asymmetry in PO arms (Fig. 5A; PO: $F_{1,8}=6.17$, $p=0.02$; PD: $F_{1,8}=2.81$, $p=0.11$; ALA: $F_{1,8}=1.04$, $p=0.32$). We summarize the ANOVA results for these single and double rudiment larvae in Supplementary material.

DISCUSSION

Metamorphosis in extant echinoderms involves a transformation from a bilateral larva to a pentameral adult [30, 31]. Because the common ancestor of echinoderms and other deuterostomes is hypothesized to have had an adult with bilateral symmetry [reviewed in 32], this bilateral-to-pentameral shift during echinoderm ontogeny is considered key to understanding the evolution of this unique group [33-35].

In all living echinoderms with indirect development (sensu McEdward [6]), the adult body plan develops in a curious fashion with respect to that of the larva, via a directional asymmetry: the juvenile forms on the left side of the otherwise bilaterally symmetrical larva [18-22]. Here we show for the first time that in two disparate echinoids, a sea urchin and a sand dollar, a second directional asymmetry is apparent during late larval development: the majority of the larval arms, which are supported by
calcium carbonate skeletal rods, are substantially (approximately 11%; see Figs. 1 and 2) shorter on the rudiment side of the larva.

Although asymmetries in larval arms have been noted previously in echinoid larvae [36-38], ours is the first demonstration of such a unidirectional asymmetry in multiple arms. As in the only other reported case of a directional asymmetry in advanced pluteus larval arms [37, 38], we show evidence here for a link between formation of the rudiment and the arm length asymmetry, with shorter larval arms specifically on the rudiment (left) side. Our demonstration of this asymmetry in both an irregular and a regular echinoid, as well as our casual observations of such asymmetries in the larvae of several other echinoid taxa which we have reared (data not shown), suggest to us that this directional asymmetry in arm length is likely a generic feature of late-stage, echinoid larval development, and as such, it calls out for a functional explanation.

Possible developmental mechanisms underlying larval arm asymmetry

The molecular and developmental mechanisms establishing rudiment formation on the left side of the larva have been studied in some detail. Specifically, Nodal and BMP signaling are the primary factors responsible for the development of the juvenile rudiment on the left side of the sea urchin larva. Whereas BMP signaling on the left side of the larva is required for the activation of other asymmetrically-expressed genes, Nodal signal on the right side of the larva inhibits BMP signaling there, thus directing the
formation of the rudiment to the left side [24, 25, 39, 40]. Still, the consequences of this asymmetry for development of the larval arms is unclear.

If the BMP-Nodal gene network is likewise involved in our reported arm length asymmetries, then we would predict that the arm length asymmetries would first appear at approximately the same developmental stage as rudiment formation begins. This is not what we have observed. Indeed, at soft tissue stage iv [22] (stage bin A, day 25 in this experiment) rudiment formation was already well underway and we did not observe any directional asymmetries in arm length, which we first detected at skeletal stage 5-6 [22] (stage bin D, day 33 in this experiment; Figure 2).

These observations suggest that if the BMP-Nodal gene network that regulates asymmetric rudiment formation is also involved in the directional arm asymmetry, then its involvement in the latter is likely to be either indirect, or to involve subsequent signaling steps. Still, our data reported here on double rudiments do suggest that there is a mechanistic connection between rudiment asymmetry and the arm length directional asymmetry, as also reported by Emlet [38] for the posterolateral arm length asymmetry in the black sea urchin, *Stomopneustes variolaris* (Echinoidea: Stomopneustidae). Evaluating the nature of this mechanistic connection could involve examining arm asymmetries in the context of reduction-of-function manipulations (e.g., using morpholino oligonucleotides) directed against BMP or nodal signaling components. Specifically, one could experimentally generate double rudiment larvae, for example by inhibiting Nodal signaling [24], and test whether such a manipulation results in loss of the directional asymmetry, as we saw in our naturally-occurring double rudiment larvae. If BMP-Nodal signaling is indeed connected to the arm length
asymmetries, then it is possible that the expression of skeletal elongation genes such as p58b and p16 [41, 42], and the skeletogenic gene network to which they belong [43], may integrate with the BMP-nodal gene network in an as yet unrecognized fashion.

Functional implications of the asymmetry for larval biology

The antagonistic relationship between rudiment growth and arm length in feeding pluteus larvae has been well-described in numerous taxa. Specifically, echinoid larvae show adaptive phenotypic plasticity for arm growth relative to growth of the rudiment: under high food conditions, arm length is reduced relative to rudiment growth; under low food, arms grow longer and rudiment growth is delayed [9, 23, 44-62]. The adaptive nature of this plasticity is indicated by the increased food capture efficiency in larvae with longer arms [9].

Therefore, there is presumably a mechanistic connection between rudiment growth and arm length, and it may be that this same antagonistic connection underlies the late stage arm-length asymmetries reported here and by Yanagisawa [37] and Emlet [38]. But why would such an antagonistic connection only manifest on the left side of the larva during these late stages? Here we consider two functional hypotheses in turn for our observed directional asymmetries in arm length. According to the developmental constraint hypothesis, the asymmetries that we report here result from some type of local developmental constraint or trade-off, in which the rudiment grows at the expense of only the adjacent larval arms. Alternatively, the swimming stability
hypothesis predicts that this link is driven by selection on a specific, asymmetric larval shape that facilitates stability or some other aspect of performance in the water column.

The developmental constraint hypothesis envisions a scenario where some factor utilized in the construction of both the rudiment and the larval arms is in limited supply. The increasing demands in the rapidly growing rudiment for this hypothesized factor leaves less of it available for the growth of nearby larval arms, and the result is shorter larval arms adjacent to the rudiment.

Although our experiments are not sufficient to falsify this hypothesis, our results are not wholly consistent with it. On the one hand, the left larval arm that is most distant from the rudiment—the left preoral (PRO) arm—shows no evidence for directional asymmetry in either *D. excentricus* or *S. purpuratus*, thus offering some support for a local constraint related to the rudiment. Furthermore, the constraint hypothesis would predict that the local competition would become more pronounced as the rudiment grows ever larger and more complex as ontogeny proceeds; our developmental time series with *S. purpuratus* (see Figure 2) is more or less consistent with this prediction. However, under a reduced food scenario, where arms grow longer and rudiment growth is delayed, then the constraint hypothesis would seemingly predict an even more dramatic asymmetry in such larvae. This is not what we observed; we saw no difference in the observed asymmetry between food treatments.

Therefore, our data provide mixed support for the constraint hypothesis. Still there is some precedence in other developing organisms for such a hypothesized local competition for factors or resources. For example, the wing-reproduction tradeoff in monarch butterflies (*Danaus plexippus*) has been proposed to be related to a position-
dependent mechanism, where juvenile hormone produced in the brain travels in the hemolymph past the wings and through the thorax, ultimately reaching the gonads in the posterior. Lessman and Herman [63] hypothesized that the highly active thorax during long-distance flight acts like a gauntlet, breaking down active juvenile hormone as it passes, leaving lower levels to arrive at the gonad, and therefore leading to reduced gonadal growth during flight. A second proposed example is in horned beetles (Onthophagus spp.) and other insects, where a factor such as insulin may be in limited supply, and could account for the apparent trade-off in the sizes of adjacent structures [64-66; but see 67].

In the urchin pluteus example, what could such a factor be? One appealing possibility relates to the availability of calcium, which is used to construct both the skeletal rods that support the larval arms and the diverse juvenile skeletal elements forming in the rudiment at late larval stages; these processes may thus be in direct, local competition. A simple experiment that would test for calcium limitation would be to add additional calcium to the sea water in which the larvae are grown – if calcium within the larvae at these late stages is normally in limited supply, then the asymmetry could be attenuated or disappear if excess calcium is provided. Alternatively, one could block calcium transport into the tissues of the rudiment through morpholino microinjection [68] or treatment with chemical inhibitors, and test whether such treatments result in more symmetrical larvae than in controls. Indirect evidence for this calcium limitation hypothesis follows from the findings of Byrne et al. [69], who reported that Heliocidaris tuberculata (Echinoidea: Echinometridae) larvae reared at low pH showed notable asymmetries (presumably FA) at early larval stages.
A second intriguing possibility is that the local rudiment-arm length antagonism is regulated by thyroid hormone (TH) signaling. Our previous studies [57] demonstrated that TH treatment results in a phenotype similar to that previously described for low food treatments, where juvenile structures grow faster, and larval arm growth is suppressed. Experiments with TH synthesis inhibitors [32, 70-72] indicate that feeding larvae have the capacity to produce TH internally, and our unpublished immuno-labeling experiments indicate that the source for TH may be structures within the rudiment. Therefore, if the rudiment is indeed the source for TH in plutei, and if TH levels correlate negatively with arm growth, then one might expect arms near to the rudiment to be shorter than ones more distant: this is precisely what we have observed here. A simple test of this scenario would be to provide excess TH exogenously and see if the observed asymmetries disappear.

Finally, additional support for biased L-R allocation of materials in pluteus larvae comes from some intriguing observations on sea star bipinnaria larvae, whose feeding larvae are considered homologous to echinoid plutei [73]. Circulation of fluid in the blastocoel cavity of bipinnaria larvae has been described as largely unidirectional: from the stomach, along the left side of the larva, and then around the mouth to the right side of the larva [74; Jaeckle, pers. comm.]. Furthermore, coelomic fluid flow out of the left hydrocoel via the pore canal and hydropore to the exterior of the larva [72] would tend to draw blastocoelar fluid towards the left side [76], with the growing rudiment in later stage larvae thus being a possible sink for blastocoelar substances. This biased directional flow could therefore represent a mechanistic basis for uneven distribution of blastocoelar substances, leading to the asymmetries that we report here.
As for the swimming stability hypothesis, it proposes that the asymmetry in larval arms would provide a selective advantage to larvae, whereby asymmetrical larvae would, for example, sink more slowly (and thus be retained in the water column more efficiently) than symmetrical larvae. Such an asymmetry would be predictably directional due to the substantial asymmetry in ballast provided by the rapidly growing and calcifying rudiment, predominantly on the left side of the larva.

Indirect support for this hypothesis comes from several examples in the literature. First, Collin [36] found evidence for fluctuating asymmetry (FA) in early, pre-rudiment larval stages of the sand dollar D. excentricus. FA is widely viewed as a measure of developmental stability and perturbations to the developmental process can have both genetic and environmental causes [77]. Nevertheless, the apparent continuity between FA in larval arms earlier in ontogeny [36], and then directional asymmetry (DA) later (our data reported here) may indicate that the asymmetries themselves may be functional throughout ontogeny. In this case, the forming rudiment in late stages (which were beyond Collin's final time point in her data set) might impose additional constraints that could lead to predictably shorter arms on the left.

Chan [78] reviewed a number of studies on pluteus larval morphology as it relates to stability and swimming, under different flow regimes and through ontogeny. The basic pattern that Chan reports is a slight tilt in the orientation of the larval body in flow increases the chances that a larva can maintain upward swimming (and thus presumably stay in surface waters), rather than being carried downward. In an unpublished study, Miyashita (pers. comm.) discovered that modeled, asymmetrical D.
*excentricus* larvae at the 4-arm stage (pre-rudiment growth) are able to maintain upward swimming more effectively than symmetrical ones.

The models that Chan and Miyashita employed were based upon those developed by Clay and Grunbaum [10], again using the sand dollar *D. excentricus*, but once again focusing on early (4 arm) larval stages before the development of the rudiment. One important set of parameters in this model relate to the centers of gravity and buoyancy, which would clearly change with the addition of two more pairs of larval arms and an asymmetric, calcified rudiment, as seen in the stages that we examined. Furthermore, drag on larvae increases with both arm length and arm number [15], which would be predicted to impact the stability of larvae in different flow regimes [8].

Taken together, the studies to date suggest that larval shape, orientation and asymmetries all can contribute to position in the water column, which can have important consequences for dispersal, prey encounter and predator avoidance throughout larval development, and encountering the substrate in late stage larvae preparing to settle to the benthos. Nevertheless, it is difficult to extrapolate from the previous modeling studies on much simpler larval morphologies to those in fully formed, 8-arm larvae with an asymmetrically growing and calcifying rudiment in realistic flow conditions.

Our experiments reported here do not directly address the swimming stability hypothesis. However, one intriguing observation is that the three arm pairs that protrude the furthest from the larval midline—the postoral, posterodorsal and anterolateral arms—are the three pairs that showed clear directional asymmetries in one or both species. By contrast, the arm pair that runs closest to the midline—the preoral arms—showed no
signs of directional asymmetry in either species at any age or stage that we examined. Likewise, the observations by Yanagisawa [37] and Emlet [38] of a dramatic asymmetry in late stage larvae of the sea urchin *Stomopneustes variolaris* was specifically in a unique pair of arms that project off the posterior end of those larvae, the posterolateral arms. All of these observations suggest that asymmetric arm growth is not simply a generic feature of late-stage pluteus larval arm growth common to all arms, and may thus point towards a functional explanation such as envisioned by the swimming stability hypothesis.

