Evolutionary conservation and divergence of the transcriptional regulation of bivalve shell secretion across life history stages

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Abstract

Adult molluscs produce shells with diverse morphologies and ornamentations, different colour patterns and microstructures. The larval shell however, is a phenotypically more conserved structure. How do developmental and evolutionary processes generate varying diversity at different life history stages? Using live-imaging, histology, scanning electron microscopy and transcriptomic profiling, we have described shell development in a heteroconchian bivalve the Antarctic clam, *Laternula elliptica* and compared it to adult shell secretion processes in the same species. Adult downstream shell genes, such as those encoding extracellular matrix proteins and biomineralisation enzymes, were largely not expressed during shell development, and instead, a development-specific downstream gene repertoire was expressed. Upstream regulatory genes such as transcription factors and signalling molecules were conserved between developmental and adult shell secretion. Comparing heteroconchian transcriptomic data with recently reported pteriomorphian larval shell proteome data suggests that, despite being phenotypically more conserved, the downstream effectors constituting the larval shell "tool-kit" may be as diverse as that of adults. Overall, our new data suggests that a larval shell formed using development-specific downstream effector genes is a conserved and ancestral feature of the bivalve lineage, and possibly more broadly across the molluscs.

Introduction

Molluscan shells are environmentally, economically and evolutionarily important (McDougall and Degnan 2018). The hard multifunctional external shells of molluscs are often attributed to the evolutionary success of this group. Shells are exquisitely preserved in the fossil record and their expansive extant adaptive diversity of form, as well as remarkable phenotypic plasticity, provides a powerful system to study morphological evolution (Thompson 1992). In the last decade, deciphering the molecular mechanisms that control shell secretion has received particular focus (Clark et al. 2020). A range of gastropod (Herlitze et al. 2018; Marie et al. 2010), cephalopod (Marie et al. 2009; Setiamarga et al. 2020) and bivalve (Arivalagan et al. 2017; Sleight et al. 2016a) shells have been subject to proteomic sequencing, coupled to the transcriptomic investigation of the shell-secreting mantle tissue (Arivalagan et al. 2016; Sleight et al. 2016b), resulting in large lists of candidate shell-forming genes and proteins. Comparative methods have found that despite the “deep” homology of molluscan shells and shell plates (Vinther 2015), the molecular mechanisms that control biomineralisation in molluscs – particularly the downstream effectors such as the shell matrix proteins - are extraordinarily diverse (Jackson et al. 2006). Features such as repeat low complexity domains (RLCD), domain shuffling and co-option (Aguilera et al. 2017), gene family expansions and subsequent subfunctionalisation (Aguilera et al. 2014) drive much of the observed molecular diversity. A handful of core protein domains however, are essential for all molluscan biomineralisation, regardless of morphologies, microstructures or polymorphs (carbonic anhydrase, chitin-binding, VWA and tyrosinase domains; Arivalagan et al. 2017). Due to the likelihood of fossilization, ease of study and ability to collect sufficiently large samples, there is an overwhelming bias towards adult shell studies, particularly in proteomic and transcriptomic studies. In developmental studies, some candidate gene approaches have been successfully used (Hohagen and Jackson 2013; Liu et al. 2017; 2020; Nederbragt et al. 2002), but comparatively little is known about the molecular processes that pattern and generate the embryonic shell field, larval mantle and early shell secretion.

Phenotypically, larval mollusc shells are more conserved than adult shells in terms of morphology, crystal polymorph and microstructure; they are unsculptured simplified forms composed of an organic outer layer and aragonite mineralised layer (Weiss et al. 2002). More generally, developmental processes are highly conserved and under strong selective constraints (Prud'homme and Gompel 2010; Smith et al. 1985) and so, if there are deeply homologous molecular mechanisms directing the production of the molluscan shell, they are more likely present during developmental stages. Although limited in number and taxonomic coverage, studies on the molecular control of shell development have uncovered intriguing insights. Recent work in pteriomorphian bivalves (oysters and mussels) compared the

proteomes of larval shells to that of adults in the same species and showed that the repertoire of extracellular matrix proteins in the larval shell is almost completely different to that of the adult shell (Carini et al. 2019; Zhao et al. 2018). Only the handful of core protein domains that have been previously described as the core "tool-kit" for molluscan biomineralisation were shared between the two life history stages (carbonic anhydrase, chitin-binding and VWA). To date, intraspecific comparisons of the molecular mechanisms governing larval versus adult shell production are restricted to one bivalve infraclass (Pteriomorphia, e.g. oysters and mussels). These studies exclusively used proteomics in a qualitative presence vs absence approach, with non-replicated estimates of transcript abundance through ontogeny. They were the first studies to reveal the striking difference between larval and adult shells at the molecular level and they focus solely on the post-translationally modified downstream effectors, ie the shell matrix proteins. Much less is known about the transcriptional mechanisms that regulate early shell development, in comparison with the adult shell of the same species.

Here we studied shell development using imaging and quantitative transcriptomics in a member of the Heteroconchia infraclass, the Antarctic clam, *Laternula elliptica*. Heteroconchia split from the rest of the Autobranchia in the Cambrian Period, around 500 million years ago (mya; Plazzi and Passamonti 2010), and so comparisons within the Autobranchia but between Heteroconchia and Pteriomorphia shed light on features that are deeply conserved within the bivalves over 500 million years of evolutionary time.

Results

Morphological mantle and shell landmarks through development in *Laternula elliptica*

To phenotypically characterise the development of the shell field, larval mantle and shell in *L. elliptica*, a combination of live light imaging, histological staining and scanning electron microscopy was used (Fig. 1). Phenotypic characterisations allowed us to assign key mantle and shell landmarks to each of the development stages studied (mantle fold appearance, organic vs mineralised shell, prodissoconch I vs prodissoconch II vs dissoconch I shell) to use as a framework to decipher the transcriptional regulation of shell secretion.

