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Transcriptomic analysis of Nodal – and BMP- associated genes during development to the juvenile seastar in *Parvulastra exigua* (Asterinidae)

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ABSTRACT

The molecular mechanisms underlying development of the pentameral body of adult echinoderms are poorly understood but are important to solve with respect to evolution of a unique body plan that contrasts with the bilateral body plan of other deuterostomes. As Nodal and BMP2/4 signalling is involved in axis formation in larvae and development of the echinoderm body plan, we used the developmental transcriptome generated for the asterinid seastar *Parvulastra exigua* to investigate the temporal expression patterns of Nodal and BMP2/4 genes from the embryo and across metamorphosis to the juvenile. For echinoderms, the Asteroidea represents the basal-type body architecture with a distinct (separated) ray structure. *Parvulastra exigua* has lecithotrophic development forming the juvenile soon after gastrulation providing ready access to the developing adult stage. We identified 39 genes associated with the *Nodal* and *BMP2/4* network in the *P. exigua* developmental transcriptome. Clustering analysis of these genes resulted in 6 clusters with similar temporal expression patterns across development. A co-expression analysis revealed genes that have similar expression profiles as *Nodal* and *BMP2/4*. These results indicated genes that may have a regulatory relationship in patterning morphogenesis of the juvenile seastar. Developmental RNA-seq analyses of *Parvulastra exigua* show changes in *Nodal* and *BMP2/4* signalling genes across the metamorphic transition. We provide the foundation for detailed analyses of this cascade in the evolution of the unusual pentameral echinoderm body and its deuterostome affinities.

1. Introduction

The molecular mechanisms underlying patterning of body axes in development are remarkably conserved across invertebrates and vertebrates, despite the great diversity of animal body plans. In most animals, the major body axes, the anteroposterior, dorsoventral and left-right are readily apparent in a bilateral body organisation, facilitating comparative analysis of axial patterning mechanisms. Within the deuterostomes however, the echinoderms have evolved a radial body plan characterised by 5-fold symmetry with many hypotheses proposed as to how this evolved and relates to the bilateral plan of groups in the chordate line to which they are related (Raff and Popodi, 1996; Morris, 2012; Lacalli, 2014; Arnone et al., 2015; Byrne et al., 2016). Echinoderms have a bilateral body organisation as larvae and the earliest echinoderms in Cambrian fossils were bilateral as adults, indicating that adult radial symmetry is secondarily evolved (Zamora and Smith, 2012; Smith et al., 2013). While the evolutionary origin of this body plan has been the subject of debate, the molecular mechanisms underlying its development are poorly understood. Understanding how pentamery is patterned in development is important in tracing its evolution.

Most molecular studies of echinoderm development have focussed on early embryos and larvae and the gene regulatory networks (GRNs) involved with patterning the primary germ layers and the sea urchin larval skeleton (Peter and Davidson, 2011; Arnone et al., 2015; Cheatle Jarvela et al., 2016; Cary and Hinmann, 2017; Gildor et al., 2017, 2019). Investigation of molecular and cellular mechanisms underlying adult development requires access to the developing juvenile, which in most species, requires long-term rearing of feeding larvae making it difficult to access the juvenile stage (Wray et al., 2004; Hodin et al., 2019). To address this challenge, rapidly developing species that have evolved non-feeding lecithotrophic larvae and metamorphose soon after gastrulation provide model systems to investigate development of the

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adult body plan (Raff, 1992; Wray, 1996; Byrne, 2006, 2013; Raff and Byrne, 2006). The evolution of lecithotrophy in echinoderms has long been the focus of studies of alternative larval life history modes (Raff, 1992; Wray, 1996; Raff and Byrne, 2006). Recent generation of transcriptomes that incorporate developmental stages through metamorphosis and adult body formation for lecithotrophic species including the sea urchin *Heliocidaris erythrogramma* and the seastar *Parvulastra exigua*, provides an important foundation for analysis of the molecular processes underlying development of pentamery (Wygoda et al., 2014; Byrne et al., 2015, 2018, 2020; Israel et al., 2016; Koop et al., 2017).

