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RESEARCH ARTICLE

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Substantial gene expression shifts during larval transitions in the pearl oyster *Pinctada margaritifera*

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Abstract

Early development stages in marine bivalve are critical periods where larvae transition from pelagic free-life to sessile mature individuals. The successive metamorphosis requires the expression of key genes, the functions of which might be under high selective pressure, hence understanding larval development represents key knowledge for both fundamental and applied research. Phenotypic larvae development is well known, but the underlying molecular mechanisms such as associated gene expression dynamic and molecular cross-talks remains poorly described for several nonmodel species, such as *P. margaritifera*. We designed a whole transcriptome RNA-sequencing analysis to describe such gene expression dynamics following four larval developmental stages: D-shape, Veliger, Umbo and Eye-spot. Larval gene expression and annotated functions drastically diverge. Metabolic function (gene expression related to lipid, amino acid and carbohydrate use) is highly upregulated in the first development stages, with increasing demand from D-shape to umbo. Morphogenesis and larval transition are partly ordered by Thyroid hormones and Wnt signaling. While larvae shells show some similar characteristic to adult shells, the cause of initialization of biomineralization differ from the one found in adults. The present study provides a global overview of *Pinctada margaritifera* larval stages *transitioning* through gene expression dynamics, molecular mechanisms and ontogeny of biomineralization, immune system, and sensory perception processes.

KEYWORDS

biomineralization, environmental sensing, immune system, larval development, *Pinctada margaritifera*, RNA-seq

1 | BACKGROUND

Pinctada margaritifera is the pearl oyster species exploited in French Polynesia for cultured pearl production and represent the most important economical income after tourism for the territory. As many bivalves, *P. margaritifera* passes two main stages in its life: a pelagic free-living larva to sessile mature oyster. Larval developmental and phenotypic characterization of *Pinctada* spp. species, including *P. margaritifera*, has been largely explored (Alagarswami et al., 1989; Liu et al., 2015; Mao Che et al., 2001; Sangare et al., 2020; Zheng et al., 2019). The entire larval cycle takes around 4 weeks at 25-27°C in hatchery production systems.

Briefly, beginning a few hours after fertilization, *P. margaritifera* fertilized eggs divide, passe from blastula, gastrula to trochophore stage and rapidly develop the velum, an organ which allow them to swim and feed through their filtration system. This stage is followed by D-shape, Velyger, Umbo and Eye-stage (pediveliger). During these transitions, larvae will start feeding, develop prodissochonchs I and II (early shells) an appendix to sense the environment and settle.

Early developmental stages are considered as the most vulnerable and sensitive period characterized by the highest selective pressure (Plough, 2018). Impacts during these stages might have lifelong effects, even extending beyond individuals' lifetime (multi- or trans-generational effect) (Byrne, 2011). Consequently, larval development is as a unique time window to analyze the onset and the actors involved in vital biological processes, as evidenced by the detection of vital QTLs (Nascimento-Schulze et al., 2021; Plough & Hedgecock, 2011). For both ecological and aquaculture purposes, several mechanisms, among which biomineralization, immune defense and settlement behavior, have been explored during early life stages.

Environment perception is also a crucial function, which allow the larvae to select microenvironment and trigger settlement behavior (Wang et al., 2016). Despite substantial progress in testing for physical or chemical means to improve settlement yield in hatcheries (Joyce & Vogeler, 2018), our knowledge of the mechanisms conditioning the metamorphosis are still missing and restrict our ability to properly tease apart hypotheses of settlement induced by mandatory chemical cues and/or by energy limitation (Bishop et al., 2006). In French Polynesia, most of the pearl oysters supply still relies on wild spat collection, with success extremely variable in time, seasons and locations, suggesting important effect of environmental factors influencing larvae fixation and/or spat survival (Sangare et al., 2020). With recent development of hatcheries, much effort has to be undertaken for optimization with regard to larvae cycle, biology and to sustainability of the production. Hence, understanding the immune defense or the onset of biomineralization mechanisms in *P. margaritifera* may thus contribute to the implementation of novel hatchery policies to manage and monitor diseases emergence (Wang et al., 2016).

Here we sequenced the transcriptome of *P. margaritifera* at four critical and different larval stages (D-shaped, veliger, umbo and eye-spot) with the aim to identify key genes and functions associated with metamorphic transitions with a special focus on major biological process and cross-talks.