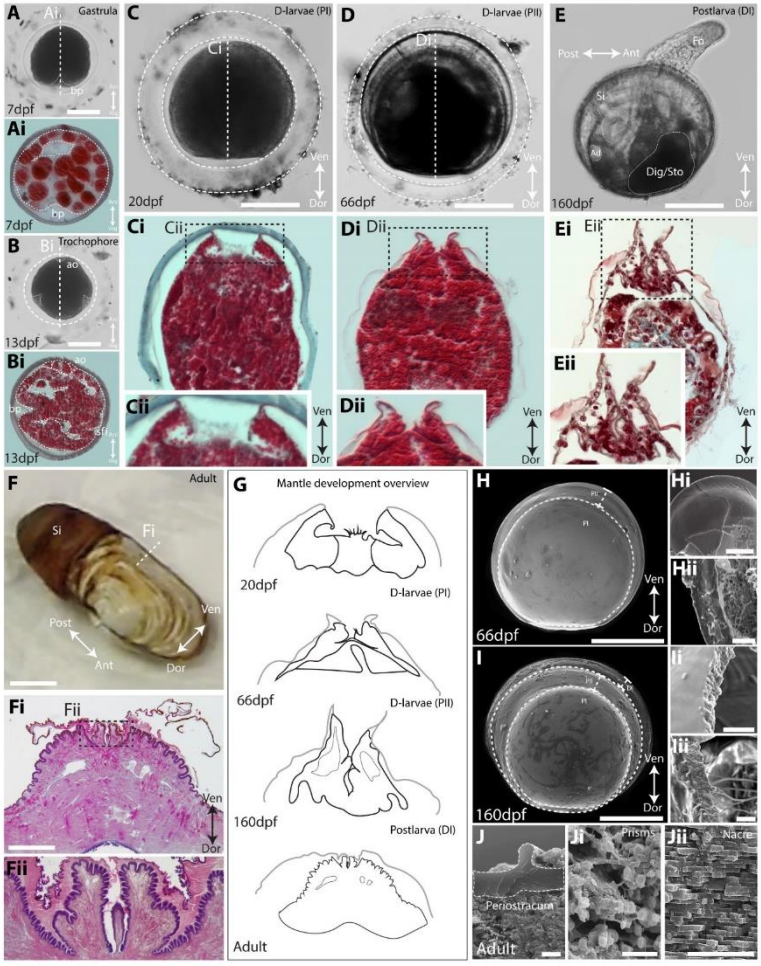

Figure 1. Shell and mantle development in *Laternula elliptica* characterised using live imaging, histology and Scanning Electron Microscopy (SEM). A-Ai Invagination of the blastopore (bp) during gastrulation 7 days post fertilization (dpf) forming the archenteron prior to shell field induction (encapsulated, scale bar 60µm). **B-Bi** Early trochophore 13dpf apical organ (ao) and shell field invagination (sfi) (encapsulated, scale bar 60µm). **C-Cii** Early D-stage larvae 20dpf, first prodissoconch I organic shell (PI) secreted by unfolded larval mantle (Ci-Cii), reduced ciliary velum resorbing (Cii) (encapsulated, scale bar 60µm). **D-Dii** Late D-stage larvae 66dpf with prodissoconch II (PII) that is mineralized, mantle folds appear and fuse to form the early fused inner mantle fold (capsule disintegrating, scale bar 60µm). **E-Eii** Hatchling postlarva 166dpf with mineralised dissoconch (DI) secreted by one cell thick folded mantle (Ei-Eii), siphon (Si), adductor muscle attachment (Ad) and digestive gland/stomach (Dig/sto) are visible through the transparent shell, foot (fo) active (scale bar 100µm). **F-Fii**

Adult mantle with outer folds and fused inner fold, periostracal grooves secreting two-layered periostracum, scale bar = 2mm, reproduced with permission from Sleight et al. (2016a). **G** Schematised overview of mantle morphogenesis, mantle tissue black, periostracum grey. At late D-larvae stage PII (66dpf) mineralisation coincides with formation of folds in larval mantle edge. **H-Hii** SEM of late D-larval shell 66dpf showing delineated PI and PII shells, polygonal mottling on the dorsal surface suggestive of the calcification nucleation points, Hi & Hii show the shell is brittle and hence mineralised, H scale bar 100µm, Hi scale bar 35µm, Hii scale bar 10µm. **I-Iii** SEM of postlarval shell 160dpf DI delineated from PI and PII shells, arenophilic secretions and intraperiostracal spikes present. I scale bar 100µm, Ii scale bar 5µm, Iii scale bar 3µm. **J-Jii** SEM cross section of adult shell microstructure, a relatively think periostracum (J), an outer layer of aragonitic granular prisms (Ji) and an inner layer of aragonitic nacreous sheets (Jii), scale bars 5µm.

Transcriptional characterisation of shell development in *Laternula elliptica*

Frist, a candidate gene approach was used to ask if the larval shell is built using the same protein coding genes as the adult shell. The expression of six genes encoding upstream regulatory proteins such as transcription factors and signalling molecules, and five downstream effector genes encoding extracellular matrix proteins and biomineralisation enzymes, were quantified over developmental time. All candidates had previously been characterised in adult shell secretion and repair in this species (via transcriptomics, computational gene network predictions, shell proteomics, qPCR and mRNA *in situ* hybridisations (Sleight et al. 2020; Sleight et al. 2016a; Sleight et al. 2015)). All of the upstream regulatory candidates were expressed during development, either increasing over time, or oscillating between stages (Fig. 2Bi). Only one of the downstream candidates, *le-meg,* was expressed during early shell development and the remaining four (*mytilin, pif, tyrA* and *tyrB*) were only expressed in the latest postlarva stage (Fig. 2Bii).

Next, genes with developmental expression profiles indicating likely involvement in regulating shell development were screened. A trait-based approach, using Weighted Correlation Network Analysis (WGCNA) was used to cluster expression profiles into modules (Fig. 2C). Module-trait relationships were then calculated to identify sets of genes (eigengenes) whose expression significantly correlated to the previously identified mantle and shell development landmarks. Five eigengene modules (M1-5) were extracted based on their significant positive correlation to traits of interest ($P<0.001$, $R>0.65$, Fig. 2D-E). M1 contained 352 genes and was significantly positively correlated to the postlarva stage (DI, mineralised, intraperiostracal spikes and arenophillic secretions) and the average expression profile of two downstream adult candidate shell genes (*pif* and *tyrA*). M1 was enriched in genes related to the cytoskeleton, protein transport (dynein) and calmodulin-binding (conserved developmental genes in this module include: *arm*, *ltbp4*, *foxj1*, *vwa3a*, *iqcg*, *camk4*). M2 contained 337 genes whose expression profiles significantly positively correlated to veliger stage 17dpf (first organic PI initially deposited) and the average expression profile of one downstream adult candidate

gene (*le-meg*). M2 was enriched in genes related to methylation and hydrogen ion transport (conserved developmental genes in this module include: *alpl*, *chs2*, *sbp1*, *h2b*). M3 contained 647 genes and was significantly positively correlated to early D-larvae (PI) stage 20dpf (first organic shell) but none of the adult candidate shell genes. M3 was enriched in genes related to protein folding and protein transport (conserved developmental genes in this module include: *hsp70*, *cpn601*, *hsp90*, *btf3*, *ccb23*). M4 contained 2,875 genes whose expression significantly positively correlated to late D-larvae (PII) stage 66dpf (mineralised shell) but none of the adult candidate shell genes. M4 was enriched in genes related to proliferation/growth, hox code transcription factors and multi-functional enzymes (conserved developmental genes in this module include: *a-somp*, *fgf1*, *abd-a*, *abd-b*, *nkx2.6*, *mox1*). M5 contained 6,369 genes significantly positively correlated to postlarva stage (DI) 160dpf and the average expression profile of four of the adult biomineralisation candidate genes (*pif, mytilin, tyrA* and *tyrB*). M5 was enriched in genes related to calcification, egf-like domains and vitamin C (conserved developmental genes in this module include: *bmp2*, *bmp3*, *bmp7*, *fgfl-1*, *ca*, *fz4*, *notch1*, *wnt4*, *wnt2b*, *tyr2*, *perl*). Modules 2-4 contain genes whose expression dynamics suggest they are involved in the regulation of early larval shell, where as M1 and M5 are both correlated to the downstream adult shell genes and show an increase in expression only in the latest stage, the postlarva (Supplementary file 1).