To adequately test this hypothesis experimentally, one would need to examine larvae under realistic flow conditions, and see if the degree of asymmetry in late stage larvae is related to their position in the water column. In addition, one could develop more complex models of the pluteus larval form that would extend from the Clay & Grunbaum [10] model, but include all four arm pairs and a growing and calcifying, asymmetrical rudiment. The swimming stability hypothesis would predict that arm asymmetries at these later stages would have clear consequences for position in the water column and/or swimming ability.

Finally, we note that the developmental constraint and swimming stability hypotheses are not the only two possible explanations for our observed asymmetries (and these themselves are not necessarily mutually exclusive). For example, in recent years it has become clear that asexual larval reproduction is widespread in echinoids (which again, has been particularly well-documented in *D. excentricus*), and one method of such asexual budding in sea stars [79, 80] – as yet undocumented in echinoids [but see 28] – is a loss of the arm tips. It is possible that such budding occurs
preferentially on the left side of late-stage echinoid larvae, which would lead to a directional asymmetry pattern such as we observed. Furthermore, Emlet [38] suggested that the asymmetry in posterolateral arms in *Stomopneustes variolaris* may be an adaptation for more effective settlement to the benthos: a long left posterolateral arm could interfere with substrate contact. A similar mechanical interference scenario might promote the evolution of shorter arms in late stages in other urchins as well, as we have observed here.

**CONCLUSIONS**

We here identify and characterize the ontogeny of a directional asymmetry in echinoid pluteus larvae that is visible at late stages, alongside the well-known directional asymmetry in the growing juvenile rudiment. Our data from two disparate echinoids, separated by approximately 250 million years of evolution, suggests that this asymmetry, though previously undescribed, may in fact be a common feature of late stage echinopluteus ontogeny. By examining rare, aberrant plutei with juvenile rudiments on both the left and the right side, we show that the asymmetry is mechanistically and/or functionally connected to rudiment development. We explore several hypotheses to account for this asymmetry, focusing on two main hypotheses: that the asymmetry aids in swimming stability in the water column or that the asymmetry is a result of a developmental constraint on material deposition in arms versus the rudiment.
One feature of echinoids that makes them such a valuable taxon for comparative studies is their great morphological diversity in the context of a relatively robust understanding of their phylogeny. As with adult features, sea urchin larvae also show remarkable diversity: for example, in arm number, their lengths relative to the body and their position [81]. Furthermore, there are many independently evolved instances of loss of larval feeding, accompanied by partial to complete loss of these larval arms [82]. And finally, functional and anatomical studies indicate that the similar larval morphology in the brittle stars (class Ophiuroidea) represents a completely independent evolutionary acquisition of the pluteus form [12, 13]. Such diversity in form, with independent evolutionary events and an easily quantifiable morphology, provides ample material for detailed comparative investigations into this directional asymmetry: a tractable aspect of functional morphology that can be studied in the context of the multiple ecological requirements facing feeding and dispersing larvae in the ocean.
ETHICS STATEMENT

The research presented here does not require any approval and therefore complies with all necessary regulations.

AVAILABILITY OF SUPPORTING DATA

All relevant data is included in the manuscript and appendices.

LIST OF ABBREVIATIONS USED

L-left; R-right; PO-postoral arms; PD-posterodorsal arms; ALA-anterolateral arms; PRO-preoral arms; dPF-days post-fertilization; FA-fluctuating asymmetry; DA-directional asymmetry; PC-principle component; MFSW-0.45 µm Millipore filtered natural sea water; MFASW-0.45 µm Millipore filtered artificial sea water; KCl-potassium chloride; TH-thyroid hormone; BMP-Bone Morphogenetic Protein; FHL-Friday Harbor Laboratories (University of Washington).

COMPETING INTERESTS

The authors declare that they have no competing interests, financial or otherwise.

AUTHORS' CONTRIBUTIONS

J.H. and A.H. contributed equally. All authors discussed and commented on the manuscript. J.H. conducted the experiments in Seattle and FHL. A.H. supervised the
experiments in Guelph, and A.H and K.L conducted the experiments there. J.H. and A.H. designed the experiments. A.H. ran the statistical analyses. A.H. prepared the figures. J.H. and A.H. wrote the paper.

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REFERENCES


FIGURE LEGENDS

Figure 1: Larvae of the sand dollar *Dendraster excentricus* show directional asymmetry in postoral arms. A) schematic of a sand dollar pluteus larva with four pairs of larval arms indicating the measurements taken for this study. Note that the left side of the larva is the side of the juvenile rudiment; as this is a ventral view, the “left” side of the larvae is seen here on the right side of the cartoon, and vice-versa. B) Representative larva at 9 days post-fertilization, oriented as in A. The darker region (Rud) on the left side of the larva (right side of the photo) shows the well-developed juvenile rudiment (scale bar: 100µm). C) Larval arm length in µm for all four larval arms at day 9; dark grey-left arm, lighter grey-right arm. PO: postoral arms; PD: posterodorsal arms; ALA: anterolateral arms; PRO: prooral arms. Asterisk indicates significant differences ($p < 0.05$) between left and right side, and therefore directional asymmetry.

Figure 2: Directional asymmetry increases in postoral and posterodorsal arms as purple urchin larval development proceeds. We analyzed left-right asymmetry in developing purple urchin (*Strongylocentrotus purpuratus*) larval arms as a function of age (A) and binned stage (B). The schematic in (C) indicates the larval arms measured; abbreviations and orientation as in Figure 1. The cross-polarized light micrograph in (D) shows a representative early stage larva (day 15, stage bin A), and in (E), a representative late stage larva (day 39, stage bin F). Note the visible juvenile skeleton (on the left side; pictured in the right side of these ventral views) and clear L-R arm asymmetry in (E) but not (D). Time is in days post fertilization (dPF), for stage we binned juvenile rudiment stages after [22] as follows: Bin A - skeletogenic stage 0; Bin B...
– skeletogenic stages 1-2; Bin C – skeletogenic stages 3-4; Bin D – skeletogenic stages 5-6; Bin E – skeletogenic stages 7-8; Bin F – skeletogenic stages 9-10. Scale bars in D,E: 150µm. Asterisks indicate $p<0.05$.

**Figure 3:** Reduced larval food does not alter directional asymmetries in arm length over time. We detected asymmetry in arm lengths with time under both high and low food conditions in purple urchins, with no detectable differences in any arm pair between high and low food (A-D). Abbreviations as in Figure 1. Time is days post fertilization (dPF). Larvae in the high food (HF) treatment had reached metamorphic competence by day 45, as indicated by the juvenile cartoons in A-D. Asterisks indicate $p<0.05$.

**Figure 4:** Reduced larval food does not alter directional asymmetries in arm length across stages. (A-D) We detected asymmetry in arm lengths with stage under both high and low food conditions in purple urchins, with no detectable differences in any arm pair between high and low food (A-F). Abbreviations as in Figure 1; stages binned as in Figure 2; asterisks indicate $p<0.05$.

**Figure 5:** Larval arms of aberrant purple urchin larvae with double rudiments are symmetrical. We analyzed arm length asymmetries (A-C) in naturally occurring larvae with juvenile rudiments on both the left and right sides (double rudiments; D), as compared to their full siblings with single rudiments (E). A-C: Whereas larvae with single rudiments in this experiment showed directional asymmetry in postoral (PO) arm length (asterisk in A), larvae with double rudiments did not show asymmetry in any of their arm
pairs. Abbreviations and orientation of larvae as in Figure 1. Scale bars in D,E: 150µm.
Asterisk indicates $p<0.05$. 
Larvae of the sand dollar *Dendraster excentricus* show directional asymmetry in postoral arms. A) schematic of sand dollar pluteus larva with four pairs of larval arms indicating the measurements taken for this study. Note that the left side of the larva is the side of the juvenile rudiment; as this is a ventral view, the “left” side of the larvae is seen here on the right side of the cartoon, and vice-versa. B) Representative larva at 9 days post-fertilization, oriented as in A. The darker region (Rud) on the left side of the larva (right side of the photo) shows the well-developed juvenile rudiment (scale bar: 100µm). C) Larval arm length in µm for all four larval arms at day 9; dark grey: left arm, lighter grey: right arm. PO: postoral arms; PD: posterodorsal arms; ALA: anterolateral arms; PRO: preoral arms. Asterisk indicates significant differences (p < 0.05) between left and right side, and therefore directional asymmetry.
Directional asymmetry increases in postoral and posterodorsal arms as purple urchin larval development proceeds. We analyzed left-right asymmetry in developing purple urchin (Strongylocentrotus purpuratus) larval arms as a function of age (A) and binned stage (B). The schematic in (C) indicates the larval arms measured; abbreviations and orientation as in Figure 1. The cross-polarized light micrograph in (D) shows a representative early stage larva (day 15, stage bin A), and in (E), a representative late stage larva (day 39, stage bin F). Note the visible juvenile skeleton (on the left side; pictured in the right side of these ventral views) and clear L-R arm asymmetry in (E) but not (D). Time is in days post fertilization (dPF), for stage we binned juvenile rudiment stages after [22] as follows: Bin A – skeletogenic stages 0; Bin B – skeletogenic stages 1-2; Bin C – skeletogenic stages 3-4; Bin D – skeletogenic stages 5-6; Bin E – skeletogenic stages 7-8; Bin F – skeletogenic stages 9-10. Scale bars in D,E: 150µm. Asterisks indicate p<0.05.
Reduced larval food does not alter directional asymmetries in arm length over time. We detected asymmetry in arm lengths with time under both high and low food conditions in purple urchins, with no detectable differences in any arm pair between high and low food (A-D). Abbreviations as in Figure 1. Time is days post fertilization (dPF). Larvae in the high food (HF) treatment had reached metamorphic competence by day 45, as indicated by the juvenile cartoons in A-D. Asterisks indicate p<0.05.
Reduced larval food does not alter directional asymmetries in arm length across stages. (A-D) We detected asymmetry in arm lengths with stage under both high and low food conditions in purple urchins, with no detectable differences in any arm pair between high and low food (A-F). Abbreviations as in Figure 1; stages binned as in Figure 2; asterisks indicate $p<0.05$. 140x128mm (150 x 150 DPI)
Larval arms of aberrant purple urchin larvae with double rudiments are symmetrical. We analyzed arm length asymmetries (A-C) in naturally occurring larvae with juvenile rudiments on both the left and right sides (double rudiments; D), as compared to their full siblings with single rudiments (E). A-C: Whereas larvae with single rudiments in this experiment showed directional asymmetry in postoral (PO) arm length (asterisk in A), larvae with double rudiments did not show asymmetry in any of their arm pairs. Abbreviations and orientation of larvae as in Figure 1. Scale bars in D,E: 150µm. Asterisk indicates p<0.05. 150x145mm (150 x 150 DPI)
GENERAL COMMENTS

I) Results and analyses. I am puzzled why the authors present and analyse the mean±SE of the right and left sides of arms (Figs. 1-5), because this greatly reduces their ability to detect left-right asymmetry. For example, some individual larvae of a given age/bin will have longer arms than others in the same age/bin, which will increase the among-individual variation for each side measurement, even though the right arm might consistently be longer than the left in all of those individuals.

I suggest re-doing the graphs and analyses of DA using mean \((R-L)/[(R+L)/2]\) or it’s mathematical equivalent mean \([\ln(R) - \ln(L) = \text{mean} (\ln(R/L))]\) [Appendix I of Palmer & Strobeck 2003. Fluctuating asymmetry analyses revisited. In Developmental Instability (DI): Causes and Consequences, M. Polak, ed. Oxford University Press, Oxford. pp. 279-319]. Then, for each arm, the data will be actual mean asymmetry in that arm rather than mean lengths of R and L. The statistical test for DA is then simple: does the mean, signed asymmetry \{mean (ln(R) - ln(L))]\} differ significantly from zero? The difference \(\ln(R) - \ln(L)\) has the added desirable property that it expresses asymmetry as a proportion of the mean (a value of 0.1 would mean that the difference between sides is 10% of the mean arm length).

II) Figures. In the graphs (Fig. 2A, B; Fig. 3, Fig. 4, Fig. 5), I suggest graphing on a double-Y graph i) average arm length and ii) average asymmetry \[\text{mean} (\ln(R/L))]\] for each time period or stage bin. This way readers can see how both arm length and arm asymmetry differ or change over time.

III) Supplementary results. ALL references to the "Supplementary Material" should refer to specific, numbered tables in the Supplementary Material file (see more detailed comments under Supplementary Information file below).

III) Citations. Are all these citations needed? In several places where blocks of citations that support a single point are only mentioned once, might a single citation work to support the statement? For example, no less than 18(!) references [44-62] are cited only once to support the point that arm lengths are plastic in response to food levels. Maybe choose the one citation that is also cited elsewhere in the text.

Having said this, I'm a tad surprised the authors don't cite:


Eaves presents extensive data on the prevalence of right vestibules in both Strongylocentrotus purpuratus and Dendraster excentricus larvae (see her Table 1 footnote).
SPECIFIC COMMENTS (page/line)

2/9: 'profound' here seems rather over the top to me

2/23-27: 'For the first time' is not correct because, as the authors note later, such arm asymmetries have been described by Emlet 2009 and Collin 1997.