2 | METHODS

2.1 | Animal tissue sampling, RNA extraction and sequencing

A data set of twelve pools were obtained from four different *P. margaritifera* larval stages harvested in triplicate: D-shape (4 dpf), Veliger (8 dpf), Umbo (13 dpf) and Eye-spot (22 dpf) coming from the same mass breeding. Larvae were fed ad libitum with a mix (1:1) of *Isochrysis galbana* and *Chaetoceros minus* and maintained in throughflow system in UV-treated water at ambient temperature (mean 27°C). After sampling, whole individuals were preserved in ribonucleic acid (RNA) later (Qiagen) and kept at −80°C until RNA extraction. For all samples, total RNA was extracted with TRIzol Reagent (Life Technologies) following manufacturer's recommendations. High-quality RNA was dried in RNA-stable solution (ThermoFisher Scientific) following manufacturer's recommendations and shipped to McGill sequencing platform services (Montreal, Canada). *RNA quality was tested in the lab using both Nanodrop and bioanalyzer. RIN > 7 were used as lower threshold. RNA quality was further assessed after shipping at the sequencing platform using Bioanalyzer*. TruSeq Sample Prep. (Illumina, San Diego, California CA, USA) RNA libraries were sequenced on a single lane of HiSeq. 4000 100-bp paired-end (PE) sequencing device.

2.2 | Differential expression analysis

Raw reads were trimmed using Trimmomatic v0.36 (Bolger et al., 2014) with following parameters Illuminaclip:2:20:7, Leading:20, Trailing:20, Slidingwindow:30:30 and Minlen:60. Only paired-end reads were retained and mapped to the reference genome (Le Luyer et al., 2019), using GSNAP v2017-03-17 (Wu et al., 2016) with default parameters but allowing a minimum mismatch value of 2 and a minimum coverage of 0.9. A matrix of raw counts was built using HTSeq-count v0.6.1 (Anders et al., 2015). Pairwise differential expression was assessed using the DESeq. 2 *v1.38.1* R package with Wald test tests (Love et al., 2014). PCA was constructed for the entire data set based on the variance-stabilizing transformation (vst) of genes expression using the plotPCA function implemented in the DESeq. 2 v1.38.1 R package (Love et al., 2014). Differentially expressed genes (DEGs) were considered significant when FDR < 0.01 and |log2FC| > 2. EuKaryotic Orthologous Group (KOG) enrichment was explored using Mann-Whitney U tests implemented in KOGMWU *v1.2* R package (Dixon et al., 2015) with KOG annotations retrieved with eggNOG-mapper v2 (Cantalapiedra et al., 2021) against the eggNOG 5.0 database (Huerta-Cepas et al., 2019) following default parameters.

2.3 | Genes co-expression analyses

Signed co-expression network was built based on the variance stabilized data (VSD) using the R package *WGCNA* following the protocol developed by Langfelder and Horvath (Langfelder & Horvath, 2008). Briefly, we filtered the data for keeping only genes with at least 5% of total variance, as recommended. We fixed a "soft" threshold power of 14 following recommendations (Langfelder & Horvath, 2008). The modules were defined using

the "*cutreeDynamic*" function (minimum of 50 genes by module and default cutting-height = 0.99) based on the topological overlap matrix, and a module Eigengene distance threshold of 0.25 was used to merge highly similar modules. For each module, we defined the module membership (kME, correlation between module Eigengene value and gene expression values). Only modules with significant correlation to Timing (chronological continuous value, 1–4), or specific stage (discrete values, 0–1) were conserved for downstream analyses. GO enrichment were conducted for each module using two-tailed Mann-Whitney U tests implemented in GO_MWU (Dixon et al., 2015) with module membership values (kME) used as continuous variable. Significant GO terms are discussed when *Padj* < 0.05.

3 | RESULTS

3.1 | Sequencing

A total of 357.07 M PE raw reads were obtained for the whole data set. After trimming, 328.50 M PE cleaned reads were retained for mapping yielding a total of 283.37 M mapped PE reads to the *P. margaritifera* genome. No difference in mapping rates across conditions was observed (mean mapping rates = 79.31% +/− 0.04%), suggesting no library bias effect. Trimming and mapping statistics are provided in Supplementary table S1.