Genes associated with Nodal and bone morphogenetic protein (BMP) signalling are integral to axis formation in patterning animal development (Bier and De Robertis, 2015). These signalling pathways have been a focus in comparing the molecular mechanisms patterning body axes across the Metazoa and in identifying potential homologies (Shen, 2007; Bier and De Robertis, 2015). In sea urchins and seastars the role of the Nodal-BMP cascade in larval axis formation is well documented (Smith et al., 2008; Raff and Smith, 2009; Duboc et al., 2010; Angerer et al., 2011; Luo and Su, 2012; Bessodes et al., 2012; Molina et al., 2013; Yankura et al., 2013; Su, 2014; Sasaki and Kominami, 2017; Fresques and Wessel, 2018). For *H. erythrogramma*, temporal and spatial expression patterns revealed that genes of the Nodal-BMP cascade are also expressed during metamorphosis and juvenile development (Wygoda et al., 2014; Byrne et al., 2015, 2018; Koop et al., 2017). *BMP2/4* and several downstream genes are located in the five hydrocoele lobes, the first morphological expression of pentamery, and core of the echinoderm body plan (Koop et al., 2017). This indicates a role for Nodal-and BMP signalling in patterning body plan development. The 5-fold expression of Nodal and BMP2/4 signalling genes in the hydrocoel as well as pentameral expression of genes of the *Drosophila* retinal GRN indicates that pentamery may have evolved through duplication of the ancestral single anterior-posterior axis (Byrne et al., 2007; Koop et al., 2017), as hypothesised (Raff and Popodi, 1996; Morris, 2012; Byrne et al., 2016).

As genes associated with Nodal and BMP2/4 signalling appear to be involved in patterning development of pentamery in sea urchins, we investigated the expression of these genes in development to the juvenile seastar, *Parvulastra exigua*. For the Echinodermata, the asteroid model represents the basal-type body architecture with a distinct (separated) ray structure and are likely to be a better system to understand evolution of pentamery than the highly modified sea urchin body, which has cohesion between the rays and other derived features including the most radical echinoderm metamorphosis forming the juvenile almost entirely from axial elements (skeleton associated with the water vascular system) (David and Mooi, 1998; Byrne et al., 2016). *Parvulastra exigua* releases its large eggs (360 μm diameter) in benthic masses that adhere to the substratum with their sticky jelly coat, a hypertrophied extracellular matrix that attaches the embryos to the substrate (Cerra and Byrne, 1995). The larval feeding program has been deleted as the blastopore closes soon after gastrulation. *Parvulastra exigua* has holobenthic development and hatch as larvae that maintain a tenacious attachment to the substratum (Fig. 1). The larvae have the tripod morphology of benthic seastar larvae with a hypertrophied attachment complex. Metamorphosis ensues as the larval body degenerates and the juvenile tube feet take over the role of attachment over approximately 2 weeks. Evolution of development in *P. exigua* has been the focus of studies of

