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Cells to Shells

Transcriptomics provides insight into *Mytilus galloprovincialis* (Mollusca: Bivalvia) mantle function and its role in biomineralisation

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ABSTRACT

The mantle is an organ common to all molluscs and is at the forefront of the biomineralisation process. The present study used the Mediterranean mussel (*Mytilus galloprovincialis*) as a model species to investigate the structural and functional role of the mantle in shell formation. The transcriptomes of three regions of the mantle edge (umbo to posterior edge) were sequenced using Illumina technology which yielded a total of 61,674,325 reads after adapter trimming and filtering. The raw reads assembled into 179,879 transcripts with an N50 value of 1086 bp. A total of 1363 transcripts (321, 223 and 816 in regions 1, 2 and 3, respectively) that differed in abundance in the three mantle regions were identified and putative function was assigned to 54% using BLAST sequence similarity searches (cut-off less than 1 e^{-10}). Morphological differences detected by histology of the three mantle regions was linked to functional heterogeneity by selecting the top five most abundant Pfam domains in the annotated 1363 differentially abundant transcripts across the three mantle regions. Calcium binding domains dominated region two (middle segment of the mantle edge). Candidate biomineralisation genes were mined and tested by qPCR. This revealed that Flp-like, a penicillin binding protein potentially involved in shell matrix maintenance of the Pacific oyster (*Crassostrea gigas*), had significantly higher expression in the posterior end of the mantle edge (region one). Our findings are intriguing as they indicate that the mantle edge appears to be a heterogeneous tissue, displaying structural and functional bias.

1. Introduction

Biomineralisation is a biologically controlled process through which living organisms generate mineralised structures (Wilbur and Saleuddin, 1983). The minerals are naturally occurring, of inorganic origin and possess a crystalline structure. Biomineralisation is a widespread and ancient process, occurring in both prokaryotes and eukaryotes, in plants and in animals (Yoshida et al., 2010; Knoll, 2003). The structures produced can have multiple functions, including tissue support, protection from the external environment, feeding and sensing (Miyamoto et al., 2013; Wilbur and Saleuddin, 1983). According to the pioneers of the field of biomineralisation, the process of shell mineralisation occurs in two main steps. First, ions are transported across an epithelium, proteins are then synthesized and secreted. Second, calcium carbonate ($CaCO_3$) crystals are deposited and grown concomitantly with an organic matrix which envelops the crystals (Wilbur and Saleuddin, 1983).

When one thinks of shells, in general one immediately thinks of sea shells; the shells of molluscs. Phylum Mollusca is a highly successful and species-rich group of organisms, yet much of their biology remains poorly understood despite their importance from an ecological and socio-economic perspective. Shellfish account for 22.8% of global aquaculture and mussels are the third most produced family of bivalves (The State of World Fisheries and Aquaculture, Food and Agriculture Organization of the United Nations, 2014). In addition, due to their sessile and filter-feeding qualities, mussels are also used in environmental monitoring programmes as biological indicators of pollution and other changes to the marine environment, such as ocean acidification (De Zwaan and Eertman, 1996; Fitzer et al., 2014; Dupont and Pörtner, 2013; Huning et al., 2013). Therefore, understanding the underlying mechanisms of biomineralisation and the effects of external stress are vital for the maintenance of food security (Cheung et al., 2010), ecological stability (Bijma et al., 2013) and a sustainable shellfish industry (Narita et al., 2012).

The *Mytilus* shell is composed of an organic matrix, two forms (aragonite and calcite) of $CaCO_3$ and an uncalcified periostracum that covers the whole structure (De Paula and Silveira, 2009). The organic matrix, which surrounds the calcite and aragonite crystals, is made up largely of polysaccharides (i.e. chitin) and glycoproteins (De Paula and Silveira, 2009). The organic matrix is synthesized in the mantle and secreted into the extrapallial cavity of the animal, where it becomes a template for the nucleation, orientation and growth of $CaCO_3$ crystals (Nudelman et al., 2006; Simkiss and Wilbur, 1989). This controlled

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biological process, whereby a hard structure is formed from the synthesis of organic molecules, is known as biomineralisation (Yoshida et al., 2010).

Bivalves generally uptake calcium (Ca^{2+}) and bicarbonate (HCO^{-}_{3}) from their environment and metabolize them to form the $CaCO_3$ that is found in the shell, however the exact mechanism and regulation of this process is still ambiguous. The mantle tissue appears to be important for shell formation; it is where proteins such as chitin and silk fibroin are produced and secreted to form the organic matrix (Freer et al., 2014; Marin et al., 2000, 2008; Miyamoto et al., 2013; Suzuki et al., 2009). This tissue has several known functions including sensory activities, secretory activities, shell growth and shell repair (Watabe, 1983), however the expression of genes associated with these functions has never been mapped to specific regions along the mantle edge. Gardner et al. (2011) used microarrays to identify clusters of transcripts in different regions of *Pinctada maxima*'s mantle and Jolly et al. (2004) localized zones where shell matrix proteins were expressed in the *Haliotis tuberculata* mantle. Nonetheless, despite the existence of several large mantle transcriptome datasets generated by next generation sequencing (NGS) (Artigaud et al., 2014; Clark et al., 2010; Craft et al., 2010; Freer et al., 2014; Philipp et al., 2012), none have contemplated the reported functional variations and consider the mantle edge as a homogeneous tissue.