3.2 | Differential expression analysis

Most of the expression variation was explained by difference across developmental stages as shown by the principal component analysis (PCA) (Figure 1a) with 63% and 21% of the total variation explained by the first and second axis, respectively. Repartition of the group along the first PC axis (D-shape, Veliger, Umbo, Eye-spot) shows that major differences are observed across more distant development stages and suggests a progressive but distinguishable change across developmental stages with relatively low intra-group dispersion (Figure 1a).

A pairwise differential expression analysis was performed to identify genes implicated in each of the larval transition changes. A total of 3126 differentially expressed genes (DEGs) were identified (|log2FC| > 2; FDR < 0.01). Wald tests for pairwise comparisons reveal increasing number of DEGs for each successive larval transition with: (1) 52 upregulated and 336 downregulated DEGs in D-shape versus Veliger, respectively, (2) 157 upregulated and 456 downregulated DEGs in Veliger versus Umbo, respectively and (3) 231 upregulated and 689 upregulated DEGs in Umbo versus Eye-spot, respectively. Most of the DEGs where specific to each transition (Figure S1). KOG enrichment and correlations show that biological functions necessary for each larval stage largely differs (Figure 1b,c). For instance, we noted that cell motility is largely upregulated in Veliger compared to D-shape stages, while genes related to metabolism (especially of carbohydrate, amino acid and lipids) are higher in D-shape stages. Metabolism enrichment decreases in subsequent developmental stages, except for Eye-stage that solicits carbohydrate transport and metabolism more than Umbo (Figure 1b).

To further dissect the global response along larval development, we implemented a genes co-expression network approach. We explored the functional role of the genes within each module based on the Gene Ontology enrichment (Table S2). The 11 modules grouped in three main clusters (Figure 2a,b).

3.3 | Clusters and modules enrichments

Cluster 1 shows reduced expression at eyed-stage compared to earlier stages, notably expression at D-shape, with module brown showing a perfect linear decrease from D-shape to Eyed stage as marked by the negative correlation with Timing ($r = -1.0$; $p < 0.001$, Figure 2b). The brown module shows enrichment for functions related to signalization (cGMP-mediated signaling, ncRNA processing and second-messenger mediated signaling), tissue modeling (microtubule-based process, cellular component assembly) but also for energy production (glycerol ether, fatty acids and carbohydrate derivative biosynthetic processes). Brown module also encompasses different genes that include Von Wilebrand factor type A domain (vWFA) and chitin binding related genes as well as immune related genes including defensin, MIF, viperins or interferon. The darkturquoise module shows enrichment for glutamate homeostasis, serine family amino acid biosynthetic process and retinoic acid catabolic process, and energy derivation by oxidation of organic compounds. Darkgrey module shows enrichment for several energy processes, including pentose metabolic process, proteolysis via protein deubiquitination or insulin secretion, and DNA methylation. These processes are concomitant with enrichment for DNA methylation and thyroid hormone generation coupled with vitamin B6 metabolic process. Thyroid-stimulating hormone receptor (TSHR) is also found in the brown module, and decrease in expression over the course of development.

3.4 | Mid larval development transition

Cluster 2 is marked by a negative peak of D-shape and in some extent at Veliger stage (except module royalblue), compared to later developmental stages. Among the enriched functions, we note the strong contribution of cell, appendage and animal organ morphogenesis as well as collagen fibril organization in the blue module which is marked by a peak at Umbo and Eye-spot compared to earlier *stages*.