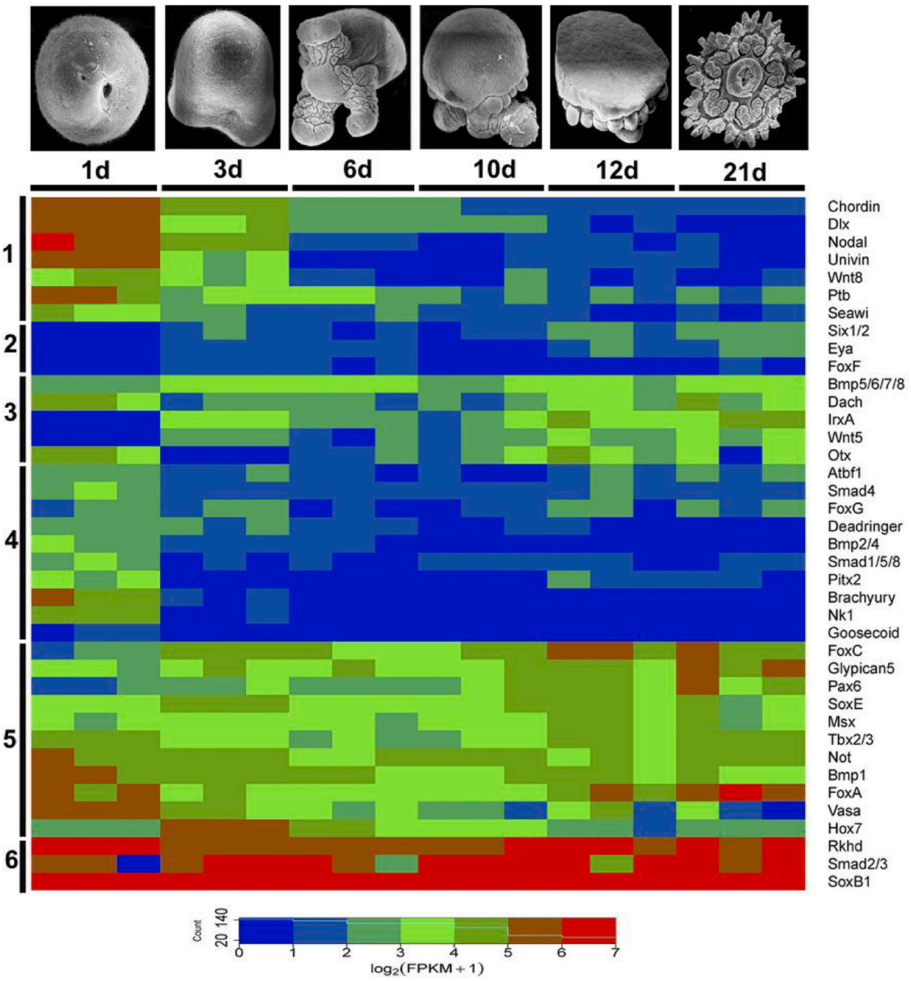

Fig. 1. Heatmap analysis showing expression of 39 genes of the Nodal and BMP regulatory network in the transcriptome of *Parvulastra exigua* at six developmental time point from the gastrula to the juvenile. Scanning electron microscopy images along the top illustrate the developmental stages: gastrula (1-day post fertilization- dpf), early brachiolaria (3 dpf), hatched brachiolaria (6 dpf), flexed brachiolaria with a flattening rudiment region (early metamorphosis) (10 dpf), metamorphic juvenile (12 dpf) and the fully developed juvenile (21 dpf). Gene expression levels (heatmap colour code) indicates level of expression in log2 FPKM (fragments per kilobase of transcript per million mapped reads). Genes are grouped according to expression profile over the entire time course (see Supp Fig 1 for group dendrogram).

evolution of benthic larvae in asteroids (Byrne, 1995, 2006, 2013).

We used the de novo developmental transcriptome generated for *Parvulastra exigua*, from the embryo through metamorphosis, incorporating 6 stages from gastrula (1-day post fertilization - dpf) to the definitive juvenile (21 dpf) to determine the expression profiles of genes associated with Nodal and BMP2/4 signalling. A recent investigation of global variation in gene expression in *P. exigua* showed that marked changes occur during metamorphosis and gene ontology analysis revealed dynamic changes in gene expression through the transition to pentamery (Byrne et al., 2020). Many of the terms enriched in late metamorphosis included signalling genes (Byrne et al., 2020). In situ hybridisation shows that axial patterning genes (e.g. *hox4*, *eng*, *otx*, *eya*, *pax6*), including genes associated with Nodal and BMP2/4 signalling, are expressed in coelomic, neural and sensory structures (Byrne et al., 2005, 2020; Cisternas and Byrne, 2009). Thus, as for sea urchins, gene expression in development of the pentameral seastar shows 5-fold expression including in structures core to the adult body plan (Byrne et al., 2005, 2020; Cisternas and Byrne, 2009).

We identified 39 genes associated with the *Nodal* and *BMP2/4* network in the *P. exigua* transcriptome and determined their temporal expression patterns and profiles through metamorphosis to the pentameral juvenile. Our results revealed complex changes in gene expression through development and that groups of genes change their expression in concert. The expression profiles point to genes likely to be involved in metamorphosis and juvenile development. Our results were compared with those from a similar study of Nodal and BMP2/4 associated genes in sea urchin juvenile development (Byrne et al., 2015).

2. Materials and methods

2.1. Analysis of temporal expression of Nodal/BMP genes

Transcriptome assembly, library construction and de novo transcript assembly of the *P. exigua* developmental transcriptome are detailed in Byrne et al. (2020). The transcriptome was searched for genes known to be associated with Nodal- and BMP2/4-mediated patterning in sea urchin development and, to some extent, in sea star development (Duboc et al., 2010; Angerer et al., 2011; Luo and Su, 2012; Molina et al., 2013; Koop et al., 2017; Sasaki and Kominami, 2017; Fresques and Wessel, 2018), resulting in 39 genes. Expression values were calculated as the log2 transformation of FPKM (fragments per kilobase of transcript per million mapped reads) plus 1. For genes with more than one assembled transcript, the variance of all timepoints was calculated using the `var` function in R (Team, 2012) and the particular transcript with the largest variance value was chosen as the representative transcript of the gene. To generate the clustered heatmap, hierarchical clustering using the complete linkage approach was used. A dendrogram of the clustering analysis was also produced (Supp. Fig. 1). To create clusters, the dendrogram was cut at a height of 36, resulting in 6 clusters (Supp. Fig. 1). Bootstrap resampling of replicates within timepoints was carried out 1000 times and clustering of the resampled datasets was performed (Hubert and Arabie, 1985).