2/32: If you believe Eaves 2005 (see above), right vestibules appear to be rather common, they are just not as well developed as left ones in both species studied here.

3/21: 'which' is generally preceded by a comma, otherwise use 'that'

11/29-30: Levene’s test is used to test for differences in FA (a variance) among groups, not to test for the presence of FA. The significance of FA relative to measurement error is tested with an ANOVA on repeat measurements (see comments under Supplementary Information file below). Also, here the authors should refer readers to specific numbered tables in the Supplementary Info. file.

12/25: Refer readers to specific numbered tables in the Supplementary Info. file.

12/28: It's not clear to me why the authors do a PCA here. To test for the presence of asymmetry for each arm the test would merely be: is the mean \[\ln(R) - \ln(L)\] significantly different from zero. A simple ANOVA would test whether asymmetry differed among arms: Factor 1= arm, Factor 2= Fertilization group, dependent= \[\ln(R) - \ln(L)\] (see General Comments I).

13-14: I suggest presenting these results for S. purpuratus as a) mean arm length and b) mean asymmetry \[\ln(R) - \ln(L)\], as a function of time or larval stage bin (see General Comments I).

14/28-32: This statement cannot be correct. There is ALWAYS some fluctuating asymmetry (random deviations from perfect symmetry, or random variation about some average directional asymmetry). In some cases there may also be directional asymmetry (DA; significant average deviation from symmetry).

15/35-50: This test would be much more straightforward if asymmetry is measured as \[\ln(R) - \ln(L)\] (see General Comments I). Then a simple ANOVA would test for food effects: Factor 1= arm number, Factor 2= food treatment, dependent= \[\ln(R) - \ln(L)\]. This ANOVA could also be done using age/larval stage bin as a third factor, to test for age/stage dependence.

16/6-26: Again (see General Comments I), this is a weak test for differences in asymmetry. I would suggest re-doing these analyses as a 1-factor ANOVA for each arm using a more sensitive measure of asymmetry: Factor 1= normal v double rudiment, dependent= \[\ln(R) - \ln(L)\].
17/11-21: Perhaps the authors should acknowledge that Collin 1997 reports "significant right-bias directional asymmetry" in the posterodorsal arm rods of 8 day old *Dendraster* larvae (her Table 3 statistical results support her statement in line 5 of her results section).

17/42-19/8: This speculation about potential signaling pathways seems longer than needed. I would suggest condensing it about half a page.

23/30: DA appearing later in development, and FA earlier, was also reported by Collin 1997 (her Table 3, and line 5 in her results section).

26/6-13: Emlet 2009 (p. 172) also notes that a larva with a right-side rudiment had reversed larval arm asymmetry and one with no rudiment at all had symmetrical arms. These observations seem important and worth mentioning here.

26/36: 'Previously undescribed' is not quite correct because, as the authors note, such asymmetries have been described by Emlet 2009 and Collin 1997. Perhaps this could be reworded.

27: Is this last paragraph needed? It does not seem to add much to the paper.

34/11: State that ‘colored lines’ indicate the linear measurements taken.

35/ Fig 5: Replace lengths of left and right arms with actual measures of asymmetry (e.g., ln(R) - ln(L)) and average arm length (see General Comments I), or indicate in the legend what the dark and light bars represent.

Results, general: Throughout, report results of the present study in past tense.

Figures, general: In both the PDF and on-line versions, I had difficulty viewing the figures in the submitted MS; panel labels, and text that spanned outside a figure box, did not show against the black background. The authors should be more careful to proof their submitted MS for proper figure visibility.

Figure legends: In each legend, indicate a) that the bars are ±SE and b) what the sample sizes were.

Fig. 1 legend: Panels A and B are reversed from what the legend says.

**Supplementary Information file:**

- A 93 page Supplementary Information file seems truly excessive and irresponsible to me. The authors should be more judicious in their choice of tests and presentation of statistical results. Mostly, these look like raw output files from a shopping list of statistical tests. If the authors aren't willing to make the effort to condense these results into sensible tables, why should the referees and readers bother spending time trying to do so?
- ALL tables should be individually numbered and have legends, pages should be numbered.

- **Error analysis.** The error analysis has not been done correctly to test for the significance of FA or DA relative to measurement error. The design should be: Factor 1= side, Factor 2= individual larva, dependent= repeated arm measurements. When done this way 1) the significance of the sides x individuals interaction term is a test of the size of FA relative to measurement error (i.e., did the difference between sides in individuals exceed that expected given the observed level of measurement error?) and 2) the F value of the ‘sides’ mean square divided by the ‘sides x individuals’ mean square is a test of the significance of DA.

  This should be done on a trait-by-trait basis (it is important to test for the significance of FA for each trait separately). The combined analysis (Table 2) seems meaningless to me.

- If the measurements for the right side of one larva in the error analysis were missing (Table 1), that individual should be excluded from the analysis.

- As a courtesy to readers, the order of traits in tables should be the same (e.g., Tables 4 & 5).

A.R. Palmer
HODIN ET AL. RSOS-160139, RESPONSE TO REVIEWERS

We thank both reviewers for their extremely helpful and insightful comments; in addressing them we feel that the revised manuscript is greatly improved. In the sections below we list all of the relevant comments of each of the two reviewers, indented and in italics. We respond in point-by-point fashion in unindented, unitalicized text. We refer throughout the document below to Page and Line number references in the revised version of our manuscript. Please note that our page number references are to the numbers printed on the lower right of every page, not the page numbers as listed in the compiled PDF in the upper left corner (“page x of y”).

(Please ignore the other stray numbers in the upper and lower left corners of alternating pages; this must be a glitch in the page numbering program as it relates to header and footer text. We were unable to delete these numbers without deleting the correct line numbers as well; our apologies).

May 19, 2016

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REVIEWER 1

One is ... left wondering what is the significance of the work especially as it appears to involve a very low sample size – with regard to the independent source of data – the parent or family. I would like to see the stats revisited. Because I am most concerned about the methods and how the data were handled I will focus on that aspect – with a few comments elsewhere.

1. Dendraster – crosses were set up with 2 females. Thus in my view here n=2 so that all they can do is a t-test. Larvae are not independent data sources – they are nested in female. For the other species n=4 so that is a bit better. Thus the conclusion on pg. 26 will need to be toned down – unless they can avail of data from the literature to extend their inferences on application of the phenomenon in disparate echinoids.

We thank the reviewer for his/her careful attention to the manuscript and our experimental design. We would note that in the Dendraster data set, we are reporting on our rearing under standard laboratory conditions of a cohort of larvae (full-sib design), and performing morphometrics on a haphazardly chosen subsample of these full-sib larvae. We note that both are standard procedure for larval morphometrics (see for example papers by Drs. Richard Strathmann, Larry McEdward and Justin McAlister as examples – in our reference list, references 48, 51, 54 and 61).

We have here added a second female to this design as a way of ensuring that the asymmetries that we report are not simply due to an aberrant female or genotype, and we have demonstrated this statistically (see Page 13 Lines 281-284 of the revised manuscript).

We do feel that the above rationale for our use of the second female was not clear in the manuscript as originally submitted, and we apologize for that oversight. We have now clarified this point both in the Methods (Page 6 Line 118-121) and the Results sections (Page 13 Lines 278-280).

2. ANOVA is the statistic used, but the assumptions of ANOVA would have been violated. ANOVA requires the data to be independent and with the degrees of freedom required only data
for the second species can be analysed $df = 4 - 1$. Also ANOVA requires data to be normal and have homogeneity of variance.

As explained above, the experimental design we used in this study is a full sib design with independent culture vessels, and the large majority of studies on larval morphometrics have been performed this way. While there are limitations of this design (for example, in the absence of multiple, independent genetic crosses) it is the standard in the field, and is indeed suitable for ANOVA analysis, as the references cited above confirm.

The reviewer is correct to point out that ANOVA requires normality of data, and we apologize for not addressing this point in the methods as originally submitted. We have now clarified that all of our data is normally distributed as confirmed by p-p and q-q plots (see Page 12 Line 250 in the revised manuscript).

3. What the authors might do is focus their study on the species for which they have the most data S. purpuratus and use the one where $n = 2$ as additional information. I can not see what can be done with $n = 2$.

We agree with the reviewer that the purple urchin data is much stronger for a variety of reasons. This is why we have limited our description of our Dendraster results to a single section in the results and one figure. By contrast, we present our purple urchin data in 5 figures, encompassing three separate experiments.

4. The double rudiments were obtained from brood stock maintained in an artificial sea water system. Artificial sea salts can be toxic or have negative affects on aspects of development – especially skeleton formation. Have a read of a paper by Roberta Challener in an aquaculture journal a couple of years ago.

This is a valid concern of the reviewer. The artificial sea water (ASW) system in Guelph is a more advanced mixture than some typical preparations (e.g., InstantOcean or simple seawater protocols like MBL sea water), is well buffered, subject to regular chemical testing, and includes essential trace compounds. If our ASW system were toxic to larvae, we would expect to see a range of abnormalities in all of our rearings, and we have not observed this at all in many years (>8 years) of our use of this system. Furthermore, we have recently published a paper in which we explicitly traced the progression of larval development in purple urchins from fertilization through settlement with a detailed staging scheme (see ref. 22 in our manuscript), comparing larvae reared in Guelph to those reared on natural sea water in several different west coast locales. We observed no differences, indicating that our ASW-reared larvae in Guelph develop normally all the way thru larval development.

Nevertheless, in order to address the concern of the reviewer, we have expanded our description in the Methods of the conditions that are correlated with our observations of enhanced double rudiments in Guelph. Briefly, we only observed it in a certain percentage of our rearings, and we stopped observing it at all recently after we had some natural attrition of adults in our colony. These observations point to the fact that the occasional enhanced proportion of double rudiment larvae in our cultures was associated with a specific female and/or genotype, as we now explain on Page 8 Line 171 through Page 9 Line 183.
Finally, on the paper of Challener et al. mentioned by the reviewer, we believe that the reviewer is referring to their paper on ocean acidification (OA) effects on urchin larvae using their artificial sea water system (Journal of Applied Aquaculture, Volume 25, Issue 2, 2013, pp 154-175). The “abnormalities” reported by Challener and colleagues were due to the change in sea water chemistry associated with their future OA treatment (i.e., pH 7.8), not in their control treatment, which was ASW with ambient ocean chemistry conditions (i.e., pH 8.2). As the authors state in their discussion (p167): “Studies have shown that the use of synthetic sea salts, in general, does not impact early development in echinoids (Hovanec et al. 2005) and the use of commercial artificial seawater has many benefits such as cost and logistics, particularly for those facilities not located near the coast.”

5. Asymmetrical arm length of sea urchin larvae is a response variable often scored in ecotoxicology studies – it would be interesting if the asymmetry in those studies was one sided. I wonder if you might be able to tell from photos. These type of studies should be cited on pg 17 L 10.

We thank the reviewer for this suggestion, but we would not feel comfortable drawing conclusions from measurements taken on any such photos for several reasons, most notably that the sample sizes would be too low, the length of the arms cannot be measured accurately enough in the X-Y plane from such photos, and because we would have no information at all about the Z plane.

Nevertheless, we do find it quite striking that of the dozens of papers reporting detailed larval morphometrics in echinoids, very few of them have even reported L-R measurements separately (we cite all three of the exceptions of which we are aware). Indeed this striking gap in the literature is exactly the reason why we wanted to be sure to cite all of the aforementioned echinoid larval morphometrics papers that failed to measure left and right arms separately, as we see this as the most likely explanation for why the striking asymmetries that we report here have not been previously noted. See also our response to Reviewer 2 on this point (Response to Reviewer 2, General Comment IV, below).

We thank the reviewer for pointing us to toxicological studies on asymmetry in urchins; we have chosen two recent papers to highlight in our revised manuscript (see new References 80 and 81). We found a slightly different location in the discussion to include these references than the the location suggested by the reviewer; see Page 26 Lines 558-560.

6. There is considerable text on the potential biomechanical impacts of the asymmetry. One wonders what is the magnitude of this asymmetry in the study species and if it is significant ecologically. How much shorter are the arms – how many microns? Would one expect a small difference to matter? The authors need to provide data on the magnitude of difference, range etc. For the species that you have n=4 does this differ between families?

We agree that we should have reported the magnitude of the asymmetry in explicit terms. As suggested by Reviewer 2, we have now undertaken a new analysis of our data computing the ln(R/L) value as an index of the magnitude of the asymmetry. In so doing, we see that the magnitude of asymmetry in our larvae can get as high as a 20-25% difference between left and right [i.e., ln(R/L) = 0.25; see Figures 1-3]. We feel this difference is substantial, justifying our speculations on the ecological/biomechanical implications of a directional asymmetry of this magnitude.
We also would like to note that we mixed the larvae from each of the four purple urchin fertilizations precisely to create a larval cohort in which, if we saw a difference between left and right, we could conclude that this was a general phenomenon, not limited to particular aberrant genotypes (n.b., this “mixed parentage” design is the main alternative to the “full sib” design in the larval morphometrics field). In other words, our mixed parentage design supports our conclusion that the phenomenon is likely general for the two widely divergent species we tested, and perhaps echinoids in general.