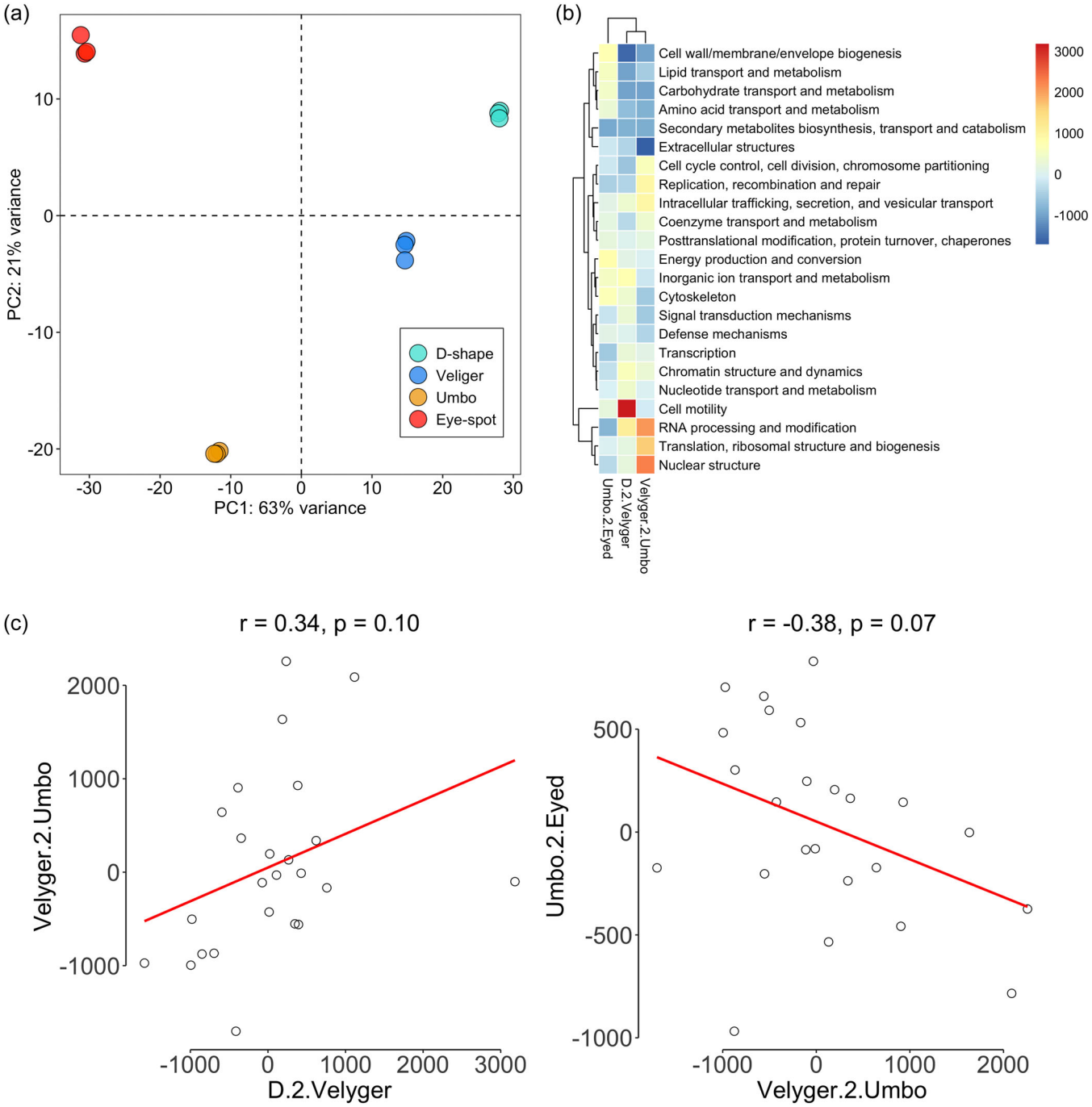

FIGURE 1 Exploration of genes expression and biological functions variations across stages. Principal component analysis (PCA) of gene expression across larval stages in *P. margaritifera* using variance stabilized data (VSD) (a), hierarchical clustering of KOG enrichments for each transition to different larval stages with black outline indicating *P*adj < 0.05 (b) and the correlation of KOG delta rank values for each successive larval stage transition (c).

The same module also shows enrichment for Norrin and Wnt signaling pathway as well as genes involved in the development of central nervous system, which is indicative of putative cross-talks (Chang et al., 2015). The blue module also shows enrichment for signaling pathways involved in immune defense, including the induction of apoptosis though the NIK/NF kapa B signaling pathways and complement activation and immune related genes among the main drivers, notably C1Q coding genes. Finally, the blue module is notably driven by expression of *diacylglycerol O-acyltransferase 1*, a gene responsible for production of triacylglycerol, the principal source of energy for bivalve larvae (Marshall et al., 2010). Finally, we found several biomineralization related genes, including *perlucin*, *perlucin-like* and *temptin* among the main hub genes in the blue module.