A co-expression analysis of Nodal and BMP 2/4 was also performed. Co-expression plots were constructed for eleven genes identified to have time series expression profiles with the smallest distance to the profiles of *Nodal* and *BMP2/4*. These were determined by calculating the Manhattan distance between all gene pairs of expression profiles. Genes beginning with Hypp are hypothetical proteins in the *Patiria miniata* genome (legacy.echinobase.org/Echinobase/PmAbout).

2.2. Microscopy

For scanning electron microscopy, developmental stages were fixed in 2.5% paraformaldehyde (ProSciTech, Australia) in FSW for 1–2 h and washed in FSW and rinsed in 2.5% (*v*/v) sodium bicarbonate (pH 7.2). They were then post-fixed in 2% osmium tetroxide in 1.25% sodium bicarbonate buffer for 1 h at room temperature. Samples were then dehydrated in an ethanol series to 100%, critical-point dried, and sputter-coated before viewing using a JOEL JSM-35C scanning electron microscope.

3. Results and discussion

The *P. exigua* transcriptome revealed expression of 39 genes associated with Nodal and BMP2/4 many of which are known to be important in larval axis development in sea urchin and seastars with feeding larvae (Duboc et al., 2010; Angerer et al., 2011; Luo and Su, 2012; Molina et al., 2013; Koop et al., 2017; Sasaki and Kominami, 2017; Fresques and Wessel, 2018). In seastar development Nodal plays a role in patterning the left/right and dorsal/ventral axes as in sea urchins, although with some difference in expression patterns of associated genes and a delay in timing compared to sea urchins (Sasaki and Kominami, 2017; Fresques and Wessel, 2018). In support of the role of Nodal in patterning asteroid embryonic axes, BMP signalling occurs in the dorsal side of the embryo (Yankura et al., 2013). Here we extend these studies across metamorphosis to the developing juvenile seastar.

3.1. Nodal and BMP associated genes expression profiles from the gastrula through to the juvenile

We identified 39 genes associated with Nodal and BMP signalling in the *P. exigua* transcriptome (Fig. 1). The heat map shows that most genes were expressed in the gastrula stage (1 dpf) while others had various patterns of down regulation post gastrulation or expression through development to the juvenile (Fig. 1). Several genes likely to be involved in Nodal signalling were expressed from gastrula through the early larva during formation of the hydrocoele (Morris et al., 2009, 2011; Byrne et al., 2020). The analysis of similarity between genes based on their temporal expression profiles resulted in six groups of genes as shown in the heatmap (Fig. 1). Bootstrap resampling resulted in a mean adjusted Rand index of 0.64 (Supp Fig. 1) providing support for the clustering (see Freyhult et al., 2010).

Group 1 included seven genes (e.g. *Chordin*, *Dlx*, *Nodal*, *Univin*, *Wnt8*, *Ptb*, *Seawi*) that were highly expressed in early development at the gastrula stage (1 dpf) and then reduced during larval development (3 and 6 dpf) followed by low or no expression at metamorphosis (10 and 12 dpf) and in the juvenile (21 dpf).

Group 2 included genes (*Six1/2, Eya, FoxF*) that had low or no expression through development with some upregulation in the juvenile stage. *Six1/2* and *Eya* were primarily expressed from late metamorphosis onwards.

Group 3 included five genes, some of which (e.g. *BMP5/6/7/8, Dach, Otx*) were expressed at the gastrula stage (1 dpf) while the others (*IrxA, Wnt5*) had little to no expression at this stage. All of these genes where subsequently expressed fairly consistently in larvae and through to the juvenile.

Group 4 included ten genes including key transcription factors (e.g. *BMP2/4, Smad4, Pitx2, Brachyury*) that were are all expressed at the gastrula stage and are thereafter down regulated with *BMP 2/4*, *Deadringer* and *Goosecoid* strongly downregulated post gastrulation.