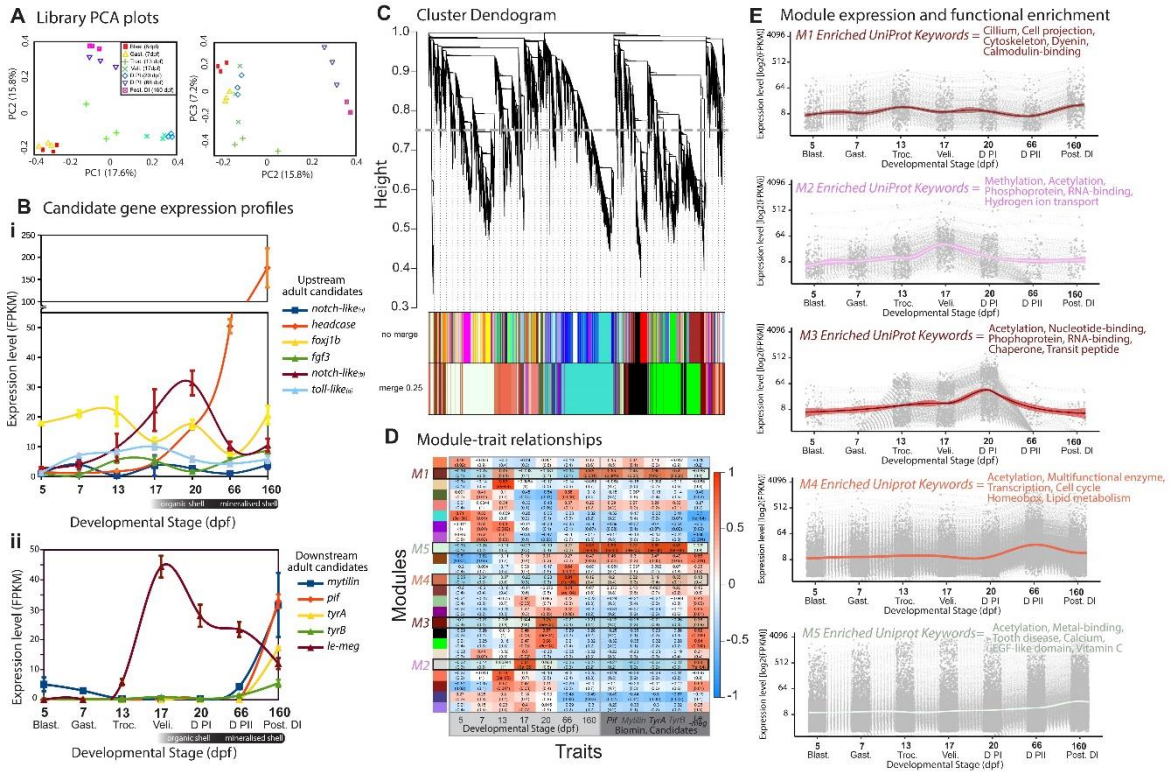

Figure 2. Candidate gene and trait-based approach to decipher transcriptional regulation of mantle and shell development in *Laternula elliptica* using bulk RNA-Seq. **A** PCA plots (PC1-3) of each RNA-seq library clustering by developmental stage (n=3 libraries

per stage). **B** Average expression (Fragments Per Kilobase of transcript per Million [FPKM], mean average, +/- S.E., n=3) at each stage of shell development of candidate upstream regulatory and downstream effector genes. **C** Dendrogram obtained by average linkage hierarchical clustering using the Weighted Correlation Network Analysis (WGCNA) R package, module assignment determined by the Dynamic Tree Cut after merging at 75% cut-off. **D** Correlation of eigengene modules to shell development traits (dpf and expression of candidate downstream adult biomineralisation genes). Each row corresponds to an eigengene module and each column a trait, color-coded by direction and degree of the correlation (Pearson R, red = positive correlation; blue = negative correlation). Five eigengene modules are highlighted (M1-M5) as they significantly positively correlate to shell development traits of interest. **E** All transcripts in each eigengene module of interest (M1-M5) extracted and mean average FPKM plotted over developmental time (n=3, shaded +/- 95% confidence interval, method = loess). Selected biologically interesting significantly enriched Uniprot keywords highlighted (calculated using String-DB, supplementary file 1).

A second unbiased statistical approach was used to identify genes that were upregulated at each consecutive stage of shell development. Significantly upregulated genes were screened for functional categories relating to shell secretion (Fig. 3), extracted, and plotted over time in clustered heatplots (Fig. S1). Genes upregulated at the D-larvae (PII) stage included a striking number of transcription factors and signalling molecules, such as components of the hh and wnt signalling pathways (*gli*, *ptch*, *fzd4* and *wnt6*). The stage that had the fewest upregulated genes was D-larvae (PI) with 25 genes upregulated including genes involved in signalling (*rhoj* and *apolpp*), ion transport (*orct*) and the extracellular shell matrix (*pif*) (Supplementary file 1). Many of the screened biomineralisation genes had a stage-specific expression pattern. For example, genes that were highly upregulated in the postlarva stage (when the downstream adult shell candidates are also highly expressed) were expressed at very low levels through all of the earlier stages of shell development. Some of the stage-specific expression patterns were explained by ontogenetic partitioning of isoforms and/or paralogues (indicated with arrows Fig. S1), for example we found an isoform of *ptch3* that was expressed only in early shell development (significantly upregulated in the veliger stage) and a different isoform only expressed late in shell development (significantly upregulated in D-larvae PII stage).

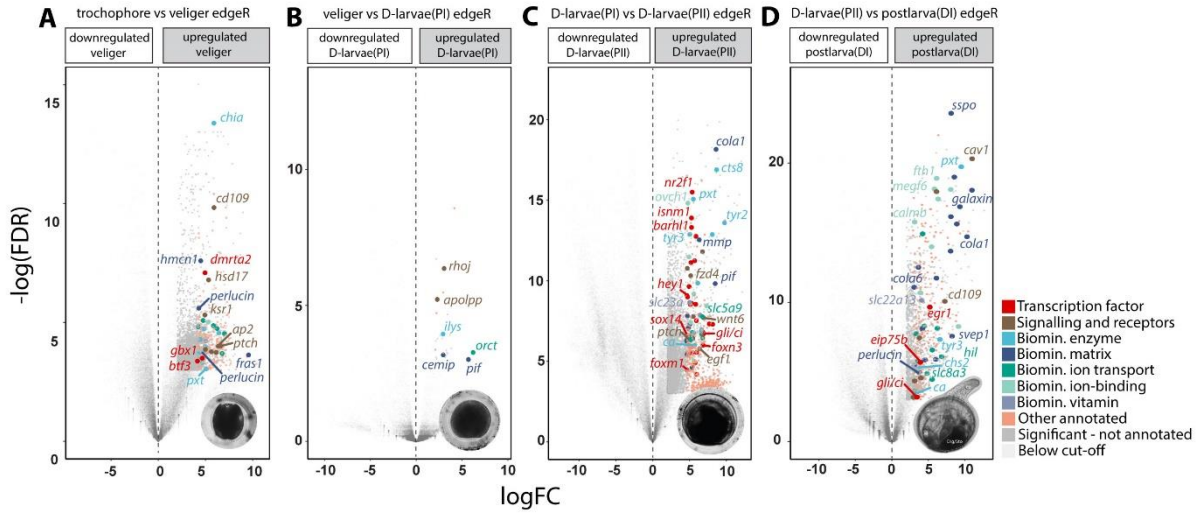

Figure 3. Unbiased pairwise differential expression approach to decipher transcriptional regulation of shell development in *Laternula elliptica*. Differential expression was determined using edgeR with a negative binomial additive general linear model and quasi-likelihood F-test, corrected for multiple testing using the Benjamin-Hochberg method to control the false discovery rate (FDR). Upregulated genes at each stage screened for statistical significance (FDR<0.05), magnitude ($log_2FC>2$) and functional categories related to shell development, as per colour-coded key. Specific genes of biological interest are labelled. **A** upregulated genes at initial organic shell stage, veliger 17dpf. **B** upregulated genes at early D-larvae (PI) stage 20dpf with full organic PI shell. **C** upregulated genes at late D-larvae (PII) stage 66dpf when larval shell first begins to be mineralised and folds established in the larval mantle. **D** upregulated genes in hatchling postlarva (DI) stage 160dpf.