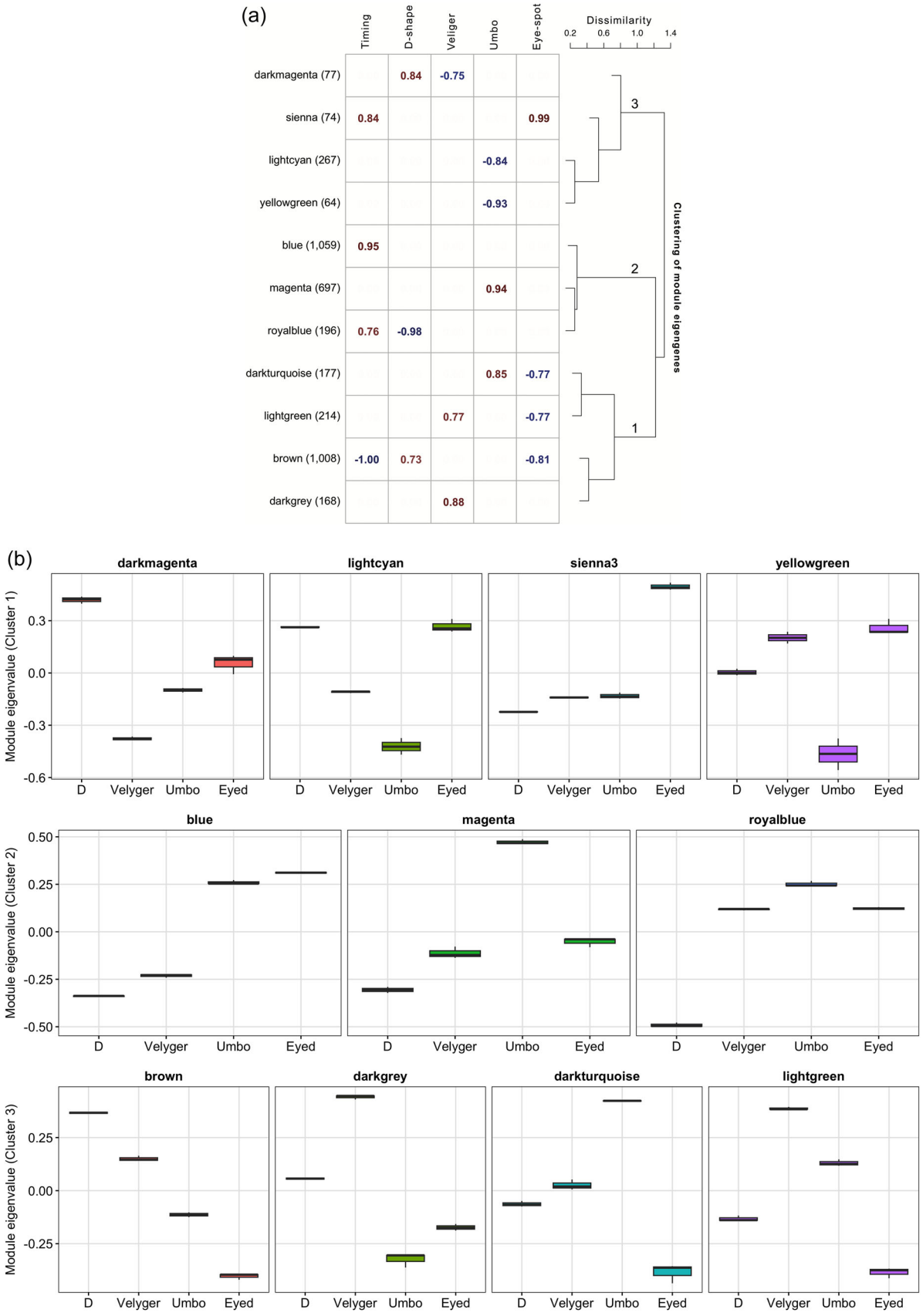

FIGURE 2 Genes network analysis. (a) Correlation matrix WGCNA. Stages are defined as discrete variable (0 or 1). Timing is a continuous variable (1–4) for each ordered *P. margaritifera* developmental stages (from D-shape to Eye-spot). Numbers on the tree (right) indicate cluster sections. (b) Module eigenvalue per module.