Group 5 included key transcription factors, *Fox C*, *Pax6, SoxE, Bmp1, Hox 7*, that were expressed at the gastrula stage and remained expressed through development. They were all expressed at relatively constant levels through the premetamorphic, metamorphic and juvenile stages, with a one gene (*Vasa*) being down-regulated following metamorphosis. Some of these genes (e.g. *Not, Bmp1, FoxA, Vasa*) had their highest expression in gastrulae with *FoxA* also exhibiting high expression in the juvenile.

Group 6, three genes (e.g. *Rkhd*, *Smad2/3*, *SoxB1*) exhibited high levels of expression through development.

3.2. Expression similarity analysis of Nodal and BMP 2/4

The co-expression analysis of *Nodal* and *BMP 2/4* in the *P. exigua* transcriptome (Fig. 2) identified the eleven genes with the most similar pattern of expression to each of these genes. For *Nodal* these included *Chordin*, *Wnt 8*, and *Dlx*, genes known to be involved in regulatory relationships with *Nodal* in echinoid development (Bessodes et al., 2012; Luo and Su, 2012; Molina et al., 2013). This result supports the veracity of the transcriptome analysis.

For *BMP 2/4* the co-expression analysis identified nine genes including two Hypp genes but did not identify genes known to be involved in regulatory relationships with *BMP 2/4*. The Hypp genes are hypothetical proteins predicted from the *Patiria miniata* genome and, based on their expression pattern, may be undiscovered genes which have functions in the BMP 2/4 network.

For *P. miniata* which has the ancestral feeding type larva, Nodal and BMP2/4 signalling are involved in development of the ciliary band associated larval nervous system and the GRN model presented by Yankura et al. (2013) points to some gene relationships. How these might relate to development in *P. exigua* which lacks ciliary bands and has a highly modified larval nervous system (Elia et al., 2009) remain to be explored through functional analysis.

3.3. Comparison with expression of Nodal and BMP 2/4 associated genes in juvenile sea urchin development

The heatmap generated for Nodal-BMP2/4 network genes across development of *P. exigua* is similar to that generated for *H. erythrogramma* (Byrne et al., 2015). While it is difficult to compare the patterns of gene expression in the two heatmaps in absolute terms, comparisons of the most similar developmental stages, gastrula, larva and juvenile, highlights conserved and divergent trajectories of