The signalling components upregulated during the shell development stages include hh and wnt pathways (Fig. 4A-B). To further explore the possible role of these signalling pathways in shell development we extracted genes relating to the canonical transduction of the hh and wnt pathways and plotted them over developmental time (Fig. S2). Most of the hh and wnt signalling genes were expressed prior to the secretion of the larval shell, followed by very low levels during early shell development stages– veliger and D larvae (PI) stages. One exception was a *ptch* isoform, which was upregulated again at the late D (PII) stage, coinciding with the beginning of larval mantle folding and onset of mineralisation.

<u>Interspecies comparison of the molecular control of larval shell development</u>

To test if larval shell matrix proteins are more conserved than those of adults, genes identified as likely larval shell downstream effectors in *L. elliptica* were compared with the three publicly available larval shell proteomes (*Crassostrea gigas*, *Pinctada fucata* and *Mytilus edulis*). Protein domains were compared across the four species to search for conserved vs lineage

specific domains, as per Arivalagan et al. (2017). Only two protein domains were present in all four species larval shell datasets, VWA and chitin-binding, carbonic anhydrase was present in three out of four species (Fig. 4A).

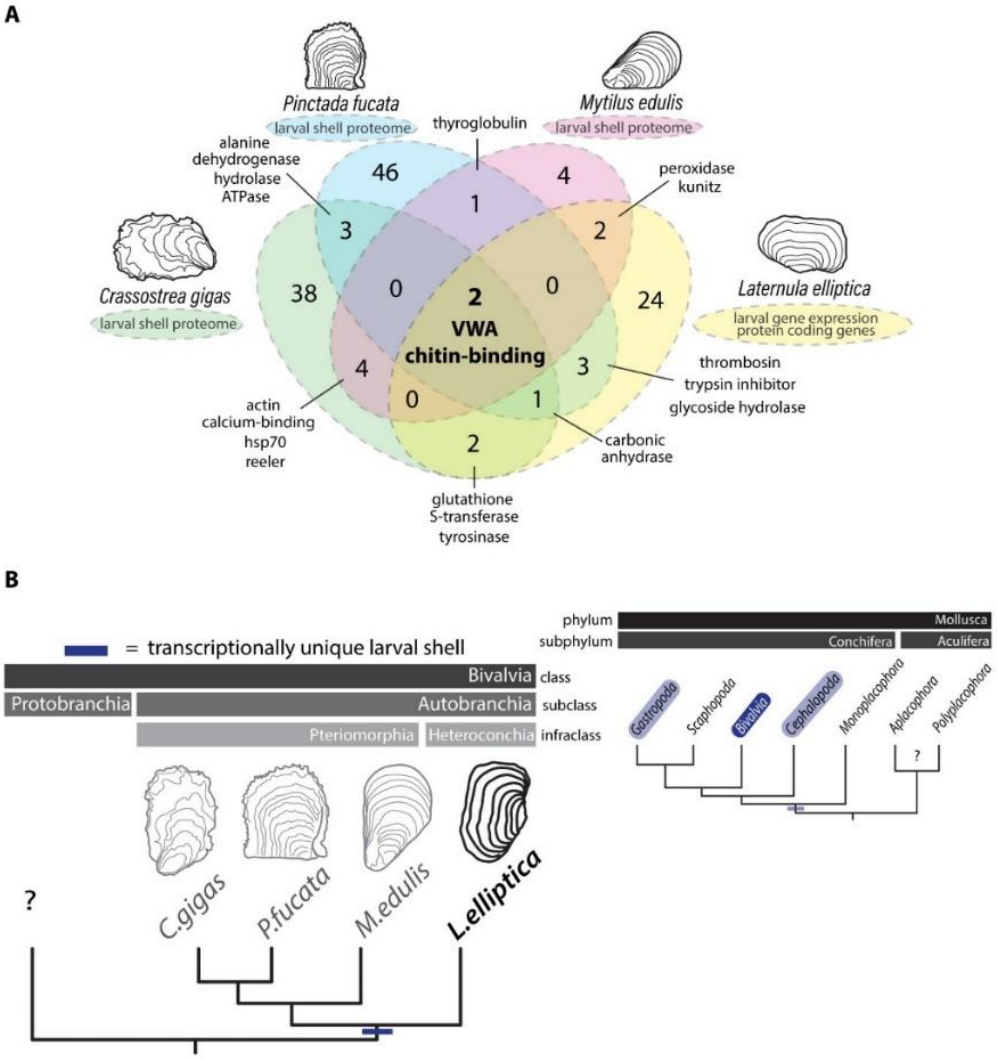

Figure 4. Development-specific downstream effectors control larval shell production in autobranchians, and more broadly across conchiferans. A Larval downstream effectors, such as shell matrix proteins and biomineralisation enzymes translated to amino acid sequences and surveyed for known domains. Protein domains compared against larval shell proteomes to search for conserved vs lineage specific domains using OrthoVenn2. **B** A development-specific larval shell proteome (versus adult shell proteome) in three species of Pteriomorhpia and, presented here, transcriptionally unique, development-specific downstream shell genes in a representative from Heteroconchia points to an ancestral condition to the Autobranchia subclass. Development-specific shell genes and isoforms have also been reported in gastropods. Taken together with many conchiferan molluscs retaining a larval shell but secondarily loosing, or reducing, an adult shell leads us to hypothesise that a transcriptionally unique larval shell is likely a deeply conserved trait to the Conchifera (no known developmental data for Aculifera or Protobranchia).

Discussion

We have quantified the ontogenetic expression dynamics of genes putatively involved in shell development for the first time in a heteroconchian bivalve, the Antarctic clam *L. elliptica.* These genes can be broadly categorised into two groups: downstream effectors that code for proteins involved in the extracellular shell matrix, enzymes and ion transporters (such as peroxidases, chitinases, matrix metalloproteinases, galaxins, ion and vitamin transporters) and upstream regulatory genes that code for transcription factors and signalling components (wnt and hh signalling components, hox, fox and sox transcription factors). Owing to a lack of genetically tractable model systems to conduct gene function experiments in molluscs (and more broadly spiralians), data on the gene regulatory networks that drive processes such as shell development and biomineralisation in this group are sparse, either focussing on two or three candidates (Kin et al. 2009; Liu et al. 2017; 2020; Nederbragt et al. 2002; Samadi and Steiner 2009; Tan et al. 2018), or use only computational methods (Sleight et al. 2020). Previous work has implicated bmp signalling in the regulation of molluscan shell secretion using *in situ* expression data and perturbations such as RNAi, DMH1 (a bmp inhibitor) or addition of bmp protein. Results have been variable; work in bivalves suggest bmp signalling could play a role in controlling adult shell secretion (Zhao et al. 2016) and shell development (Liu et al. 2020; Nederbragt et al. 2002). More recent experiments in the gastropod *Crepidula fornicata* found that when bmp signalling was disrupted during development, there was no observed effect on shell phenotype (Lyons et al. 2020) and single-cell RNAseq data from the trochophore stage of the heteroconchian bivalve *Dreissena rostriformis* suggests no bmp gene expression in shell-field cells (Salamanca-Díaz et al. 2022). The WGCNA analysis presented here found bmp pathway components were correlated only to the postlarva stage and the upregulation of the downstream adult shell candidates, but no bmp components were found using the differential expression approach. Taken together with previous reports in other groups, we hypothesise that bmp signalling is not involved in early shell development in heteroconchian bivalves, but could play a role in controlling the growth and maintenance of shell secretion at later stages.