**REVIEWER 2 – Dr. Rich Palmer**

**GENERAL COMMENTS**

I) Results and analyses. I am puzzled why the authors present and analyse the mean±SE of the right and left sides of arms (Figs. 1-5), because this greatly reduces their ability to detect left-right asymmetry....

We absolutely agree with Dr. Palmer that our method of reporting the data as originally submitted was inadequate. We have now completely reanalyzed the data along the lines that he suggested below, and have, in the process, reworked every single one of our (now six) Figures. We believe that these changes have dramatically improved the manuscript, even though it has only resulted in marginal changes to our conclusions.

II) Figures. In the graphs (Fig. 2A, B; Fig. 3, Fig. 4, Fig. 5), I suggest graphing on a double-Y graph i) average arm length and ii) average asymmetry [mean (ln(R) - ln(L))] for each time period or stage bin. This way readers can see how both arm length and arm asymmetry differ or change over time.

We have followed this excellent suggestion (see our new Figures 2-5), as it much more clearly shows the magnitude of the directional asymmetries that we have observed. Indeed we have now abandoned our previous statistical analysis and (unless noted) have restricted our statistical analyses of L-R differences to an analysis of the ln(R/L) index of asymmetry. We have decided to retain the left and right arm lengths in the figures, and provide total arm length in separate figures for each of our datasets (see Fig. 4 and supplemental figure S1).

III) Supplementary results. ALL references to the "Supplementary Material" should refer to specific, numbered tables in the Supplementary Material file (see more detailed comments under Supplementary Information file below).

We apologize for our unclear citations in the text to the supplementary material. We have rectified this in the revised version, and note that our supplementary material is now much reduced as well (see below).
IV) Citations. Are all these citations needed? In several places where blocks of citations that support a single point are only mentioned once, might a single citation work to support the statement? For example, no less than 18 (!) references [44-62] are cited only once to support the point that arm lengths are plastic in response to food levels. Maybe choose the one citation that is also cited elsewhere in the text.

We understand that many journals limit numbers of references, and this is surely understandable in the context of printed journals. Nevertheless, in the context of our online only journal submission here, we would like to retain all of these references. In particular, Dr. Palmer pointed to our large number of references in the echinoid larval morphometrics literature (now references 46-64 in the revised version). As we stated in our response to Reviewer 1 (see our response to Reviewer 1, Comment 5, above), we found it intriguing that the directional asymmetries that we report here have not been previously noted. We can only conclude that this is due to the fact that, surprisingly, none of these 19 studies reported left and right side measurements separately (one reference had a note saying they did not detect L-R differences as “data not shown”)?! We felt that we needed to cite all 19 of these papers as justification for the description of our findings as a “newly identified... asymmetry.”

Having said this, I’m a tad surprised the authors don’t cite:
Eaves presents extensive data on the prevalence of right vestibules in both Strongylocentrotus purpuratus and Dendraster excentricus larvae (see her Table 1 footnote).

We apologize for this oversight, and now cite Eaves 2005 (as our new reference 29) in the Methods section, as part of our now-expanded account of our enhanced double rudiment cohorts in Guelph (see Page 9 Line 176 in the revised manuscript, as well as our response to Reviewer 1, Comment 4, above).

SPECIFIC COMMENTS (page/line)

2/9: ’profound’ here seems rather over the top to me

We have removed this word as suggested (now page 2 line 21).

2/23-27: ’For the first time’ is not correct because, as the authors note later, such arm asymmetries have been described by Emlet 2009 and Collin 1997.

We thank Dr. Palmer for noticing our misleading wording. We have now settled on modified wording that we believe indicates the novelty of our observations while not understating the key observations of Emlet, Yanisagawa and Collin in this context, and we now use this modified wording throughout the manuscript (see, e.g., Page 2 Line 28, Page 5 Lines 93-95 and Page 19 Lines 404-409).

We note that the asymmetry reported by Emlet & Yanisagawa, while possibly related to our DA observations herein, are on a unique set of larval arms (the extremely long posterolateral arms) that project off of the posterior in Stomopneustes larvae. Indeed these arms are so unusually long that it led Emlet to propose that the reduction in posterolateral arm length on the left side is an absolute functional requirement for these unique larvae to be able to interact with the benthos at settlement! We discuss the observations of Collin further below.
2/32: If you believe Eaves 2005 (see above), right vestibules appear to be rather common, they are just not as well developed as left ones in both species studied here.

We agree with Eaves' observations (and we have repeatedly observed exactly what she reported), yet we do not feel that it warrants a change in wording in our abstract, as even Eaves herself notes that there is still a profound directional L-R asymmetry in rudiment invagination: the left side invagination always happens first and it is always bigger and more developed than the right side invagination (with the exception of the very rare *situs inversus* larvae, which we do not discuss in our manuscript), even in double rudiment larvae.

We do nevertheless agree that Eaves observations are definitely worth mentioning in our paper, and as indicated above, we now refer to Eaves 2005 in our methods section (new Ref. 29), alongside our expanded account of our enhanced double rudiment larval cohorts.

11/29-30: Levene’s test is used to test for differences in FA (a variance) among groups, not to test for the presence of FA. The significance of FA relative to measurement error is tested with an ANOVA on repeat measurements (see comments under Supplementary Information file below). Also, here the authors should refer readers to specific numbered tables in the Supplementary Info. file.

We appreciate the clarification here. We did repeat the analysis of FA relative to measurement error and report it in the revised version of the manuscript (see top of Page 16).

12/25: Refer readers to specific numbered tables in the Supplementary Info. file.

See our response to Dr. Palmer's General Comment III, above.

12/28: It's not clear to me why the authors do a PCA here. To test for the presence of asymmetry for each arm the test would merely be: is the mean \([\ln(R) - \ln(L)]\) significantly different from zero. A simple ANOVA would test whether asymmetry differed among arms: Factor 1 = arm, Factor 2 = Fertilization group, dependent = \(\ln(R) - \ln(L)\) (see General Comments I).

We absolutely agree with Dr. Palmer's comment here. We have now removed the PCA from the manuscript and replaced this analysis with the ANOVA as suggested. The conclusions have not changed profoundly as a result, but it has yielded a few new insights.

13-14: I suggest presenting these results for *S. purpuratus* as a) mean arm length and b) mean asymmetry \([\ln(R) - \ln(L)]\), as a function of time or larval stage bin (see General Comments I).

See our response to Dr. Palmer's General Comment I, above.

14/28-32: This statement cannot be correct. There is ALWAYS some fluctuating asymmetry (random deviations from perfect symmetry, or random variation about some average directional asymmetry). In some cases there may also be directional asymmetry (DA; significant average deviation from symmetry).
Via Dr. Palmer's review, we discovered that we had not done the FA test correctly. Once we repeated it using the method suggested, we indeed found evidence for FA in urchin larval arms, and now report this result as such (now top of Page 16).

15/35-50: This test would be much more straightforward if asymmetry is measured as ln(R) - ln(L) (see General Comments I). Then a simple ANOVA would test for food effects: Factor 1 = arm number, Factor 2 = food treatment, dependent = ln(R) - ln(L). This ANOVA could also be done using age/larval stage bin as a third factor, to test for age/stage dependence.

16/6-26: Again (see General Comments I), this is a weak test for differences in asymmetry. I would suggest re-doing these analyses as a 1-factor ANOVA for each arm using a more sensitive measure of asymmetry: Factor 1 = normal v double rudiment, dependent = ln(R) – ln(L).

See our response to Dr. Palmer's General Comment I, above.

17/11-21: Perhaps the authors should acknowledge that Collin 1997 reports "significant right-bias directional asymmetry" in the posterodorsal arm rods of 8 day old Dendraster larvae (her Table 3 statistical results support her statement in line 5 of her results section).

Yes indeed, and we regret having failed to acknowledge this finding properly in our originally submitted manuscript. We have rectified this in the revised version (see Page 19 Line 410-413, Page 22 Line 464 and top of Page 26).

We would note that the scope of asymmetry was still “subtle” (in the words of Dr. Collin) in her singular day 8 posterodorsal observation, 4-5x lower in magnitude than the asymmetries that we observed, and in only one arm as opposed to our observed DA in 2 pairs of arms in Dendraster (see our new Fig. 1). We suspect that this difference may be due to Dr. Collin having stopped her experiment on Day 8, and thus at an earlier stage than our observations on Day 11. But without representative photos of her day 8 larvae (which were fed at much higher levels than we typically do), we are unsure how advanced they were with respect to rudiment stage (i.e., how close they were to competence), thus it is difficult to explicitly compare her results to ours in this context.

17/42-19/8: This speculation about potential signaling pathways seems longer than needed. I would suggest condensing it about half a page.

We hope that our manuscript, if published, will interest a broad range of readers, but we suspect that the urchin development community will find our observations particularly interesting, and will be keen to know what the signaling mechanism might be. Therefore, we feel strongly about retaining the bulk of this discussion on signaling pathways. Nevertheless, to accommodate the reviewers suggestion, we have shortened this section by almost the amount suggested (now middle Page 20 – middle Page 21)

23/30: DA appearing later in development, and FA earlier, was also reported by Collin 1997 (her Table 3, and line 5 in her results section).

Indeed, and we have now modified this sentence to clarify this point (see Page 26, top).
26/6-13: Emlet 2009 (p. 172) also notes that a larva with a right-side rudiment had reversed larval arm asymmetry and one with no rudiment at all had symmetrical arms. These observations seem important and worth mentioning here.

We agree that these were key observations, and we have included them in the revised manuscript on Page 21 Line 441.

26/36: 'Previously undescribed' is not quite correct because, as the authors note, such asymmetries have been described by Emlet 2009 and Collin 1997. Perhaps this could be reworded.

See our response to Dr. Palmer's second Specific Comment, above.

27: Is this last paragraph needed? It does not seem to add much to the paper.

We respectfully disagree with Dr. Palmer here. We feel that the broad readership of RSOS warrants broad (while still relevant) conclusions. We believe our last paragraph is thus appropriate and would like to retain it.

34/11: State that ‘colored lines’ indicate the linear measurements taken.

We have done as suggested, see new legend to Figure 1.

35/ Fig 5: Replace lengths of left and right arms with actual measures of asymmetry (e.g., ln(R) - ln(L)) and average arm length (see General Comments I), or indicate in the legend what the dark and light bars represent.

We have done as suggested, as we discussed above (Response to Dr. Palmer, General Comment II)

Results, general: Throughout, report results of the present study in past tense.

We have done as suggested.

Figures, general: In both the PDF and on-line versions, I had difficulty viewing the figures in the submitted MS; panel labels, and text that spanned outside a figure box, did not show against the black background. The authors should be more careful to proof their submitted MS for proper figure visibility.

We did submit full, high quality, stand alone images with the first submission, and we have done so in this submission as well. In the original submission, we must not have noticed that the figures rendered poorly once uploaded. In the revised submission we have double-checked the quality of the uploaded figures (both as standalone and in the PDF); the issues that Dr. Palmer described above should not be a problem in this round.

Figure legends: In each legend, indicate a) that the bars are ±SE and b) what the sample sizes were.
We now have made sure to reference the error bars and sample sizes in each figure legend.

*Fig. 1 legend: Panels A and B are reversed from what the legend says.*

We regret this error, which we rectified in the revised Figure 1.

*Supplementary Information file:
A 93 page Supplementary Information file seems truly excessive and irresponsible to me. The authors should be more judicious in their choice of tests and presentation of statistical results. Mostly, these look like raw output files from a shopping list of statistical tests. If the authors aren't willing to make the effort to condense these results into sensible tables, why should the referees and readers bother spending time trying to do so?

We apologize for this extensive supplemental in the original submission, and thank Dr. Palmer for taking the time to wade through our many statistical analyses. We were under the mistaken impression that all such data needed to be included in the paper per journal requirements. We have since realized that there is a separate repository for such data, and that this does not need to be included in the paper per se, and explicitly shared with the reviewers. After submission of the original manuscript we uploaded all the raw data to the repository (dryad) which greatly reduces the need for providing supplementary information.

Dr. Palmer can rest assured that the revised supplemental (with a singular figure and brief legend) is much more streamlined than in the previous submission. We have also now included two tables in the manuscript that contain the key statistics all in one location. We hope that these changes will be seen as satisfactory.

*ALL tables should be individually numbered and have legends, pages should be numbered.*

We have numbered all pages in the revised version; we have removed the tables from the supplement, placed all ancillary data and analyses in the dryad repository, and now only have two tables, both in the main text, with table legends.