Similarly, the royalblue module, characterized by a distinctive peak from Veliger onward, also shows enrichment for primarily metabolism regulation, notably the carbohydrates catabolic process and lipid oxidation, and fatty acid alpha-oxidation. This module also encompasses regulation of immune system process, of defense response and cytokine production involved in inflammatory response and developmental pigmentation.

3.5 | Transition to metamorphosis (environment perception)

Modules within cluster 3 show relatively low levels of expression at Umbo stage but high levels at Eyed-stage, notably in the yellowgreen, lightcyan and sienna3 modules. The step from Umbo to Eyed-shape is also marked by activation of immune related functions, such as Toll signaling and B-cell mediated immunity or cytokines (Interferon- type I). Apoptosis related immune functions are also enriched in the sienna module through the TRAIL-activated signaling pathways.

Immune induction is accompanied by the development of nervous system related process, including activation of synaptic transmission and the G-protein coupled receptor signaling pathway, detection of light stimuli, or nerve development in the lightcyan module (Supplementary File S1). The yellowgreen module shows enrichment for tyrosine catabolic and serotonin biosynthetic processes. Similar observations are made in the sienna3 module, with enrichment for neurogenesis, midbrain and cerebellum development. The sienna3 module eigenvector seems to be driven by *Achaete-scute homolog 1* transcription factor, combined with expression of Tenascin and Neural cell adhesion molecule 1. *Achaete-scute* is involved in neurogenesis and serotonergic phenotypes (Simionato et al., 2008). Finally, the sienna3 module also includes a main driver of larval settlement in annelids, an ortholog of the sex peptide receptor (SPR) in *Drosophila* (Conzelmann et al., 2013).

4 | DISCUSSION

Larval stages transitions are critical periods, where several vital functions will be activated during morphogenesis, and represent a period of high selective pressure. Our study shows that each developmental transition encompasses largely stage specific genes, even if some main biological functions are recurrently solicited such as those implicated in energy metabolism. This is a conserved pattern observed for different marine bivalves (Li et al., 2016; Liu et al., 2015; Yurchenko et al., 2018; Zheng et al., 2019). Hence, exploring transitions provides a potential strategy for looking at the onset of specific physiological functions.

4.1 | Immune development

The first day of larval life are often decisive for survival and immune system establishment (Wang et al., 2018). Deciphering the initialization of immune competencies are relevant *for* species ecology and hatchery productions, where high densities favors disease emergence (Dubert et al., 2017). Early detection of MIF and other related genes, some peaking at early stages, confirms that innate immune system is present and activated early in the development (Rojas et al., 2021a; 2021b). Nonetheless, *regulation* of antigen recognition differed from earlier stages to spats, as observed in other marine bivalves (Balseiro et al., 2013), suggesting that competency for pathogens recognition (PRs) intervened later in the development. Prior antigen recognition activity might be possible in the earliest stages with the presence of actors from maternal origins (Balseiro et al., 2013), but this observation has not been confirmed for *P. margaritifera* in this study.

4.2 | Biomineralization related genes

Similarities in shells microstructures (i.e. aragonite and calcite) are observed between adults and larvae; hence, one would expect that larval development is key for deciphering the onset of biominerals deposit. Observation in *P. fucata* reported that biomineralization activity is detected as soon as Trochophore and D-stages larvae, but peak from Umbo onward (Li et al., 2020; Liu et al., 2015; Miyazaki et al., 2010). Supporting these observations, we found several biomineralization related genes with higher expression in latter stages, notably *perlucin* and *perlucin-like* (Blank et al., 2003; Le Luyer et al., 2019). These genes expression profiles suggest that a peak or higher upregulation of genes involved in mineral nucleation and shell growth occurs from the Umbo (Blank et al., 2003). Nevertheless, most of genes known to be involved in shell or pearl formation in adults were not or lowly expressed during early development in *P. margaritifera*. A possible reason for the larval biomineralization genes repertoire might be strikingly different from adults (Zhao et al., 2018). We indeed found biomineralization related genes highly expressed in earlier stages, with perfectly decreasing expression across larval development. These genes with chitin binding or Von Willebrand factor type A domains (vWFA) were not reported as genes with a major role in adults biomineralization (Le Luyer et al., 2019).