The transcriptomic profiling data presented here suggests that hh and wnt signalling pathways are involved in shell development. These signalling pathways have well characterised roles in animal dorsal-ventral (wnt) and anterior-posterior patterning (hh), and the outgrowth of appendages in vertebrates and arthropods, as well as osteoblast differentiation and digit patterning in vertebrate endoskeletons (Hiscock et al. 2017; Riddle et al. 1993; Witte et al. 2009). Previous work has linked wnt signalling genes to biomineralisation in bivalves (Gao et al. 2016), and most recently, wnt and hh signalling have been shown to pattern cephalopod arm development (Tarazona et al. 2019). If cephalopod “limbs” evolved by parallel activation

of a gene regulatory network for appendage development that was present in the bilaterian common ancestor (Tarazona et al. 2019), our data also suggest that this genetic program for appendage development may have been co-opted for shell development. More data is required on the spatial expression and function of hh and wnt genes in relation to mollusc shell development, and future work in species more suited to such methods should prioritise these studies.

Recent larval shell proteomes of three pteriomorphian bivalve species report that a development-specific repertoire of downstream effector proteins are found in the extracellular matrix of the larval shell when compared to the adult shell (Carini et al. 2019; Zhao et al. 2018). Here, we asked if this pattern holds true in a heteroconchian representative, and aimed to understand the transcriptional regulation of larval shell deposition versus that of adults. We find that previously characterised downstream adult shell genes are largely not involved in larval shell secretion (except for *le-meg*), neither the first organic stage nor early mineralised stages, but that the upstream regulatory genes, such as signalling molecules and transcription factors, are more conserved between life history stages. Our data suggest that global transcriptional mechanisms underpinning the unique larval shell proteins include ontogenetic partitioning of isoforms and paralogues, in addition to some *bona fide* unique larval genes (such as *btf3*, *pap18*, *chid1*, *cemip* and *nsf*). We hypothesise that, similar to other developmental systems, such as *svb*-driven gene regulatory networks in the formation of diverse actin rich projections in arthropods (Smith et al. 2018), conserved gene regulatory networks drive shell deposition in larval and adult life history stages, but the downstream effectors activated by the network depends on local factors in different life history and cellular contexts. As is the case with many systems studying morphological evolution, questions regarding the connection between gene regulatory networks and downstream effectors, the generation of temporal and local contexts and how different downstream effectors exert influences on phenotypic outcomes, remain unanswered.

Terminal downstream effectors in adult shells, such as extracellular shell matrix proteins, are rapidly evolving, lineage-specific and surprisingly diverse, with just four domains critical for all molluscan biomineralisation (carbonic anhydrase, chitin-binding, VWA and tyrosinase (Arivalagan et al. 2017)). To test if this pattern holds true for early life history stages where, phenotypically at least, shells are more conserved between species, we carried out comparative domain analysis between heterochoncian and pteriomorphian bivalves. Similar to the pattern in adult life history stages for these groups, we find only two domains shared between the four species (chitin-binding and VWA domains), despite the similarity in shell phenotype across species at this stage. In our analysis we compared data generated by different groups using different methods, in addition, our data is a prediction of

biomineralisation effectors and hence, it is likely that the number of conserved domains are underestimated. For example, it is unlikely that carbonic anhydrase is truly absent in larval mussel shells as reported Carini et al., (2019), but rather it was technologically difficult to detect all proteins present in larval shell with the small input material that is available from developmental stages.

For the first time, we have compared the transcriptional regulation of shell secretion at different life history stages of a heteroconchian bivalve. Studying a heterconchian representative, and comparing it to data available for pteromorphians allows us to hypothesise that a development-specific transcriptionally unique larval shell is likely an ancestral feature of autobranchians, or perhaps even more broadly to conchiferans (Fig. 5B). Ontogenetic partitioning of some specific shell gene isoforms has been reported in gastropods, as well as alternative splicing to generate diverse shell matrix proteins from a single genomic loci (*Halitosis asinine* and *Lymnaea stagnalis* (Herlitze et al. 2018; Jackson et al. 2006)). In addition, some conchiferan molluscs have a larval shell but have secondarily lost, or reduced, an adult shell (Knutson et al. 2020). There is a huge diversity of form and function between different life history stages in molluscs. Taken together, these findings lead us to hypothesise that a development-specific transcriptionally unique larval shell may be a deeply conserved trait to the Conchifera, but more data from diverse taxa, especially the Acuilifera, are needed to resolve questions on the evolution of biomineralisation in the molluscs.

Methods

Animal husbandry, spawning and developmental staging

Embryos were obtained from an adult broodstock of sexually mature *L. elliptica* individuals and divided into three independent closed-system 1L tanks. Embryos were maintained at 0°C ± 0.5°C, aerated with an airstone with 50% water changes every two days using autoclaved seawater. Embryos were staged as per Peck et al. (2007).

Fixation, histology and imaging

For histology and Scanning Electron Microscopy (SEM), embryos were fixed in 500 µL of 2.5% glutaraldehyde in phosphate buffered saline (PBS) at room temperature for 30 minutes, rinsed twice in PBS with 0.1% tween, dehydrated into 100% ethanol and stored at 4°C.

For histology, embryos were cleared in Histosol (National Diagnostics, 3 × 20 min, room temperature), transitioned through 1:1 Histosol:molten paraffin (2 × 30 mins, 60°C) then pure molten paraffin (RA Lamb Wax – Fisher Scientific, 60°C overnight). Five changes of molten paraffin (each >1 hr) were conducted before embedding in a Peel-A-Way embedding mold (Sigma). Wax blocks were serially sectioned at 8 µm on a Leica RM2125 rotary microtome. Sections were stained with a modified Masson's trichrome stain as per Witten and Hall (2003). All histology was conducted on a minimum of 3 individuals per stage. Sections were imaged on a Zeiss Axioscope A1 with Zen software (at the University of Cambridge).

For SEM of the shell late D (66dpf) and postlarval (160dpf) stages, larvae were transferred from ethanol to electron microscope stubs, attached using carbon adhesive discs,carbon coated and examined using a QEMSCAN 650F (at the University of Cambridge) at accelerating voltages of 10 kV.

Each stage was also live imaged on an upright compound light microscope fitted with a camera (Olympus BX50 microscope fitted with Olympus PM-C35 camera using Olympus U-CMAD-2 software, at the British Antarctic Survey) by mounting in seawater onto a glass slide, under a coverslip on small clay feet. All live imaging was conducted on a minimum of 3 individuals per stage.

Images were adjusted for contrast and brightness, cropped, rotated, and flipped. All plates were constructed in Adobe Illustrator software.

Sampling, RNA extraction and sequencing

Triplicate RNA-Seq samples were collected for each developmental stage, one from each independent tank. For each sample, exactly two hundred staged-matched embryos were snap frozen in a 70% ethanol dry ice slurry and stored at -80°C. Total RNA was extracted from each sample as per manufactures recommendations (Relia Miniprep kit, Promega) and tested for

quality and quantity using Nanodrop and Agilent Tapestation. All samples had an RNA Integrity Number (RIN) of over 7. Libraries were prepared by the DNA sequencing facility in the Biochemistry Department at the University of Cambridge (TruSeq Stranded mRNA, Illumina) and sequenced on an Illumina NextSeq500 generating over 300 million 150bp stranded paired-end reads. All raw data is publicly available from NCBI SRA accession number: PRJNA803976.