*Error analysis. The error analysis has not been done correctly to test for the significance of FA or DA relative to measurement error. The design should be: Factor 1 = side, Factor 2 = individual larva, dependent = repeated arm measurements. When done this way 1) the significance of the sides x individuals interaction term is a test of the size of FA relative to measurement error (i.e., did the difference between sides in individuals exceed that expected given the observed level of measurement error?) and 2) the F value of the 'sides' mean square divided by the 'sides x individuals' mean square is a test of the significance of DA. This should be done on a trait-by-trait basis (it is important to test for the significance of FA for each trait separately). The combined analysis (Table 2) seems meaningless to me.*

*If the measurements for the right side of one larva in the error analysis were missing (Table 1), that individual should be excluded from the analysis.*

We understand the shortcoming of our analysis and completely revised it according to the instructions and the very useful excel spreadsheet provided on Dr. Palmers website.
A newly-identified left-right asymmetry in larval sea urchins

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A newly-identified left-right asymmetry in larval sea urchins

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Running title: Left-right asymmetry in larval sea urchins
ABSTRACT

Directional asymmetry in body form is a widely spread phenomenon in animals and plants alike, and a functional understanding of such asymmetries can offer insights into the ways in which ecology and development interface to drive evolution. Echinoids (sea urchins, sand dollars and their kin) with planktotrophic development have a bilaterally-symmetrical feeding pluteus larva that undergoes a dramatic metamorphosis into a pentameral juvenile that enters the benthos at settlement. The earliest stage of this transformation involves a directional asymmetry: the echinus rudiment will form the oral field of the juvenile. Here we show for the first time in two echinoid species that there is a corresponding directional asymmetry in the overall shape of the larva: late stage plutei have consistently shorter arms specifically on the rudiment (left) side. We then demonstrate a mechanistic connection between the rudiment and arm length asymmetries by examining rare, anomalous purple urchin larvae that have rudiments on both the left and the right side. Our data suggest that this asymmetry is likely a broadly-shared feature characterizing ontogeny in the class Echinoidea. We propose several functional hypotheses –including developmental constraints and water column stability– to account for this newly-identified asymmetry.

Keywords: development, plasticity, fluctuating asymmetry, morphometrics, larval ecology, evolution
BACKGROUND

Many species of benthic invertebrates have a planktonic larval phase, which may allow these taxa to exploit alternative resources across life history stages, increase their dispersal ability and maintain connectivity among populations [1-3]. The echinoderms – including sea urchins, sea stars and sea cucumbers – exhibit a wide variety of such planktonic larval forms, both feeding and non-feeding [4-8]. These forms are the result of evolutionary pressures that appear to shape larval morphology within the confines of opposing functional constraints, in particular on feeding ability versus stability in the water column [9, 10]. Specifically, feeding structures generally require large surface area for particle capture, whereas stability, especially in turbulent waters, relies upon minimal surface area [8].

Consistent with these proposed trade-offs are the derived, non-feeding larval forms that have evolved independently and repeatedly across echinoderm taxa. Such larvae avoid the aforementioned functional constraints on feeding versus stability, and thus tend to be relatively simple in overall structure, with uniform ciliation or multiple ciliated bands circling their spheroid bodies to facilitate movement [11]. By contrast, feeding larvae exhibit more complex morphologies, and two classes of echinoderms – the ophiuroids (brittle stars and basket stars) and the echinoids (sea urchins, sand dollars and kin) – have independently evolved similar-looking pluteus larvae [12, 13], with 2-8 or more larval arms supported by internal skeletal rods. These arms are used for feeding and swimming, provide structural support, and might assist in passively orienting the larvae and offering protection from predation [14-17]).
The diverse echinoderm larvae described above share one key developmental feature: at a certain point in larval development, a directional asymmetry appears when juvenile structures begin to form internally on the left side in the otherwise bilaterally-symmetrical larva. In most echinoids with feeding larvae, this asymmetry is first visible as an invagination of ectoderm on the left side called the "echinus rudiment", which ultimately contacts a coelomic pouch, and they jointly transform into the oral portion of the pentamerally-symmetrical juvenile [18-22]. The juvenile structures will continue to grow and differentiate within the larva until it reaches metamorphic competence, at which point if it subsequently encounters suitable substrate, the larva will settle irreversibly on the benthos. The relationship between the juvenile and the larva is in a sense parasitic as the juvenile structures develop at the expense of larval growth [reviewed in 23].

The directionally-asymmetrical rudiment invagination is particularly well studied in the purple sea urchin, *Strongylocentrotus purpuratus*. Aihara and Amemiya [21] provided strong experimental evidence that the right side of the larva is largely responsible for differentiating the L-R axis: laterally-bisected larvae (before rudiment invagination) all regenerate and develop to competence, but the larvae developing from the left halves rarely exhibited normal L-R patterning, whereas those from the right side almost always developed normally. More targeted removals of portions of the right side also resulted in larvae with abnormal L-R patterning.

Recent molecular evidence has further supported this scenario of right side control of the L-R asymmetry. The identified genes that appear to regulate L-R asymmetry in urchins encode two secreted growth factor-like proteins – Nodal and
Lefty– and the Pitx2 paired-class homeodomain protein, all three of which are expressed primarily on the right side of the larva; their proper expression restricts rudiment formation to the left side [24]. BMP (Bone Morphogenetic Protein) signaling is then asymmetrically activated and is required for the development of left sided structures and marker genes [25]. Additionally, a H,K-ATPase-like protein also appears to be important, most likely via either H\(^+\) or K\(^+\) gradients that occur upstream of the asymmetric gene expression of *nodal*, *lefty* and *pitx2* [26].

Here we show that concomitant with this L-R asymmetry in rudiment formation is a consistent asymmetry of the larval arms in advanced echinoid pluteus larvae, the extent of which has not previously been described. We first document the asymmetry in two disparate echinoids separated by 250 million years, suggesting that this asymmetry may be a common feature among echinoids with feeding larvae. We further explore the phenomenon in *S. purpuratus* to evaluate the possible connection between the rudiment and larval arm asymmetries, and by examining anomalous larvae with rudiments on both the left and right sides. We discuss our results in the context of several hypotheses concerning the function of this newly-identified asymmetry in sea urchin plutei. In so doing, we highlight the ways in which directional asymmetries offer a unique window into how ecology and development work together to drive organismal evolution.
105 METHODS

Source populations, maintenance of adults and larval cultures

For the characterization of the arm-length asymmetries in *Dendraster excentricus* (Eschcholtz) sand dollar larvae, we used adults collected at low tide (-0.25 meters) from a large, intertidal population in East Sound (Orcas Island, WA, USA) on July 17, 2015. The adults were maintained at Friday Harbor Labs (FHL; Friday Harbor, WA, USA) in flowing sea water in sand bins until spawning. On August 26, 2015, we spawned several adults by intra-coelomic injection with 0.5M KCl. We set up crosses from two females: one by standard methods [27] using sperm collected dry that same day from a single spawning male, the second by aspirating off the eggs from the aboral surface of a second female who began spawning after we returned her to an aquarium. This second female's eggs were already fertilized, therefore we are unsure of the paternity in this second cross (several males were also spawning in the aquarium at that time, including the male from the first cross). Thus, the two crosses were either half sibs from different mothers, non-sibs, or a mixture, and we maintained them separately throughout to ensure that any results we obtained could not be explained by the larvae having been derived from an aberrant female. We conducted fertilizations and all subsequent rearing steps in 0.45 µm Millipore-filtered natural sea water (MFSW) at room temperature, which varied between 19 and 22°C.

Sand dollar embryos had hatched by 1 day after fertilization, at which point we set up one jar from each cross at approximately 1 larva/ml of MFSW, and fed them a combination of *Rhodomonas spp.* (2.5 cells/µl) and *Dunaliella tertiolecta* (3 cells/µl), and kept them gently stirred using a gyratory shaker table. We changed their water every...
two days by reverse filtration of >95% of the water volume and gave the larvae fresh
MFSW and food. On day 3 (comparable to soft tissue stage iii from [22]), we reduced
the larval density to 1 larva/5 ml MFSW, and maintained them at that density until day 9
(more or less equivalent to skeletogenic stage 10 from [22]) when we conducted all
larval arm measurements.

For the characterization of the corresponding arm-length asymmetries in purple
urchins as well as the ontogenetic characterizations and feeding trials, we used adult
Strongylocentrotus purpuratus (Stimpson), collected at Slip Point (Clallam Bay, WA,
USA) and maintained in subtidal cages suspended off the floating docks at FHL, fed
throughout the year ad libitum with drift kelp (mainly blades of Nereocystis leutkeana).

We spawned 2 males and 2 females on March 27, 2015 at FHL, by intra-coelomic
injection with 0.5M KCl. We then set up the four pairwise fertilizations in MFSW using
standard methods [27] at 11°C. We transported embryos the next day to the University
of Washington (Seattle, WA, USA) and continued to maintain the cultures at 11°C. On
day 5, when the embryos had reached the late prism/early 4-arm larval stage, we set up
a single gallon jar in MFSW at approximately 1 larva/ml from equal proportions of the 4
fertilizations, fed them a combination of Dunaliella tertiolecta and Rhodomonas spp. as
described above, and over about an hour, warmed the culture to 15°C in a shaking
water bath, where we maintained all cultures for the remainder of the experiment. Every
two days, we cleaned the cultures and fed them as described above.

On day 15, most of the larvae had reached the rudiment invagination stage (soft
tissue stage i from [22]), at which point we reduced the larval density to approximately 1
larva/1.3 ml MFSW, and then to ~1 larva/2 ml MFSW on day 17, with food at the same
concentration as previously. On day 20 (approximately soft tissue stage iv from [22]), we individually selected 1000 of the optimally developing larvae, only rejecting those (<20% of the larvae) that appeared significantly smaller than the average larva. In so doing, we reduced the density to 1 larva/6 ml, and fed them as before. This stepwise reduction in density was an attempt to limit bouts of larval cloning, which can be induced by sudden shifts in density (unpublished observations), and would be expected to increase variability in arm length within cultures [28].

On day 25 (approximately skeletogenic stage 1 from [22]), we selected out 83 larvae into each of 6 jars with 500 ml MFSW (so still at 1 larva/6 ml) and randomly assigned each jar to one of two treatments: 3 replicate jars of high food (full ration of Dunaliella:Rhodomonas at 1:4 cells/µl) and 3 replicate jars of low food (25% ration at 0.25:1 cells/µl) for the remainder of the experiment. Approximately 50% of the larvae in the high food treatment had reached metamorphic competence by day 42; the low food larvae had not quite reached metamorphic competence by the time we concluded the experiment on day 45.

For the "double rudiment" experiment we used purple urchins originally obtained from The Cultured Abalone Ltd. (Goleta, California, USA) and that we have maintained at the Hagen Aqualab at the University of Guelph (Guelph, Ontario) in an artificial seawater system on an 8:16 hr light:dark photoperiod at approximately 12°C and 34 ppt salinity, fed ad libitum with rehydrated Kombu kelp (Laminaria spp.), repeatedly spawning the same individuals as they become gravid again. Over the last several years (2011-2013), we repeatedly noted an unusually high proportion (approximately 2-5%; data not shown) of offspring of particular urchins from our Guelph colony that
exhibited rudiments on both their right and the left sides – so-called "double rudiments" – a seeming hypertrophy of the small right side invagination typical of development in at least some echinoids (including the two species we examined here; see [29]). Note that, since the death of the particular adults from which we obtained offspring with enhanced double rudiment occurrence, we have no longer observed this phenomenon in our Guelph colony, despite having made no notable changes to our water or culturing system. Therefore, we conclude that the double rudiment-enriched larval cohort that we examined here derived from a specific maternal, paternal or genetic effect, and that we typically (and since 2014, exclusively) have seen the phenomenon only at the expected rate of fewer than 1% of larvae in a colony ([30]; our unpublished observations).

In September 2013, we obtained gametes from one male and one female adult S. purpuratus by intracoelomic injection following protocols described above, whose larvae later exhibited the enhanced double rudiment phenotype. After fertilization and hatching, we set up cultures at an approximate density of 1 larva/2 ml of 0.45 µm Millipore filtered artificial sea water (MFASW), agitated to prevent the larvae from settling out of the water column. We transferred larvae three times per week to clean beakers with new water and fed them with either Dunaliella tertiolecta or Rhodomonas spp. at 12 cells/µl or 6 cells/µl respectively. In the third week of development, we noticed that this larval cohort exhibited the enhanced double rudiment phenotype. At 21 days post-fertilization, we measured nine stage-matched (see below) single- and double-rudiment sibling larvae, and assessed asymmetry as in the previous experiment.
Staging and Measurements

In FHL, we measured ten live 9-day old sand dollar larvae from each of the two crosses, gently immobilized on slides under raised cover glass, using an Olympus BH-2 microscope. In Seattle, we staged and measured live purple urchin larvae, immobilized as above, using a Leitz Wetzlar Ortholux microscope. For the purple urchins, we employed the staging scheme as outlined in [22]. Note that we used stage bins (as defined in the legend to Fig. 3) for the analyses of the urchin data in an attempt to equalize the numbers of individuals within each bin for this dataset. In both FHL and Seattle, we measured skeletal rod lengths on haphazardly-chosen larvae using a calibrated ocular micrometer, and calculated the z-axis offset of each measured skeletal rod using the gradated focus knob, which we had calibrated using a slide and cover glass of known thickness (measured with a micron caliper).