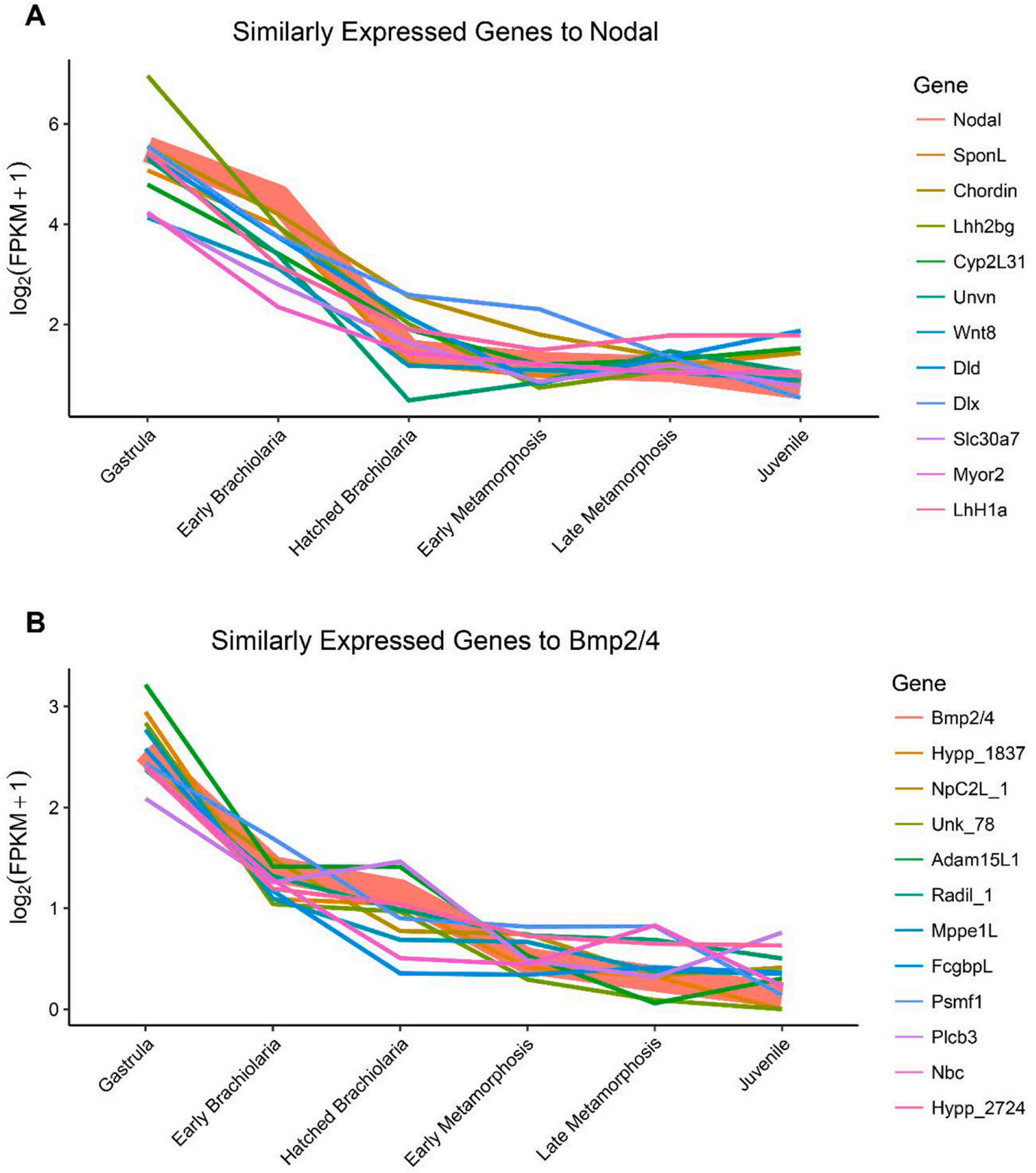

Fig. 2. Expression of 11 genes with the highest expression similarity to **a)***Nodal* and **b)***BMP 2/4* during development of *Parvulastra. exigua*. The Nodal expression profile shows a slight downregulation between gastrula and early brachiolaria, with strong downregulation from early- to hatched brachiolaria, followed by low expression levels. *BMP2/4* expression shows progressive downregulation between gastrula and early metamorphosis stages, with subsequent low expression levels. Hypp indicates that the closest Blastx match to a hypothetical gene in the starfish, *Patiria miniata* genome.

developmental gene expression between these two species (Table S1). For example, many important transcription factor genes exhibit a similar pattern of expression for all these stages (e.g. *Nodal*, *Univin*, *Fox A*, *Fox C*, *Fox G*, *Tbx2/3*, *Sox B1, Vasa*), while others completely differ (e.g. *Six1/2*, *Eya*, *Goosecoid*) and some (e.g *Smad1/5/8*) show partial similarity (Table S1).

In both species *BMP2/4* and *Chordin* are expressed in the gastrula and early larva (Table S1). This suggests that these genes may play a similar role in early development and a deep conservation of this module within the echinoderm embryonic GRN. There also are some striking differences. For example, in *H. erythrogramma BMP2/4* and *Chordin* remain highly expressed through juvenile development, but in *P. exigua* expression of these genes drops off dramatically post-gastrulation. This indicates a more limited role for these genes in patterning adult starfish development than that of the adult sea urchin.

In both *P. exigua* and *H. erythrogramma Otx* and *Nk1*, were upregulated in the gastrula and larval stages (Table S1). *Otx* upregulation during gastrulation is likely due to its conserved function in endoderm development during this embryonic stage (Hinman et al., 2003). In sea urchin and seastar larvae *Nk1* is involved in patterning the larval nervous system which is associated with ciliary bands and apical sensory organ of the feeding larva (Angerer et al., 2011; Yankura et al. 2010). Expression of this gene in *P. exigua* and *H. erythrogramma* may also be associated with development of the larval nervous system (Byrne et al., 2007; Elia et al., 2009), although this system is reduced in the non-feeding larvae of these species. Expression of *Otx* during metamorphosis and juvenile development is associated with central nervous system (CNS) development where this gene is expressed in the circumoral nerve ring and the radial nerve cord (Byrne et al., 2020). In the *H. erythrogramma* transcriptome *Otx* is also expressed in the juvenile (Byrne et al., 2015), but we do not have data on spatial expression. *Eya* was only expressed at the juvenile stage of *P. exigua* and in situ hybridisation shows that *Eya* is localised to the developing CNS (Byrne et al., 2020). In contrast, for *H. erythrogramma, Eya* expression was not detected in the juvenile stage (Byrne et al., 2015; Koop et al., 2017).