Bioinformatics and statistical analyses

Raw reads (total 309,593,642) were cleaned using ea-utils tool v1.1.2 fastq-mcf (quality –q 30, and length –l 100), after cleaning 296,480,254 reads remained. Prior to *de novo* assembly, reads were normalised using the Trinity v.2.2.0 utility script (insilico_read_normalization.pl –max_cov 50), leaving 64,355,068 reads for assembly. Clean, normalised reads were assembled using Trinity v.2.2.0 with default parameters (Grabherr et al. 2011; Haas et al. 2013). Assembly quality was assessed using Trinity utilities and the gVolante webserver tool (Table S1). Transcript abundance was estimated by alignment-based quantification using Trinity v.2.2.0 utilities. Transcripts from each cleaned library were aligned to the transcriptome using bowtie2 with default parameters and transcript abundance estimates were calculated using RNA-Seq by Expectation-Maximization (RSEM). Raw counts and Trimmed Mean of M-values [TMM] normalised Fragments Per Kilobase Of Exon Per Million Fragments Mapped [FPKM] matrices were generated.

Weighted correlation network analysis (WGCNA) was used to find clusters, termed modules, of genes with highly correlated expression across all libraries. Each cluster was then correlated to external traits of interest (Langfelder and Horvath 2008). The raw counts matrix was loaded in R and EdgeR (Robinson et al. 2010) functions were used to remove lowly expressed transcripts (keep transcripts with cpm >5 in ≥4 libraries) leaving 37,000 transcripts for WGCNA. TMM-FPKM values were used to calculate a gene dissimilarity matrix (adjacency= softpower 16 and signed, TOMsimilarity = signed) and hierarchical clustering was performed (method = average). Modules were determined using the cutreeDynamic function with a minimum gene membership threshold of 30 and dynamic tree cut-off of 25. Modules were correlated to external traits (days post fertilization or average candidate gene expression values). Modules of that were significantly correlated to traits of interest were extracted and all transcripts were putatively annotated based on sequence similarity searched using blastx against Uniprot (http://www.uniprot.org/), and tested for functional enrichment.

Pairwise differential gene expression tests were performed to find transcripts that were upregulated at each stage of shell development (compared to the previous stage). Using the EdgeR package, a negative binomial additive general linear model with a quasi-likelihood F-

test was performed and p-values were adjusted for multiple testing using the Benjamini-Hochberg method to control the false discovery rate, cut-offs for statistical significance (FDR ≤0.05) and magnitude were used (log_2FC>2). Upregulated transcripts were putatively annotated based on sequence similarity searched using blastx against Uniprot (http://www.uniprot.org/), and screened for functional categories relating to gene regulation and shell secretion.

All upregulated genes that were functionally categorised as downstream effectors, such as shell matrix proteins and biomineralisation enzymes were translated to amino acid sequences and surveyed for known domains. Protein domains were then compared against published larval shell proteomes for oyster (Zhao et al. 2018) and mussel (Carini et al. 2019) species to search for conserved vs lineage specific domains as per Arivalagan et al., (2017), using OrthoVenn2.

References

Aguilera F, McDougall C, Degnan BM. 2014. Evolution of the tyrosinase gene family in bivalve molluscs: Independent expansion of the mantle gene repertoire. Acta biomaterialia. 10(9):3855-3865.

Aguilera F, McDougall C, Degnan BM. 2017. Co-option and de novo gene evolution underlie molluscan shell diversity. Mol Biol Evol. 34(4):779-792.

Anishchenko E, Arnone MI, D'Aniello S. 2018. In sea urchin development: Implications in neurogenesis, ciliogenesis and skeletal patterning. Evodevo. 9:5.

Arivalagan J, Marie B, Sleight VA, Clark MS, Berland S, Marie A. 2016. Shell matrix proteins of the clam, *Mya truncata*: Roles beyond shell formation through proteomic study. Marine Genomics. 27:69-74.

Arivalagan J, Yarra T, Marie B, Sleight VA, Duvernois-Berthet E, Clark MS, Marie A, Berland S. 2017. Insights from the shell proteome: Biomineralization to adaptation. Mol Biol Evol. 34(1):66-77.

Carini A, Koudelka T, Tholey A, Appel E, Gorb SN, Melzner F, Ramesh K. 2019. Proteomic investigation of the blue mussel larval shell organic matrix. J Struct Biol. 208(3):107385.

Clark MS, Peck LS, Arivalagan J, Backeljau T, Berland S, Cardoso JCR, Caurcel C, Chapelle G, De Noia M, Dupont S et al. 2020. Deciphering mollusc shell production: The roles of genetic mechanisms through to ecology, aquaculture and biomimetics. Biol Rev Camb Philos Soc. 95(6):1812-1837.

Gao J, Liu J, Yang Y, Liang J, Xie J, Li S, Zheng G, Xie L, Zhang R. 2016. Identification and expression characterization of three wnt signaling genes in pearl oyster (*Pinctada fucata*). Comp Biochem Physiol B Biochem Mol Biol. 196-197:92-101.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng QD et al. 2011. Full-length transcriptome assembly from rna-seq data without a reference genome. Nature Biotechnology. 29(7):644-U130.

Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, Couger MB, Eccles D, Li B, Lieber M et al. 2013. De novo transcript sequence reconstruction from rna-seq: Reference generation and analysis with trinity. Nature protocols. 8(8):10.1038/nprot.2013.1084.

Herlitze I, Marie B, Marin F, Jackson DJ. 2018. Molecular modularity and asymmetry of the molluscan mantle revealed by a gene expression atlas. Gigascience. 7(6).

Hiscock TW, Tschopp P, Tabin CJ. 2017. On the formation of digits and joints during limb development. Dev Cell. 41(5):459-465.

Hohagen J, Jackson DJ. 2013. An ancient process in a modern mollusc: Early development of the shell in *Lymnaea stagnalis*. BMC Developmental Biology. 13:13.

Huan P, Wang Q, Tan S, Liu B. 2020. Dorsoventral decoupling of hox gene expression underpins the diversification of molluscs. Proc Natl Acad Sci U S A. 117(1):503-512.

Jackson DJ, McDougall C, Green K, Simpson F, Woerheide G, Degnan BM. 2006. A rapidly evolving secretome builds and patterns a sea shell. BMC Biology. 4.

Kin K, Kakoi S, Wada H. 2009. A novel role for dpp in the shaping of bivalve shells revealed in a conserved molluscan developmental program. Dev Biol. 329(1):152-166.

Knutson VL, Brenzinger B, Schrödl M, Wilson NG, Giribet G. 2020. Most cephalaspidea have a shell, but transcriptomes can provide them with a backbone (gastropoda: Heterobranchia). Mol Phylogenet Evol. 153:106943.

Langfelder P, Horvath S. 2008. Wgcna: An r package for weighted correlation network analysis. BMC Bioinformatics. 9:559.

Lefebvre V. 2019. Roles and regulation of sox transcription factors in skeletogenesis. Curr Top Dev Biol. 133:171-193.

Liu G, Huan P, Liu B. 2017. A soxc gene related to larval shell development and co-expression analysis of different shell formation genes in early larvae of oyster. Dev Genes Evol. 227(3):181-188.

Liu G, Huan P, Liu B. 2020. Identification of three cell populations from the shell gland of a bivalve mollusc. Dev Genes Evol. 230(1):39-45.

Lyons DC, Perry KJ, Batzel G, Henry JQ. 2020. Bmp signaling plays a role in anterior-neural/head development, but not organizer activity, in the gastropod crepidula fornicata. Dev Biol. 463(2):135-157.