To account for possible measurement error and/or bias in our measurements, we used larvae fertilized and reared in Guelph as described above, but from an August 3, 2015 fertilization. On day 21, we packed and shipped overnight approximately 200 of these live larvae to Seattle, to conduct the error measurements on the Leitz microscope set-up used for the majority of our data (see above). The larvae arrived in good condition on day 22 at approximately 13°C, and we conducted the error measurements on that day as follows. We haphazardly chose 20 larvae and placed them on individual microscope slides with raised cover glass as described above, and then staged and measured each one as above. Then, a colleague uninvolved in the study re-labeled all 20 slides and we re-measured each of the 20 larvae a second time; thus, the second measurement on these same larvae was done “blind.” We calculated measurement
error using the difference between each of the paired measurements, and used this error calculation to ensure that any reported differences in L-R asymmetry fell outside of the experimentation error range. We also used these data to assess fluctuating asymmetry (FA) as further explained below.

For the double rudiment experiment, we staged larvae under a Nikon Eclipse Ti microscope, according to our published staging scheme (see Tables 1 and 2 in [22]), and we made three-dimensional "z-stacks" (pictures taken at 10 µm steps through the larvae) with a Nikon Digital sight DS-Fi1 camera. We then conducted measurements on these z-stacks using a 3D measurement macro (calibrated to account for both the x-y and z-y plane distances covered) using ImageJ software Fiji. We measured left and right postoral (PO), posterodorsal (PD) and anterolateral (ALA) arm lengths for each larva.

As indicated in Figures 1B and 2E, the PO and PD rods are relatively straight, so our calculation of these skeletal rod lengths were straight forward. By contrast, the preoral (PRO) and ALA rods are somewhat curved in both species. Our reported PRO and ALA rod lengths represent the linear distance between the landmarks indicated in Figures 1B and 2E, and are thus are our best approximations of these rod lengths using the methods we employed.

**Statistical Analysis**

We conducted all statistical tests using SPSS v23. We analyzed all morphological comparisons using SPSS MANOVA, regression and one sample t-test.
commands. We tested for directional asymmetry (DA) using the index of asymmetry 
[ln(R)-ln(L)], which is equal to ln(R/L), and mathematically equivalent to the following 
equation:

\[
\left\{ \frac{(R-L)}{(R+L)/2} \right\}
\]

where R and L are the lengths of the right and left arm respectively [31]. For each of our 
structures measured at each time point, we then calculated the mean [ln(R/L)] and used 
a two-tailed one-sample t-test to determine if this value was significantly different from 
zero; a positive value indicates right-biased DA, a negative value indicates left biased 
DA. We used q-q and p-p plots to test for normality throughout. We further tested for 
age and stage effects on asymmetry using regression analysis. We tested for an effect 
of food on asymmetry across stages with a MANOVA using food level as a factor. We 
tested for fluctuating asymmetry (FA) using methods outlined by Palmer and Strobeck 
[31]. In all cases, we considered results “significant” if \( \alpha \) was < 0.05. We report all 
results as \( \pm \) one standard error unless stated otherwise.

RESULTS

We tested whether echinopluteus larval arms were directionally asymmetrical 
during mid- to late-larval development, and whether any detected asymmetry changed 
as a function of developmental age and stage.
Larvae of the sand dollar *Dendraster excentricus* showed directional asymmetry in larval arm growth

We measured all eight larval arms of *D. excentricus* 9 days post-fertilization at ~20°C; we analyzed differences in arm lengths between the left and right side of the larva (Fig. 1) using the index of asymmetry [ln(R/L)]. We found that the postoral (PO) and posterodorsal (PD) arms of *D. excentricus* larvae were significantly shorter on the side of the juvenile rudiment (left side) compared to the right side (PO: \( t=5.59, p<0.001; \) PD: \( t=3.05, p<0.01; n=20 \)). We did not find differences in arm length between the left and right side of *D. excentricus* larvae for the other two pairs of arms [anterolateral (ALA: \( t=0.86, p=0.4, n=20 \); and preoral (PRO: \( t=-0.89, p=0.4, n=20 \))] We also examined the index of asymmetry [ln(R/L)] for the sum of all four arm lengths (PO+PD+ALA+PRO) on each side as an indication of the overall asymmetry of the larva, and found that these larvae were indeed significantly asymmetric overall, with shorter total arm length on the left (rudiment) side [ln(R/L)=0.043 ± 0.008 (standard error), \( t=5.40, p<0.001, n=20 \)].

Note also that we set up fertilizations from two different females in this experiment, and reared their offspring and analyzed them separately, to ensure that our results were not an aberration associated with a maternal effect or a given genotype. We did not find a significant interaction between cross and the index of asymmetry for any arm pairs (PO: \( F_{1,19}=0.71, p=0.41; \) ALA: \( F_{1,19}=0.08, p=0.78; \) PD: \( F_{1,19}=1.90, p=0.19; \) F1,19=0.14, p=0.72), indicating that asymmetry patterns were not different between the two crosses.
Larvae of the purple sea urchin, *Strongylocentrotus purpuratus*, showed directional asymmetry (DA) in larval arm growth that changes through ontogeny.

To determine if the late-stage directional asymmetries (DA) we observed in sand dollar larvae are a more widespread feature among echinoids, and to examine whether such an asymmetry progresses during ontogeny, we examined DA in the purple sea urchin *S. purpuratus* at four time points post-fertilization (PF). We then analyzed arm length morphometrics as a function of age and binned stage (see Fig. 3 legend for details).

Based upon the sand dollar data and our preliminary observations on *S. purpuratus* larvae, we expected DA to first become manifest in later development, after the juvenile rudiment begins to form. Therefore, our earliest measurement date was on day 15, right around the time when we first observed rudiment invagination (Fig. 2D). We made additional measurements during the growth of the juvenile rudiment on days 25, 33 and finally on day 39 (Fig. 2E), at which point the larvae were at or near competence to transform to the juvenile stage (not shown).

Directional asymmetry (DA) as a function of age are shown in Figure 2 and Table 1. Overall, we detected DA starting on day 33 in ALA arms, and in three of the four arm pairs (PO, PD and ALA) on day 39; in each case, the arms were significantly shorter on the left side. To see if DA in each of these arms pairs varied with age, we examined linear regressions for the indices of asymmetry for each of the four sets of arms from day 15 to 39: both the ALA and PD arms showed increasing DA with age (Table 1).
In order to more clearly examine if DA in purple urchin larvae is related to the growth of the rudiment, we sorted all of the larvae (regardless of age) into one of 6 stage bins (A-F) defined by growth of skeletal structures in the rudiment (see the legend to Figure 3 for details on how stage bins A-F correspond to the rudiment staging scheme in [22]). As seen in Figure 3 and Table 2, we detected DA in ALA arms in stage bins C, D and F, and in PO and PD arms only in stage bin F (the most advanced larvae in our dataset). As with the age data, we examined linear regressions for the indices of asymmetry for each of the four sets of arms to see if DA in each of these arms pairs varied with stage. In this case, only the PD arms showed increasing DA with stage (Table 2).

As different arms appear to show different levels of DA (Figs. 2, 3; Tables 1, 2) we analyzed the index of asymmetry for the sum of all four arms) by both age (Fig. 4A, Table 1) and stage (Fig. 4B, Table 2). With respect to age (Fig. 4A, Table 1), we detected evidence for DA in total arm length on days 33 and 39. A regression analysis showed a positive relationship between index of asymmetry for total arm length and age (Table 1).

With respect to stage (Fig. 4B, Table 2), we detected evidence for DA in total arm length in stage bins C, D and F. But in this case a regression analysis showed no overall relationship between index of DA for total arm length and stage (Table 2). As can be seen in Figure 4B, DA does not monotonically increase with stage; instead the stage of peak DA for total arm length is stage bin C, which is the only stage at which our ANOVA, after Bonferroni correction, detected a significantly higher DA than in any other stage (stage bin C versus stage bin A: p=0.048).
Although it is not the focus of the current study, we undertook an analysis of fluctuating asymmetry (FA) in comparison to measurement error. We performed this analysis on a separate cohort of larvae 22 days after fertilization (all of which were in stage bin A; see Fig. 3 legend). Using a mixed model as described by Palmer and Strobeck [31], we found no evidence for directional asymmetry in PO, ALA and PD arm lengths (PO: $F_{1,37}=0.06, p=0.80$; ALA: $F_{1,37}=0.57, p=0.45$; PD: $F_{1,37}=0.98, p=0.33$); this was expected for larvae in stage bin A (c.f., Fig. 3, Table 2). Nevertheless, FA was significantly larger than measurement error (PO: $F_{1,37}=6.32, p<0.01$; ALA: $F_{1,37}=3.87, p<0.01$; PD: $F_{1,37}=19.31, p<0.01$) for all arm lengths among these larvae (note also that these larvae only had 6 arms at the stage we examined them, thus we do not have PRO data here). Although not entirely comparable, as these larvae were a separate cohort from the main experiment outlined above, we note that the scope of our measurement errors for PO, ALA and PD were 4-5x lower than the scope of L-R differences that are seen in Figures 2 and 3.

In sum, we see clear evidence for DA in multiple arms through ontogeny, whether viewed by age or stage. Arm length asymmetry in *S. purpuratus* larvae increased with age and stage in ALA and PD arms, and total arm asymmetry (i.e., asymmetry in the arms as a whole) also increased with age. When analyzed by stage, however, the pattern appears more complex: the greatest degree of total arm asymmetry occurred in our stage bin C, which falls at about the midpoint of rudiment development towards metamorphic competence.
Food did not affect larval arm asymmetry in *S. purpuratus*

One hypothesis that could explain the previously noted directional asymmetries is that there is competition for limited resources or materials between the rudiment and the nearby left arms. If so, we might expect to observe a more dramatic asymmetry in larvae raised in food limited conditions, where such materials/resources would be in reduced supply.

To test this hypothesis, we reared larvae under a high food ration for 25 days, and then shifted a subset of the larvae into a reduced food ration (25% of the high food ration) for the remainder of larval development. We then analyzed changes in arm length as a function of age and stage for the low food- and high food-reared larvae.

Plasticity in larval arm growth overall as a function of food level has been well demonstrated in *S. purpuratus* and numerous other echinoids [reviewed in 23]: in general, larvae under low food conditions have longer arms relative to growth of juvenile structures. We observed clear arm length plasticity for all arm pairs as a function of binned stage (Fig. 5; PO: $F_{1,89}=21.19, p<0.01$; ALA: $F_{1,89}=3.85, p=0.05$; PD: $F_{1,89}=11.97, p<0.01$; PR: $F_{1,89}=5.62, p=0.02$), as expected.

We then tested whether food environment would impact the extent of arm length directional asymmetry (DA) in *S. purpuratus* larvae (Fig. 5). Statistically, such a food effect on asymmetry would manifest as a significant interaction between food treatment (high or low) and the index of DA [$\ln(R/L)$]. Note that we only analyzed our data by stage, as low and high food larvae developed on quite different trajectories towards metamorphic competence (see Methods, above), and it seemed most sensible to
normalize by juvenile rudiment stage. We did not find evidence for a statistically
significant interaction between food treatment and DA for any larval arms by stage (Fig.
5; PO: F_{1,119}=0.58, p=0.45; ALA: F_{1,119}=0.01, p=0.98; PD: F_{1,119}=1.44, p=0.23; PR:
F_{1,119}=3.32, p=0.07). DA in the high and low food treated larvae for total arm length by
stage can be found in the Supplementary Material (Fig. S1).

S. purpuratus larvae with double rudiments do not exhibit right-biased directional
asymmetry

We analyzed anomalous larvae with naturally occurring double rudiments (i.e.,
larvae with “twin” juvenile rudiments developing simultaneously on the right and left
sides; Fig. 6B) to test the hypothesis that directional asymmetry (DA) in larval arms is
functionally linked to the formation of the juvenile rudiment (Figs. 3, 4B). As expected,
our single rudiment larvae (Fig. 6C) showed right-biased DA in both PO and PD arms,
with shorter arms on the left side (Fig. 6a; PO: t=2.68, p=0.028; ALA: t=1.90, p=0.093;
PD: t=2.95, p=0.018; n=8). By contrast, in our double rudiment larvae (Fig. 6B), the ALA
and PD arms did not show any evidence of DA (Fig. 6a; ALA: t=0.065, p=0.95; PD:
t=-1.08, p=0.32; n=9). In fact we detected a significant DA in the opposite direction in
the PO arms, which had slightly (~4.3%) longer arms on the left side (Fig. 6a; PO:
t=-3.377, p=0.012, n=9).
**DISCUSSION**

Metamorphosis in extant echinoderms involves a transformation from a bilateral larva to a pentameral adult [32, 33]. Because the common ancestor of echinoderms and other deuterostomes is hypothesized to have had an adult with bilateral symmetry [reviewed in 34], this bilateral-to-pentameral shift during echinoderm ontogeny is considered key to understanding the evolution of this unique group [35-37].

In all living echinoderms with indirect development (*sensu* McEdward [6]), the adult body plan develops in a curious fashion with respect to that of the larva, via an internal directional asymmetry: the juvenile forms on the left side of the otherwise bilaterally symmetrical larva [18-22]. Here we show for the first time that in two disparate echinoids, a sea urchin and a sand dollar, a second directional asymmetry is apparent during late larval development, when the overall shape of the larva changes from more or less bilaterally symmetric to directionally asymmetric during later stages: the majority of the larval arms, which are supported by calcium carbonate skeletal rods, are substantially (up to 25%; see Figs. 1 and 2) shorter on the rudiment side of the larva.