4.3 | Energy and signaling pathways leading to larval transition

Energy requirements increase during larval development and are key determinants of metamorphosis success. Eggs and larvae mainly rely on fatty acids (FAs) especially triacylglycerol reserves, during development (Labarta et al., 1999; Nascimento-Schulze et al., 2021). This high energy demand accompany several morphogenesis steps during development in larva, and are also coupled with the use of other energy resources, notably the pentose metabolic process, for initialization of shell formation (Liu et al., 2020).

Among the main molecular drivers of larval early transitions (D-shape to Veliger), we found the generation of Thyroid hormones (THs). Exogenous TH pathway is conserved in vertebrates and invertebrates (Heyland & Moroz, 2005) and endogenous synthesis of TH has already

been observed in animals devoid of dedicated morphological structure (Eales, 1997). In echinoderms, providing supplementary TH to starved larvae allows metamorphosis (Heyland et al., 2004); hence, exogenous TH is mandatory for the settlement success of this species. Here we found that endogenous production of THs decreases with successive developmental stages, yet, response to THs increases with a peak at Eye-spot stage. Conserved pathways of THs production and activation during development suggest that THs, as seen in other bivalves, is also a major contributor to larval stages transition in *P. margaritifera* (Huang et al., 2015). Further studies properly testing exogenous supplementation of TH and determining settlement yield would serve the applied sector. Subsequent transition from Veliger to later stages in *P. margaritifera*, as many other models, relies on the Wnt signaling pathways. Wnt signaling is involved in cell fate transition and embryonic development, which includes developmental transition in bivalves (Liu et al., 2015).

4.4 | Metamorphosis and perception of the environment

Perception of environment is fundamental for larvae survival and occurs in parallel with the development of the nervous system (Joyce & Vogeler, 2018). Neurogenesis in the Pacific oyster, *Crassostrea gigas*, starts even earlier in the trochophore stage a few hours after fertilization with the formation of the larval ganglionic nervous system in worms (Yurchenko et al., 2018). However, the nervous system in molluscs is rudimentary until relatively late larval stages 22 dpf for Eye-spot which coincides with their observation of fully visible cerebral and neural part (15 dpf) and is timed with our observation in *P. margaritifera* (Croll et al., 1997). At this stage, larval settlement (moving from a pelagic to a sessile lifestyle) is possible and will be orchestrated by neuroendocrine signals. Among those signals, serotonin is an important factor for settlement behavior and has been widely tested in production hatcheries, and has been shown to enhance settlement yield (Joyce & Vogeler, 2018). Specifically, suppression of the serotonin signals disrupts normal axon guidance and results in malformation of the nervous system (Yurchenko et al., 2018). The apical sensory organ (ASO) structure appearance on the pediveliger larvae is characteristic of pediveliger (Hadfield et al., 2000). A peak of expression of SRP, an ortholog myoinhibitory peptide (MIP)/allatostatin-B in annelids (Conzelmann et al., 2013), suggest that signals orchestration upon chemical cues detection is also conserved in molluscs.

5 | CONCLUSIONS

This study provides a detailed exploration of the main mechanisms involved in larval stages transitions in *P. margaritifera*. The identification of key drivers and their cross-talk allow for exploring conservation of signaling determining pathways for metamorphosis in molluscs, notably for specific physiological functions. Future studies looking at genetic background selection over specific vQTL, based on the functional identification, should help refine our knowledge of this species' early development.

AUTHORS CONTRIBUTIONS

C.-L. Ky and J. Le Luyer conceived the experiment. M. Sham Koua realized the larval production. M. Sham Koua and V. Quillien realized the sampling. T. Destanque, P. Auffrey, and J. Le Luyer analyzed the data. T. Destanque and J. Le Luyer drafted the paper. All the authors contributed to final revisions of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

These sequence data have been submitted to the EMBL Nucleotide Archive database under accession number: PRJEB60445 (ERP145507). Scripts have been deposited on Zenodo: DOI:10.5281/zenodo.7703206. The data that support the findings of this study are openly available in ENA at https://www.ebi.ac.uk/ena/browser/, reference number PRJEB60445.

ETHICS STATEMENT

The study was approved by the ethics committee of the French Polynesia's government.

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PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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