For *H. erythrogramma*, BMP2/4 signalling, through its control of *Nodal*, appears to have a role in the development of pentamery and in situ hybridisation showed that *BMP2/4*, and its putative targets genes *(Eya*, *Six1/2*, *Tbx2/3* and *Msx*) are expressed in the five hydrocoele lobes with *BMP2/4*, *Msx* and *Tbx2/3* showing earliest pentameral pattern of expression (Koop et al., 2017). However, pentameral expression of Nodal-BMP2/4 associated genes and a number of other genes (see Sly et al. 2002) are also likely to be 5-fold iterations of an existing pentameral organisation set up in earlier development and so may not be involved in setting up pentamery which is suggested to be set up in early development (Byrne et al., 2016; Koop et al., 2017). In sea urchin gastrulae, the *Nodal* signalling centre on the right side at the head of the archenteron is likely to pattern development of coelomic structures and may be involved in patterning pentamery (Smith et al. 2008; Koop et al., 2017). Development of pentamery is thus hypothesised to involve discrete five-fold expression of genes in the endomesoderm at the head of the archenteron before pentamery is evident morphologically. In this scenario, the evolution of pentamery is hypothesised to have involved a duplication or a split in the Nodal and BMP2/4 signalling cascade (Koop et al., 2017). Support for this suggestion would require investigation of the Nodal-BMP GRN during the transition to pentamery and gene function analysis.

For the seastar model we need data on spatial expression to identify where genes of the Nodal-BMP GRN that are active relative to developing anatomical structures with a focus on evidence for *Nodal* expression the head of the archenteron and expression of associated genes in coelomogenesis and hydrocoele formation, which hold promise for making connections with the chordate embryo (Morris et al., 2009; Morris, 2012).

4. Conclusion

Our results indicate that many Nodal-BMP2/4 associated genes expressed in early embryos are also expressed through metamorphosis and juvenile development in *P. exigua*. A suite of other genes are more highly or only expressed on either side of the metamorphic transition. A similar pattern was seen for some but not all Nodal and BMP2/4 associated genes in *H. erythrogramma* (Byrne et al., 2015, 2018; Koop et al., 2017), suggesting both conserved and divergent developmental regulatory roles for this set of genes in the Echinodermata. For both the seastar and the sea urchin, differences in gene expression pre and post metamorphosis highlight the distinct larval and adult developmental modules and likely differences in gene regulation. Whether gene expression during adult body plan development is driven by upstream regulators that are well characterised for the *Nodal-BMP2/4* GRN of the sea urchin embryo or a distinct set of regulatory inputs associated with metamorphosis and juvenile development remains to be explored.

This study and our previous transcriptome analysis, which focussed on neural transcription factors (Byrne et al., 2020), indicate a suite of genes that are upregulated during the metamorphic transition to the juvenile. With respect to neural transcription factors, these include, *FoxB*, *FoxD*, *FoxG*, *POU4f*, *NeuroD*, *Pax6*, *Otp*, *SoxB1* and *Eya* (Byrne et al., 2020). In addition, in situ hybridisation showed expression of *Eya* in the developing pentameral nervous system while *Pax6* and *SoxB1* were expressed in the primary and secondary podia. In the present study, several other important transcription factors were upregulated during juvenile development including *Hox7*, *FoxA*, *FoxC*, *Six1/2*, and *Smad2/3* (for full list see Table S1).

While we are building up a picture of the genes involved in adult body development in echinoderms, the molecular mechanisms underlying the evolution of pentamery in echinoderms from a bilateral deuterostome ancestor are not understood. These mechanisms are important to resolve as fundamental to understanding animal body-plan evolution. The extensive transcriptomic resources for *P. exigua* and *H. erythrogramma*, species with fast development to the juvenile provide the basis to investigate gene expression during echinoderm body plan development and its deuterostome affinities.

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CRediT author statement

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Paula Cisternas: Figure Preparation. All authors contributed to writing and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships.

that could have appeared to influence the work reported in this paper.

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