Although asymmetries in larval arms have been noted previously during normal development in echinoid larvae [38-40], ours is the first demonstration of such a unidirectional asymmetry in multiple arms, resulting in a consistent yet previously undescribed shape change during late pluteus development. As in one other reported case of a directional asymmetry in advanced pluteus larval arms [39, 40], we show evidence here for a link between formation of the rudiment and the arm length asymmetry, with shorter larval arms specifically on the rudiment (left) side. Our
demonstration of this asymmetry in both an irregular and a regular echinoid, as well as our casual observations of such asymmetries in the larvae of several other echinoid taxa which we have reared from several geographically-disparate locations (data not shown), suggest to us that this directional asymmetry in arm length is likely a generic feature of late-stage, echinoid larval development, and as such, it calls out for a functional explanation.

Possible developmental mechanisms underlying larval arm asymmetry

Based on previous studies, Nodal and BMP signaling are the primary factors responsible for the development of the juvenile rudiment on the left side of the sea urchin larva, with Nodal expression on the right inhibiting BMP signaling there, and thus directing BMP-activation of rudiment formation to the left side [24, 25, 41, 42]. Still, the consequences of this asymmetry for development of the larval arms is unclear.

If the BMP-Nodal gene network is likewise involved in our reported arm length asymmetries, then we would predict that the arm length asymmetries would coincide with rudiment formation. This is not what we have observed. Indeed, at soft tissue stage iv [22] (stage bin A, day 25 in this experiment) rudiment formation was already well underway and we did not observe any directional asymmetries in arm length, which we first detected at skeletal stage 3-4 [22] (stage bin C, ~day 31 in this experiment; Figures 2-4).

These observations suggest that if the BMP-Nodal gene network is involved in the directional arm asymmetry, then this involvement is likely to be either indirect, or to
involve subsequent signaling steps. Still, our data reported here on double rudiments do suggest that there is a mechanistic connection between rudiment asymmetry and the arm length directional asymmetry, as also reported by Emlet [40] for the posterolateral arm length asymmetry in the black sea urchin, *Stomopneustes variolaris* (Echinoidea: Stomopneustidae). Evaluating the nature of this mechanistic connection could involve examining arm asymmetries in the context of reduction-of-function manipulations (e.g., using morpholino oligonucleotides) directed against BMP or nodal signaling components. Specifically, one could experimentally generate double rudiment larvae, for example by inhibiting Nodal signaling [24], and test whether such a manipulation results in loss of the directional asymmetry, as we saw in our naturally-occurring double rudiment larvae. If BMP-Nodal signaling is indeed connected to the arm length asymmetries, then it is possible that the expression of skeletal elongation genes such as p58b and p16 [43, 44], and the skeletogenic gene network to which they belong [45], may integrate with the BMP-nodal gene network in an as yet unrecognized fashion.

### Functional implications of the asymmetry for larval biology

The antagonistic relationship between rudiment growth and arm length in feeding pluteus larvae has been well-described in numerous taxa. Specifically, echinoid larvae show adaptive phenotypic plasticity for arm growth relative to growth of the rudiment: under high food conditions, arm length is reduced relative to rudiment growth; under low food, arms grow longer and rudiment growth is delayed [9, 23, 46-64]. The adaptive nature of this plasticity is indicated by the increased food capture efficiency in larvae with longer arms [9].
Therefore, there is presumably a mechanistic connection between rudiment growth and arm length, and it may be that this same antagonistic connection underlies the late stage arm-length asymmetries reported here and by Collin [38], Yanagisawa [39] and Emlet [40]. But why would such an antagonistic connection only manifest on the left side of the larva during these late stages? Here we consider two functional hypotheses in turn for our observed directional asymmetries in arm length. According to the *developmental constraint hypothesis*, the asymmetries that we report here result from some type of local developmental constraint or trade-off, in which the rudiment grows at the expense of only the adjacent larval arms. Alternatively, the *swimming stability hypothesis* predicts that this link is driven by selection on a specific, asymmetric larval shape that facilitates stability or some other aspect of performance in the water column.

The *developmental constraint hypothesis* envisions a scenario where some factor utilized in the construction of both the rudiment and the larval arms is in limited supply. The increasing demands in the rapidly growing rudiment for this hypothesized factor leaves less of it available for the growth of nearby larval arms, and the result is shorter larval arms adjacent to the rudiment.

Although our experiments are not sufficient to falsify this hypothesis, our results are not wholly consistent with it. On the one hand, the left larval arm that is most distant from the rudiment—the left preoral (PRO) arm—shows no evidence for directional asymmetry in either *D. excentricus* or *S. purpuratus*, thus offering some support for a local constraint related to the rudiment. Furthermore, the constraint hypothesis would predict that the local competition would become more pronounced as the rudiment
grows ever larger and more complex as ontogeny proceeds; our developmental time series with *S. purpuratus* (see Fig. 2, Table 1) is more or less consistent with this prediction. However, when we analyzed our larvae grouped by rudiment development stage (see Fig. 3, Table 2), we detected no increase in asymmetry at later stages. Indeed, the stage with the most dramatic asymmetry was stage bin C, about mid-way through rudiment development, where calcification of juvenile structures in the rudiment is at an early stage [22]. Furthermore, under a reduced food scenario, where arms grow longer and rudiment growth is delayed, the constraint hypothesis would seemingly predict an even more dramatic asymmetry than in our well-fed larvae. This is not what we observed; we saw no difference in the observed asymmetry between food treatments.

Therefore, our data provide mixed support for the constraint hypothesis. Still there is some precedence in other developing organisms for such a hypothesized local competition for factors or resources. For example, the wing-reproduction tradeoff in monarch butterflies (*Danaus plexippus*) has been proposed to be related to a position-dependent mechanism, where juvenile hormone -produced in the brain- travels in the hemolymph past the wings and through the thorax, ultimately reaching the gonads in the posterior. Lessman and Herman [65] hypothesized that the highly active thorax during long-distance flight acts like a gauntlet, breaking down active juvenile hormone as it passes, leaving lower levels to arrive at the gonad, and therefore leading to reduced gonadal growth during flight. A second proposed example is in horned beetles (*Onthophagus spp.*) and other insects, where a factor such as insulin may be in limited
supply, and could account for the apparent trade-off in the sizes of adjacent structures [66-68; but see 69].

In the urchin pluteus example, what could such a factor be? One appealing possibility relates to the availability of calcium, which is used to construct both the skeletal rods that support the larval arms and the diverse juvenile skeletal elements forming in the rudiment at late larval stages; these processes may thus be in direct, local competition. A simple experiment that would test for calcium limitation would be to add additional calcium to the sea water in which the larvae are grown – if calcium within the larvae at these late stages is normally in limited supply, then the asymmetry could be attenuated or disappear if excess calcium is provided. Alternatively, one could block calcium transport into the tissues of the rudiment through morpholino microinjection [70] or treatment with chemical inhibitors, and test whether such treatments result in more symmetrical larvae than in controls. Indirect evidence for this calcium limitation hypothesis follows from the findings of Byrne et al. [71], who reported that *Heliocidaris tuberculata* (Echinoidea: Echinometridae) larvae reared at low pH showed notable asymmetries (presumably FA) at early larval stages.

A second intriguing possibility is that the local rudiment-arm length antagonism is regulated by thyroid hormone (TH) signaling. Our previous studies [59] demonstrated that TH treatment results in a phenotype similar to that previously described for low food treatments, where juvenile structures grow faster, and larval arm growth is suppressed. Experiments with TH synthesis inhibitors [34, 72-74] indicate that feeding larvae have the capacity to produce TH internally, and our unpublished immuno-labeling experiments indicate that the source for TH may be structures within the rudiment.
Therefore, if the rudiment is indeed the source for TH in plutei, and if TH levels correlate negatively with arm growth, then one might expect arms near to the rudiment to be shorter than ones more distant: this is precisely what we have observed here. A simple test of this scenario would be to provide excess TH exogenously and see if the observed asymmetries disappear.

Finally, additional support for biased L-R allocation of materials in pluteus larvae comes from some intriguing observations on sea star bipinnaria larvae, whose feeding larvae are considered homologous to echinoid plutei [75]. Circulation of fluid in the blastocoel cavity of bipinnaria larvae has been described as largely unidirectional: from the stomach, along the left side of the larva, and then around the mouth to the right side of the larva [76; Jaeckle, pers. comm.]. Furthermore, coelomic fluid flow out of the left hydrocoel via the pore canal and hydropore to the exterior of the larva [77] would tend to draw blastocoelar fluid towards the left side [78], with the growing rudiment in later stage larvae thus being a possible sink for blastocoelar substances. This biased directional flow could therefore represent a mechanistic basis for uneven distribution of blastocoelar substances, leading to the asymmetries that we report here.

The swimming stability hypothesis proposes that the asymmetry in larval arms would provide a selective advantage to larvae, whereby asymmetrical larvae would, for example, sink more slowly (and thus be retained in the water column more efficiently) than symmetrical larvae. Such an asymmetry would be predictably directional due to the substantial asymmetry in ballast provided by the rapidly growing and calcifying rudiment, predominantly on the left side of the larva.
Indirect support for this hypothesis comes from several examples in the literature. First, Collin [38] detected fluctuating asymmetry (FA) in early, pre-rudiment larval stages of the sand dollar *D. excentricus*, and documented the first indication of a subtle directional asymmetry [DA] in a single arm (posterodorsal; PD) during mid-late larval development. FA is widely viewed as a measure of developmental stability and perturbations to the developmental process, and can have both genetic and environmental causes [79]. Indeed, several studies have demonstrated or suggested an increase in FA or other asymmetries when sea urchin larvae or adults are exposed to toxins [e.g., 80, 81]. Nevertheless, the apparent continuity between FA in larval arms earlier in normal ontogeny [38], and then increasing DA later (38; our data reported here) may indicate that the asymmetries themselves may be functional throughout normal larval development. In this case, the forming rudiment in late stages might impose additional constraints that could lead to predictably shorter arms on the left.

Chan [82] reviewed a number of studies on pluteus larval morphology as it relates to stability and swimming, under different flow regimes and through ontogeny. The basic pattern that Chan reports is a slight tilt in the orientation of the larval body in flow increases the chances that a larva can maintain upward swimming (and thus presumably stay in surface waters), rather than being carried downward. In an unpublished study, Miyashita (pers. comm.) discovered that modeled, asymmetrical *D. excentricus* larvae at the 4-arm stage (pre-rudiment growth) are able to maintain upward swimming more effectively than symmetrical ones.

The models that Chan and Miyashita employed were based upon those developed by Clay and Grunbaum [10], again using the sand dollar *D. excentricus*, but...
once again focusing on early (4 arm) larval stages before the development of the rudiment. One important set of parameters in this model relates to the center of gravity, which would clearly change with the addition of an asymmetric, calcified rudiment, as seen in the stages that we examined (though pluteus larvae are surprisingly more buoyant as ontogeny proceeds; [83]). Furthermore, drag on larvae increases with both arm length and arm number [15], which would be predicted to impact the stability of larvae in different flow regimes [8].

Taken together, the studies to date suggest that larval shape, orientation and asymmetries all can contribute to position in the water column, which can have important consequences for dispersal or nearshore retention, prey encounter and predator avoidance throughout larval development, and contacting the substrate in late stage larvae preparing to settle to the benthos. Nevertheless, it is difficult to extrapolate from the previous modeling studies on much simpler larval morphologies to those in fully formed, 8-arm larvae with an asymmetrically growing and calcifying rudiment in realistic flow conditions.

Our experiments reported here do not directly address the swimming stability hypothesis. However, one intriguing observation is that the three arm pairs that protrude the furthest from the larval midline —the postoral, posterodorsal and anterolateral arms— are the three pairs that showed clear directional asymmetries in one or both species. By contrast, the arm pair that runs closest to the midline —the preoral arms— showed no signs of directional asymmetry in either species at any age or stage that we examined. Likewise, the observations by Yanagisawa [39] and Emlet [40] of a dramatic asymmetry in late stage larvae of the sea urchin *Stomopneustes variolaris* was specifically in a
unique pair of arms that project off the posterior end of those larvae, the posterolateral arms. All of these observations suggest that asymmetric arm growth is not simply a generic feature of late-stage pluteus larval arm growth common to all arms, and may thus point towards a functional explanation such as envisioned by the swimming stability hypothesis.

To adequately test this hypothesis experimentally, one would need to examine larvae under realistic flow conditions, and see if the degree of asymmetry in late stage larvae is related to their position in the water column. In addition, one could develop more complex models of the pluteus larval form that would extend from the Clay & Grunbaum [10] model, but include all four arm pairs and a growing and calcifying, asymmetrical rudiment. The swimming stability hypothesis would predict that arm asymmetries at these later stages would have clear consequences for position in the water column and/or swimming ability.