The present study was designed to test the hypothesis, the mantle edge of the Mediterranean mussel, *M. galloprovincialis*, is a functionally heterogeneous tissue from the posterior tip to the umbo. *M. galloprovincialis* was chosen as the model for the study as it has two symmetrical valves of the multi-layered $CaCO_3$ shell and is readily available in Southern Europe. We present, for the first time, RNA sequencing of three distinct regions of the mantle edge (posterior, middle region, and umbo) of *M. galloprovincialis*. The data from the present study will be a resource for future studies in which the goal is to characterize regulatory mechanisms involved in the control of shell growth/turnover. Functional heterogeneity was inferred from 1) presence/absence of candidate biomineralisation genes in the mantle regions and 2) histological analysis of the mantle edge.

2. Materials and methods

2.1. Sample preparation

M. galloprovincialis specimens (n = 4) were sampled from the Ria Formosa in Faro, Portugal on July 23, 2014. The samples were removed by hand from bridge pillars at low tide (37°0′22″N 7°58′3″W). The mussels were transported live to the Centre of Marine Sciences (CCMAR), University of the Algarve, anaesthetized using magnesium chloride (10%) and the mantle tissue dissected out. Tissue samples were taken within one hour of mussel collection. Individuals chosen for RNA extraction or histology were females 4.5 cm (±0.1 cm) in length from umbo to posterior edge. The mantle was sampled from the left valve for every individual, cut into three regions (posterior, middle, and umbo; i.e. region one, region two, and region three, respectively; Fig. 1) and placed into lysis buffer. A small portion of each of the mantle regions collected for RNA extraction was also fixed for histology.

2.2. Histological procedures

The three regions of dissected mantle edge were fixed overnight (12 h) with gentle agitation in 4% paraformaldehyde (PFA) at 4 °C. After fixation, the tissue was rinsed in water and then dehydrated through a graded ethanol series (70% to 100%), saturated in xylene and impregnated and embedded in paraffin wax (Merck, Germany). Serial transverse sections (5 μm) of the mantle edge were mounted on poly-L-lysine coated glass slides. The sections were dried overnight at 37 °C, cooled to room temperature and stained. In order to visualize possible secretory cells, histological staining was applied on dewaxed and rehydrated tissue using periodic acid-Schiff (PAS) to stain neutral muco-polysaccharides. After staining, mantle sections were rapidly dehydrated through a graded series of alcohols, cleared in xylene and mounted in Distyrene Plasticizer Xylene (DPX) (BioChemika, Sigma-Aldrich, Madrid, Spain). Stained sections were then analysed using a microscope (Leica DM2000) connected to a digital camera (Leica DFC480) and linked to a computer for digital image analysis.

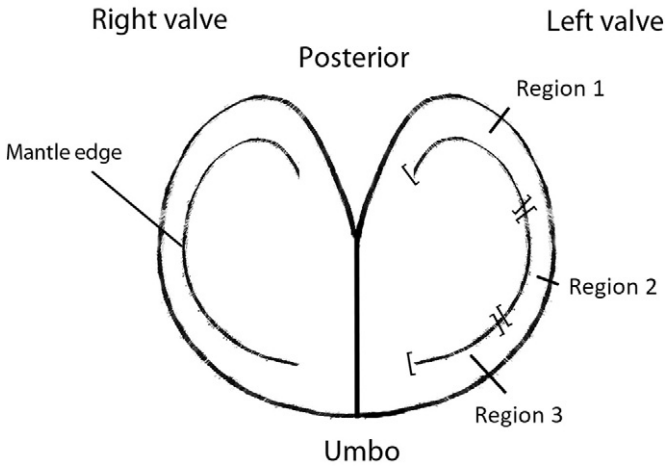

Fig. 1. Schematic diagram of an open mussel, showing how the mantle edge was divided into the 3 regions analysed in this study. The mantle edge was first dissected out (approximately 2 mm in width and approximately 3 cm in length) and then cut into three parts according to the coloration of the tissue from dark to light (region one was all dark, region three was all light and region two was a transition between light and dark coloration). Each region was approximately 1 cm in length.

2.3. RNA extraction and cDNA library preparation

Samples of mantle were homogenized using an Ultra-Turrax homogenizer (IKA) immediately after collection and RNA was extracted using a Maxwell 16 total RNA purification kit (Promega, Madrid, Spain) following the manufacturer's instructions. RNA was precipitated with ethanol and quantified using a Nanodrop (1000 Spectrophotometer, Thermo Fisher Scientific, USA). RNA samples were run on a 0.8% agarose gel for quality assessment and were treated with 1 U DNAse (DNA-free Kit, Ambion, UK) using the Turbo DNA-free kit (Ambion, UK) and quantified again using a Nanodrop.

Region specific mantle cDNA libraries were made from pools of RNA extracted from 4 individuals. Library preparation and sequencing was conducted by the Shanghai Oceans University Sequencing Service using Illumina TruSeq stranded mRNA-seq library Prep kit (RNA input 2 μg, insert size of 300–400 bps). Sequencing was carried out on an Illumina Hi-Seq 1500 and 100 base paired-end reads were generated.

2.4. Bioinformatics analysis

Adapter trimming of the reads was conducted using Trimmomatic v. 0.33 (Bolger et al., 2014) using default parameters and quality and length trimming of the reads was conducted using Fastq-mcf v. 1.04.636 (Phred score quality of 30, minimum read length 80 bp). Contaminating reads were identified by mapping the raw sequencing reads to the relevant genome (*Dicentrarchus labrax*) using TopHat v. 2.0.13 (Kim et al., 2013) with the default parameters, and removed. The cleaned sequencing reads were then *de novo* assembled using Trinity v. 2.0.6 (Grabherr et al., 2011) with default parameters.

Abundance estimates of reads were calculated using RSEM (RNA-Seq by Expectation–Maximization) v. 1.2.20 (Li and Dewey, 2011) and differentially expressed transcripts were identified using edgeR v. 3.1 (Robinson et al., 2010) with a p-value of 0.001 and a C fold change of 2. Sequence similarity and annotation of transcripts

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