Marie B, Marie A, Jackson DJ, Dubost L, Degnan BM, Milet C, Marin F. 2010. Proteomic analysis of the organic matrix of the abalone *Haliotis asinina* calcified shell. Proteome Science. 8.

Marie B, Marin F, Marie A, Bédouet L, Dubost L, Alcaraz G, Milet C, Luquet G. 2009. Evolution of nacre: Biochemistry and proteomics of the shell organic matrix of the cephalopod *Nautilus macromphalus*. Chembiochem. 10(9):1495-1506.

McDougall C, Degnan BM. 2018. The evolution of mollusc shells. Wiley Interdiscip Rev Dev Biol. 7(3):e313.

Nederbragt AJ, van Loon AE, Dictus WJ. 2002. Expression of *Patella vulgata* orthologs of engrailed and dpp-bmp2/4 in adjacent domains during molluscan shell development suggests a conserved compartment boundary mechanism. Dev Biol. 246(2):341-355.

Plazzi F, Passamonti M. 2010. Towards a molecular phylogeny of Mollusks: Bivalves' early evolution as revealed by mitochondrial genes. Molecular Phylogenetics and Evolution, 57(2):641-657.

Peck LS, Powell DK, Tyler PA. 2007. Very slow development in two antarctic bivalve molluscs, the infaunal clam *Laternula elliptica* and the scallop *Adamussium colbecki*. Marine Biology. 150(6):1191-1197.

Perry KJ, Lyons DC, Truchado-Garcia M, Fischer AH, Helfrich LW, Johansson KB, Diamond JC, Grande C, Henry JQ. 2015. Deployment of regulatory genes during gastrulation and germ layer specification in a model spiralian mollusc *Crepidula*. Dev Dyn. 244(10):1215-1248.

Prud'homme B, Gompel N. 2010. Evolutionary biology: Genomic hourglass. Nature. 468(7325):768-769.

Riddle RD, Johnson RL, Laufer E, Tabin C. 1993. Sonic hedgehog mediates the polarizing activity of the zpa. Cell. 75(7):1401-1416.

Robinson MD, McCarthy DJ, Smyth GK. 2010. Edger: A bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26(1):139-140.

Salamanca-Díaz DA, Schulreich SM, Cole AG, Wanninger A. 2022. Single-Cell RNA Sequencing Atlas From a Bivalve Larva Enhances Classical Cell Lineage Studies. Front Ecol Evol. 9:783984.

Samadi L, Steiner G. 2010. Expression of hox genes during the larval development of the snail, *Gibbula varia* (l.)-further evidence of non-colinearity in molluscs. Dev Genes Evol. 220(5-6):161-172.

Setiamarga DHE, Hirota K, Yoshida M-a, Takeda Y, Kito K, Shimizu K, Isowa Y, Ikeo K, Sasaki T, Endo K. 2020. Hydrophilic shell matrix proteins of *Nautilus pompilius* and the identification of a core set of conchiferan domains. bioRxiv.2020.2011.2014.382804.

Sleight VA, Antczak P, Falciani F, Clark MS. 2020. Computationally predicted gene regulatory networks in molluscan biomineralization identify extracellular matrix production and ion transportation pathways. Bioinformatics. 36(5):1326-1332.

Sleight VA, Marie B, Jackson DJ, Dyrynda EA, Marie A, Clark MS. 2016a. An antarctic molluscan biomineralisation tool-kit. Sci Rep. 6:36978.

Sleight VA, Thorne MA, Peck LS, Clark MS. 2015. Transcriptomic response to shell damage in the antarctic clam, laternula elliptica: Time scales and spatial localisation. Mar Genomics. 20:45-55.

Sleight VA, Thorne MAS, Peck LS, Arivalagan J, Berland S, Marie A, Clark MS. 2016b. Characterisation of the mantle transcriptome and biomineralisation genes in the blunt-gaper clam, *Mya truncata*. Marine Genomics. 27:47-55.

Smith JM, Burian R, Kauffman S, Alberch P, Campbell J, Goodwin B, Lande R, Raup D, Wolpert L. 1985. Developmental constraints and evolution: A perspective from the

mountain lake conference on development and evolution. The Quarterly Review of Biology. 60(3):265-287.

Smith SJ, Rebeiz M, Davidson L. 2018. From pattern to process: Studies at the interface of gene regulatory networks, morphogenesis, and evolution. Curr Opin Genet Dev. 51:103-110.

Tan S, Huan P, Liu B. 2018. An investigation of oyster tgf-β receptor genes and their potential roles in early molluscan development. Gene. 663:65-71.

Tarazona OA, Lopez DH, Slota LA, Cohn MJ. 2019. Evolution of limb development in cephalopod mollusks. Elife. 8:e43828.

Thompson DAW. 1992. On growth and form. Bonner JT, editor. Cambridge: Cambridge University Press.

Vinther J. 2015. The origins of molluscs. Palaeontology. 58(1):19-34.

Weiss IM, Tuross N, Addadi L, Weiner S. 2002. Mollusc larval shell formation: Amorphous calcium carbonate is a precursor phase for aragonite. J Exp Zool. 293(5):478-491.

Witte F, Dokas J, Neuendorf F, Mundlos S, Stricker S. 2009. Comprehensive expression analysis of all wnt genes and their major secreted antagonists during mouse limb development and cartilage differentiation. Gene Expr Patterns. 9(4):215-223.

Witten PE, Hall BK. 2003. Seasonal changes in the lower jaw skeleton in male atlantic salmon (salmo salar l.): Remodelling and regression of the kype after spawning. J Anat. 203(5):435-450.

Zhao M, He M, Huang X, Wang Q. 2014. A homeodomain transcription factor gene, pfmsx, activates expression of pif gene in the pearl oyster *Pinctada fucata*. PLoS One. 9(8):e103830.

Zhao M, Shi Y, He M, Huang X, Wang Q. 2016. Pfsmad4 plays a role in biomineralization and can transduce bone morphogenetic protein-2 signals in the pearl oyster *Pinctada fucata*. BMC Dev Biol. 16:9.

Zhao R, Takeuchi T, Luo YJ, Ishikawa A, Kobayashi T, Koyanagi R, Villar-Briones A, Yamada L, Sawada H, Iwanaga S et al. 2018. Dual gene repertoires for larval and adult shells reveal molecules essential for molluscan shell formation. Mol Biol Evol. 35(11):2751-2761.

Data Availability

Raw read data generated in this publication is freely available at NCBI SRA with the following accession PRJNA803976. All analysis scripts and results are available in Supplementary File 1.

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Author Contributions

AC Performed all wet-lab experiments, contributed to imaging, data analysis and interpretation, and reviewed and edited the manuscript.

MSC Contributed to supervision of AC, funded the sequencing, contributed to wet-lab experiments, imaging and data interpretation, reviewed and edited the manuscript.

EH Conducted all SEM imaging, contributed to data interpretation and reviewed and edited the manuscript.

LSP Conducted adult broodstock collection, contributed to wet-lab experiments and data interpretation, reviewed and edited the manuscript.

VAS Conceptualized and oversaw the study, supervised AC, contributed to wet-lab experiments and imaging, conducted all data analysis and interpretation, prepared all figures and wrote the manuscript.

Competing Interests statement

The authors have no competing interests to declare.