Finally, we note that the developmental constraint and swimming stability hypotheses are not the only two possible explanations for our observed asymmetries (and these themselves are not necessarily mutually exclusive). For example, in recent years it has become clear that asexual larval cloning is widespread in echinoids (which again, has been particularly well-documented in *D. excentricus*), and one method of such asexual cloning in larval sea stars [84, 85] – as yet undocumented in laval echinoids [but see 28] – is budding of the arm tips. It is possible that such budding occurs preferentially on the left side of late-stage echinoid larvae, which would lead to a directional asymmetry pattern such as we observed. Furthermore, Emlet [40] suggested that the asymmetry in posterolateral arms in *Stomopneustes variolaris* may be an
adaptation for more effective settlement to the benthos: a long left posterolateral arm could interfere with substrate contact. A similar mechanical interference scenario might promote the evolution of shorter arms in late stages in other urchins as well, as we have observed here.

CONCLUSIONS

We here identify and characterize the ontogeny of a directional asymmetry in the shape of echinoid pluteus larvae that is visible at late stages, alongside the well-known internal directional asymmetry of the growing juvenile rudiment. Our data from two disparate echinoids, separated by approximately 250 million years of evolution, suggests that this asymmetry in overall larval shape, though previously undescribed, may in fact be a common feature of late stage echinopluteus ontogeny. By examining rare, anomalous plutei with juvenile rudiments on both the left and the right side, we show that the asymmetry is mechanistically and/or functionally connected to rudiment development. We explore several hypotheses to account for this asymmetry, focusing on two main hypotheses: that the asymmetry aids in swimming stability in the water column or that it is a result of a developmental constraint on material deposition in arms versus the rudiment.

One feature of echinoids that makes them such a valuable taxon for comparative studies is their great morphological diversity in the context of a relatively robust understanding of their phylogeny. As with adult features, sea urchin larvae also show remarkable diversity: for example, in arm number, their lengths relative to the body and
their position [86]. Furthermore, there are many independently evolved instances of loss
of larval feeding, accompanied by partial to complete loss of these larval arms [87]. And
finally, functional and anatomical studies indicate that the similar larval morphology in
the brittle stars (class Ophiuroidea) represents a completely independent evolutionary
acquisition of the pluteus form [12, 13]. Such diversity in form, with independent
645 evolutionary events and an easily quantifiable morphology, provides ample material for
detailed comparative investigations into this directional asymmetry: a tractable aspect of
functional morphology that can be studied in the context of the multiple ecological
651 requirements facing feeding and dispersing larvae in the ocean.
ETHICS STATEMENT

The research presented here does not require any approval and therefore complies with all necessary regulations.

AVAILABILITY OF SUPPORTING DATA

All relevant data is included in the manuscript. We submitted all raw data used for the analysis presented in this study to Dryad (doi:10.5061/dryad.102tc) in accordance with RSOS policy.

LIST OF ABBREVIATIONS USED

L-left; R-right; PO-postoral arms; PD-posterodorsal arms; ALA-anterolateral arms; PRO-preoral arms; PF-post-fertilization; FA-fluctuating asymmetry; DA-directional asymmetry; MFSW-0.45 µm Millipore filtered natural sea water; MFASW-0.45 µm Millipore filtered artificial sea water; KCl-potassium chloride; TH-thyroid hormone; BMP-Bone Morphogenetic Protein; FHL-Friday Harbor Laboratories (University of Washington).

COMPETING INTERESTS

The authors declare that they have no competing interests, financial or otherwise.
AUTHORS' CONTRIBUTIONS

J.H. and A.H. contributed equally. All authors discussed and commented on the manuscript. J.H. conducted the experiments in Seattle and FHL. A.H. supervised the experiments in Guelph, and A.H and K.L conducted the experiments there. J.H. and A.H. designed the experiments. A.H. ran the statistical analyses. J.H and A.H. prepared the figures. J.H. and A.H. wrote and revised the paper.

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REFERENCES


FIGURE LEGENDS

Figure 1: Larvae of the sand dollar *Dendraster excentricus* show directional asymmetry in postoral arms. A) Representative *D. excentricus* larva at 9 days post-fertilization, with four pairs of larval arms. Note that the left side of the larva is the side of the well-developed juvenile rudiment (dark region labeled 'Rud' in this image); as this is a ventral view, the “left” side of the larvae is seen here on the right side of the image, and vice-versa; scale bar: 100µm. B) Schematic of a sand dollar pluteus larva with four pairs of larval arms, with the colored lines indicating the measurements taken for this study, oriented as in A. C) Mean larval arm length in µm for all four larval arms at day 9; n=20 larvae. Lighter bars: right arms; darker bars: left arms. D) Mean index of asymmetry [$\ln(R/L)$] for all arm pairs; positive values indicate right-based asymmetry (i.e., longer arms on the right side); negative values indicate left-biased asymmetry. PO: postoral arms; PD: posterodorsal arms; ALA: anterolateral arms; PRO: preoral arms. Asterisks in D indicate significant differences ($p<0.05$) between left and right side, and therefore directional asymmetry (DA). Error bars are one standard error of the mean.

Figure 2: Directional asymmetry in purple urchin larvae as a function of age. We analyzed left-right asymmetry in developing purple urchin (*Strongylocentrotus purpuratus*) larval arms as a function of age during late larval development for the (A) postoral, (B) anterolateral, (C) posterodorsal and (D) preoral arm pairs (on day 15, these larvae had only 6 arms; hence the absence of data for PRO arms on that day). Graphs in A-D have two Y-axes: the primary (left) axis shows mean larval arm length in µm for all four larval arms, as is seen in the data points connected by dark solid (left)
and dashed grey (right) lines. The secondary (right) axis shows the mean index of asymmetry $[\ln(R/L)]$, as is seen in the grey bars. (E) Schematic as in Fig. 1B repeated for convenience. The cross-polarized light micrograph in (F') shows a representative early stage larva (day 15, stage bin A; see Fig. 3), and in (F''), a representative late stage larva (day 39, stage bin F; see Fig. 3). Note the visible juvenile skeleton (on the left side; pictured in the right side of these ventral views) and clear L-R arm asymmetry in (F'') but not (F'). Time is in days post fertilization (PF), Scale bars in E,F: 150µm. Abbreviations, asterisks and error bars as in Fig. 1. See Table 1 for statistics.

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**Figure 4: Directional asymmetry in total arm length of purple urchin larvae as a function of age and juvenile rudiment stage.** We summed the length of the four pairs of arms on the right and left sides, and compared them by age (A) and juvenile rudiment stage (B). Note the lack of a monotonic increase in asymmetry as development proceeds, especially in B. Double axes and line colors as in Fig. 2. Abbreviations,
Figure 5: Reduced larval food does not alter directional asymmetries in arm length across stages. (A-D) We detected asymmetry in arm lengths with stage under both high food (HF; upper graphs in each panel) and low food (LF; lower graphs) conditions in purple urchins, with no detectable differences in any arm pair between high and low food (see the text). Double axes and line colors as in Fig. 2. Abbreviations, and error bars as in Fig. 1. Stage bins as in Fig. 3. Numbers of larvae at each stage are as follows. Stage bin A: HF, n=0; LF, n=4. Stage bin B: HF, n=3; LF, n=18. Stage bin C: HF, n=6; LF, n=9. Stage bin D: HF, n=13; LF, n=17. Stage bin E: HF, n=8; LF, n=18. Stage bin F: HF, n=15; LF, n=8. Note that there were no HF larvae in stage bin A, presumably due to their more rapid development than in the corresponding low food larval cohort.

Figure 6: Larval arms of anomalous purple urchin larvae with double rudiments are more symmetrical. We analyzed the index of arm length asymmetry (see Fig. 1 legend) in naturally occurring larvae with juvenile rudiments on both the left and right sides (double rudiments; B), as compared to their full siblings with single rudiments (C). Cartoons along bottom of figure indicate single and double rudiments. (A) Whereas larvae with single rudiments (n=9 larvae) in this experiment showed right-biased directional asymmetry (positive values) in postoral (PO) and posterodorsal (PD) arms (asterisks in right half of A), stage matched larvae with double rudiments (n=8 larvae) did not show right-biased asymmetry in any of their arm pairs; in fact PO arms in double
rudiment larvae showed left biased asymmetry (negative values; asterisk in left half of A). Scale bars in B.C: 150µm. Abbreviations, asterisks, error bars and orientation of larvae as in Fig. 1.

TABLE LEGENDS

Table 1: Directional asymmetry in Strongylocentrotus purpuratus larvae by age.
Statistics corresponding to the data shown in Figs. 2A-D and 4A. Bold rows and asterisks in the p-value columns denote all cases where \( \alpha \) was < 0.05. Abbreviations as in Figure 1. On day 15, these larvae had only 6 arms; hence the “n/a” for the preoral (PRO) arms on that day.

Table 2: Directional asymmetry in Strongylocentrotus purpuratus larvae by stage.
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480x221mm (150 x 150 DPI)
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399x443mm (150 x 150 DPI)
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153x76mm (150 x 150 DPI)
TABLE 1: Directional asymmetry in *Strongylocentrotus purpuratus* larvae by age

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<th>Arms</th>
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<th>standard error</th>
<th>n (larvae)</th>
<th>one-sample t-test</th>
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R square | F | p |
TABLE 2: Directional asymmetry in *Strongylocentrotus purpuratus* larvae by stage

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<th>Arms</th>
<th>Stage</th>
<th>mean ln(R/L)</th>
<th>standard error</th>
<th>n (larvae)</th>
<th>one-sample t-test</th>
<th>regression analysis</th>
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</table>

On November 2, 2017

https://mc.manuscriptcentral.com/rsos
We thank Reviewer 2 (Dr. Richard Palmer) for his final comments; once again, he has identified points that, when we addressed them, improved the manuscript. We would also note that since Dr. Palmer signed his review, we took the opportunity to contact him by email in the past week for clarification on two of his recent points. We make reference to this email conversation below to indicate that we feel we have now addressed these concerns in a manner satisfactory to Dr. Palmer. All told, we are now confident that the manuscript is suitable for publication as now resubmitted.

In the sections below we list all of the most recent comments of Dr. Palmer, indented and in italics. We respond in point-by-point fashion in unindented, unitalicized text. We refer throughout the document below to Page and Line number references in the revised version of our manuscript. Please note that our page number references are to the numbers printed on the lower right of every page, not the page numbers as listed in the compiled PDF in the upper left corner (“page x of y”).

(Line please ignore the other stray numbers in the upper and lower left corners of alternating pages; this must be a glitch in the page numbering program as it relates to header and footer text. We were unable to delete these numbers without deleting the correct line numbers as well; our apologies).

July 22, 2016

GENERAL COMMENT

Change in arm length with age/size. Something seems decidedly odd about the graphs in figures 2 - 5. Why does arm length not increase with increasing larval age/stage beyond day 25/stage C? Shouldn't all of these arms get longer as the larvae grow and increase in size? If these numbers are correct, the authors should comment on this near absence of any increase in arm length with age/size in the discussion.

It does indeed seem odd that larvae should get smaller arms as they age, but under high food conditions, this is exactly what is expected, and has in fact been observed multiple times in the echinoid food plasticity literature. To accommodate Dr. Palmer's suggestion, we have added an additional paragraph at the end of the feeding experiment section of the results on this point (see Page 18 Lines 380-387 in the revised manuscript).

SPECIFIC COMMENTS

Line 247- should be "two-tailed one-sample"

We have made this change, as well as all of the other orthographic corrections/suggestions that Dr. Palmer helpfully appended to the PDF file.

Line 631- As I noted in the earlier version, 'Previously undescribed' is not quite correct because, as the authors note, such asymmetries have been described by Emlet 2009 and Collin 1997.
This is one of the two points that we referred to above where we, in the past week, contacted Dr. Palmer directly for clarification. We have modified the wording in the sentence referenced above in a way that Dr. Palmer agrees now highlights the uniqueness of our contribution. See Page 29 Line 638-642 in the revised manuscript.

Fig. 3- The labeling of the lines for R and L, and the font colors, aren't consistent in these panels. Presumably R is grey and dashed throughout but this is not so in Panels B and D.

We thank Dr. Palmer for noticing this regrettable error, which we have now rectified.

Tables 1 & 2 legends- Explain to what "regression analysis" refers.

We have now added a few words to the Table legends to clarify. Specifically we added the following explanation to the legend in Table 1: “...our test to determine if DA changes as a function of age (days PF) using a linear regression”, and in the legend to Table 2: “...our test to determine if DA changes as a function of stage bin using a linear regression.”

ADDITIONAL COMMENT APPENDED TO THE PDF

Line 335- The df do not appear to be correct (and therefore the p values likely wrong) for the test for DA. In the mixed model ANOVA, the mean squares of the term "sides" (DA) should be tested over the mean squares of the interaction term "side x individual interaction" using the appropriate df for both (the listed df appears to be that for the error term).

This was the second instance where we contacted Dr. Palmer directly in the past week for clarification, and he recommended that we present all the ANOVA results in table form. To accommodate this suggestion we have now added a supplemental table (Table S1) for the FA analysis containing all required statistical parameters.

We did in fact report the incorrect degrees of freedom in our previously submitted version, and they are now properly represented in the new version. We note that our new analysis did not result in any change to our conclusion that there is FA in all three arms and no DA for any of the arms in the dataset in question.