Supplementary Figures, Tables and Files

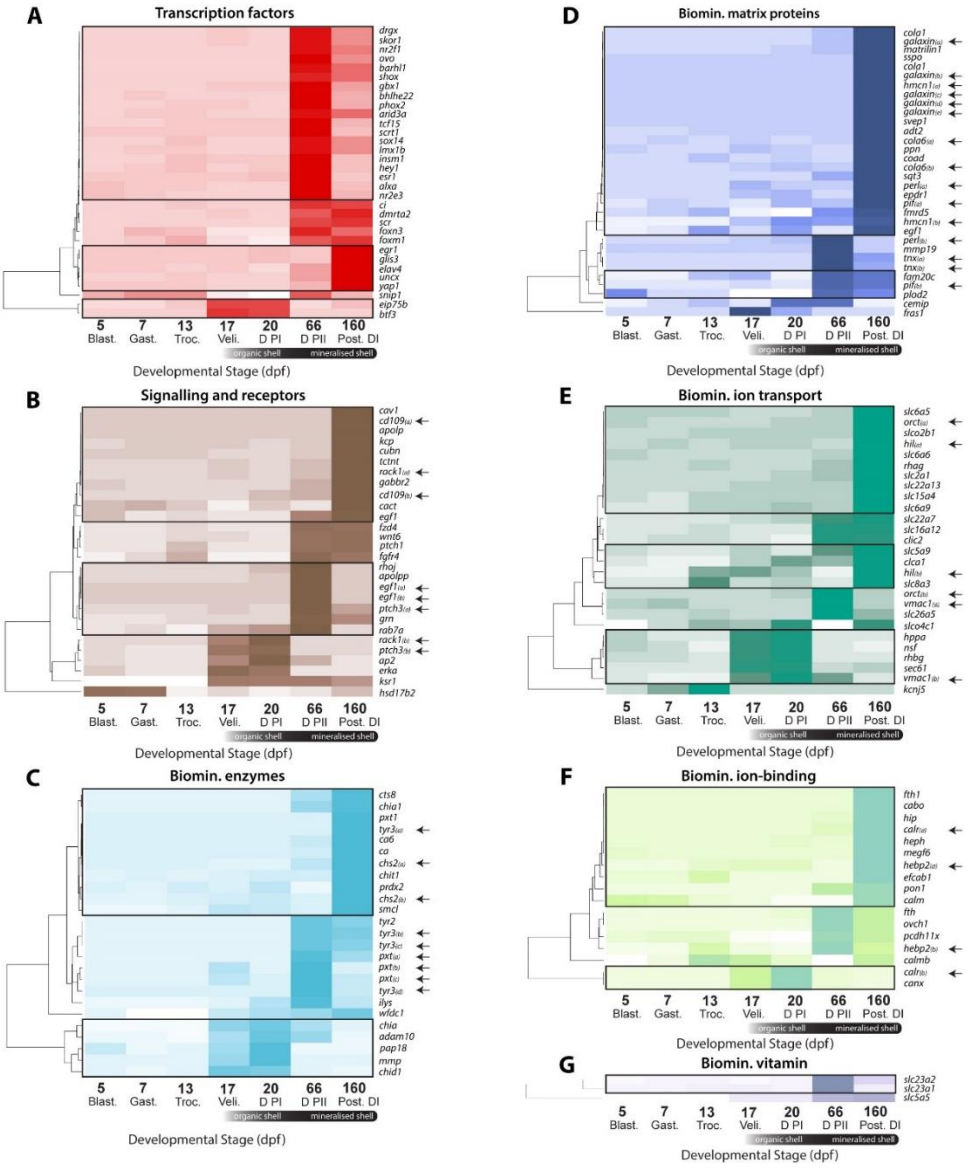

Figure S1. Clustered heatplots of significant functionally screened genes from unbiased differential expression analysis, genes typically show stage-specific expression patterns. Mean average FPKM (n=3) z-scaled over developmental time and clustered by temporal expression profile on y axis only using hclust (method = single, clustering represented by dendograms on left). Multiple isoforms of the same gene with unconfirmed nomenclature labelled with lowercase letters in parentheses and highlighted with arrows.

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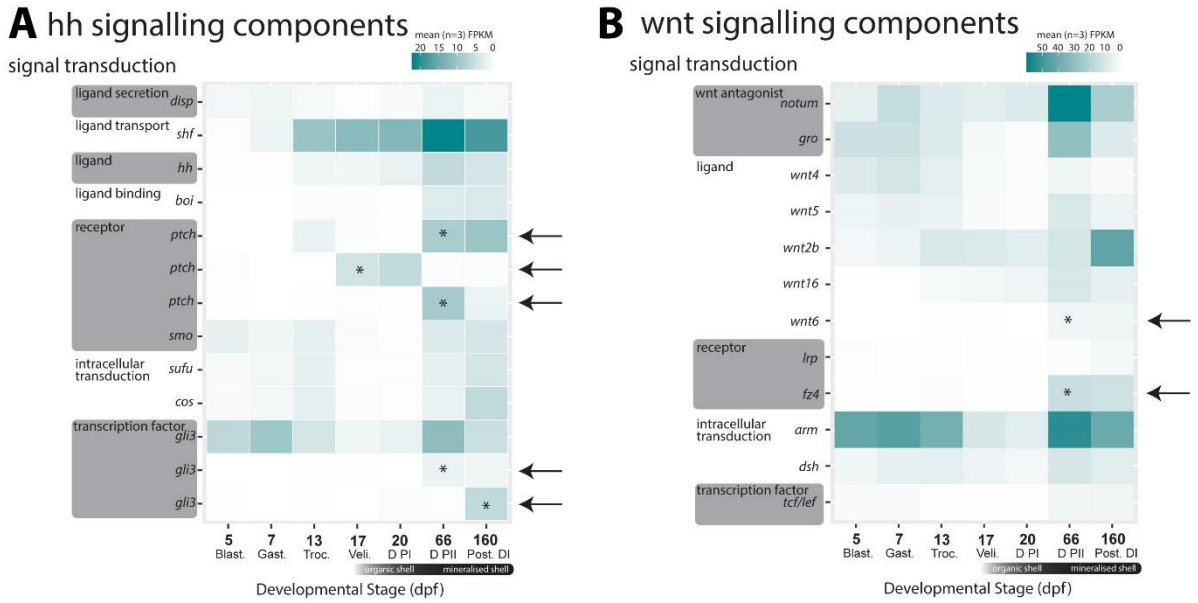

627 **Figure S2. Both hh and wnt signalling pathway genes were significantly upregulated**
628 **during shell development and could be involved in patterning and proliferation of the**
629 **larval shell field and larval mantle.** A Heatplot of hh signalling genes and B wnt signalling
630 genes. Statistically significantly upregulated genes (edgeR, FDR<0.05, $log_2FC>2$) identified
631 with arrows and stage at which significantly upregulated marked with an asterisks (*). Order
632 of genes on the y axis presented in terms of signal transduction cascades from ligand
633 secretion towards the top and recruitment of downstream transcription factors to the nucleus
634 at the bottom.

635

636 **Table S1. Summary statistics for *de novo* assembled *Laternula elliptica***
637 **developmental transcriptome.**

638

639

Reads:	
Raw reads	309,593,642
Clean reads	296,480,254
Normalised reads	64,355,068
Assembly:	
Total trinity transcripts	964,764
Total trinity genes	666,835
% GC	46.3
Statistics based on all isoforms per gene:	
N50	720
Median length	462
Mean average length	662
Read representation (average read content per library):	67%
BUSCO v3, Metazoa Gene Set (n=978)	C:99.1%[S:30%,D:69%] F:0.7%,M:0.2%

640 **Supplementary file 1.** Data archive including readme.txt file detailing curation, includes
641 annotations, statistical analysis R scripts and results tables.

642