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Transcriptome Dynamics of an Oyster Larval Response to a Conspecific Cue-Mediated Settlement Induction in the Pacific Oyster *Crassostrea gigas*

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Abstract: The molecular mechanisms underlying the conspecific cue-mediated larval settlement in *Crassostrea gigas* is not yet fully understood. In this study, we described and compared the transcriptomes of competent pediveligers (Pedi) and conspecific cue-induced postlarvae (PL). A total of 2383 candidate transcripts were identified: 740 upregulated and 1643 downregulated transcripts, after settlement. Gene Ontology analysis revealed active chitin binding, calcium ion binding, and extracellular region processes in both stages. Results showed that the differential expression trend of six candidate transcripts were consistent between the quantitative real-time PCR and transcriptome data. The differential transcript expression related to shell formation showed closely linked dynamics with a gene regulatory network that may involve the interplay of various hormone receptors, neurotransmitters, and neuropeptide receptors working together in a concerted way in the Pedi and PL stages. Our results highlight the transcriptome dynamics underlying the settlement of oysters on conspecific adult shells and demonstrate the potential use of this cue as an attractant for wild and hatchery-grown oyster larval attachment on artificial substrates. It also suggests the possible involvement of an ecdysone signal pathway that may be linked to a neuroendocrine-biomineralization crosstalk in *C. gigas* settlement.

Keywords: chemical cues; gregarious behavior; neuroendocrine; biomineralization; marine invertebrate physiology; RNA-seq

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1. Introduction

The Pacific oyster *Crassostrea gigas* is a benthic bivalve mollusk with a biphasic life cycle. It is one of the most well-studied organisms due to its biology and aquaculture importance [1,2]. The biphasic life cycle of *C. gigas* is composed of a planktonic larval stage for dispersion and a sessile adult stage, with metamorphosis marking the transition between the two life stages in a relatively short time (generally less than 48 h) [3–5]. As oyster larvae reach the pediveliger stage, they begin a series of swimming motions by sinking to the bottom and extending their foot in search of a suitable surface to settle on [6,7]. Competent pediveliger larvae manifest such behaviors. When environmental cues meet the requirements for a suitable settlement surface, larvae undergo irreversible metamorphic changes that include loss of the velum,

foot, and eyespot, development of gills, and the production of adult shells [8]. In addition, the metamorphic transition often involves morphological, physiological, structural, and functional changes that are governed by a gene regulatory network [9].

A variety of chemical cues from the natural environment have been implicated to play a role in the induction of larval settlement in many marine invertebrate species. These cues may come from microbial or bacterial biofilms [10–16], macroalgal hosts [17,18], and conspecifics [19–24]. Of the chemical cues reported from conspecifics, surface-bound cues from various oyster shells have been reported to induce larval settlement in *C. gigas* [25–27], *C. virginica* [20,28], and *C. ariakensis* [29]. These studies show that chemical cues from conspecifics may play a role in the gregarious behavior exhibited by oyster larvae upon settlement [20,25–29]. However, the exact nature of these cues is not yet fully understood and characterized. Understanding the effects of these cues on the larvae can help clarify the larval settlement mechanism of oysters, and these compounds may find application in the development of oyster aquaculture.

Several studies have also explored possible mechanisms underlying larval settlement in oysters, which include the involvement of hormones, neurotransmitters, genes, and signaling pathways [4,8,11,30–36]. The first coherent theory for settlement and metamorphosis was made by Bonar et al. [4], in which two independent nervous-system-related pathways were necessary for oyster species: first, the dopaminergic pathway, which governs settlement behavior and attachment to surfaces. This often involves larval exposure to L-DOPA, epinephrine, norepinephrine, and dopamine compounds. It is then followed by a second subsequent step, the adrenergic pathway, which regulates metamorphosis. However, they have also reported that exposure to norepinephrine or epinephrine led the oyster larvae to metamorphose directly without attaching to any surface, thereby bypassing the dopaminergic pathway. This theory was later supported by various pharmacological studies [8,11,37,38]. However, there is a lack of molecular evidence to connect these findings to actual pathways.

Recent advances in sequencing technology have paved the way for us to further explore a wider and deeper view of changes in gene expression patterns and signal pathways involved in the various larval development stages [32,39,40]. Transcriptome analysis is one of the useful technologies that may benefit us in gaining a comprehensive view of animal development and growth [30,32,41–43]. There are a growing number of molecular studies implicating the involvement of nuclear receptors and the ecdysone signaling pathway in oyster settlement [30,33,36,44]. Using the available genome and transcriptome databases, Vogeler et al. [8] have shown that the ecdysone receptor (EcR), as well as other EcR-cascade-related transcription factors, are present in *C. gigas*. They have also demonstrated that *C. gigas* EcR is highly expressed in the eyed pediveliger just before metamorphosis, suggesting its potential role in the induction of metamorphosis in bivalves. Despite this notable advance in our understanding of the gene regulatory network involved in oyster larval settlement, most of these studies use pharmacological inducing compounds, such as epinephrine, as reported in *C. gigas* [33] and *C. angulata* [31], as well as biofilm inducing cues for *C. gigas* [5]. Moreover, oysters are observed to attach and grow on top of each other's shells and build oyster reef-like structures in the natural environment. Most oyster farms still use scallops or oyster shells as settlement substrates for oyster culture. These shells are often put in cages for raft or long-line culture. Mechanisms that facilitate gregarious larval settlement under this condition are still fragmentary, especially regarding their molecular underpinnings. Previous studies by Vasquez et al. [25,26] and Sedanza et al. [27] have shown that a protein extract from the shells of conspecifics could induce larval attachment and its subsequent metamorphosis in *C. gigas* competent pediveligers. Currently, we are continuing to identify the exact nature of this conspecific cue from shells of *C. gigas* [27]. To date, there has been no available report yet that explores the effect, at a molecular level, of the exposure of competent oyster larvae to a natural settlement-inducing cue from conspecifics, such as those protein extracts found in shells, that may play a role in mediating settlement. Hence, our aim in this study is to fill in some gaps in our knowledge on the underlying specific

cellular and physiological processes involved in the development of marine invertebrates, specifically on *C. gigas*, with the aid of the 'omics' approach, such as transcriptomics. In the present study, we applied DNBseq-G400RS high-throughput sequencing technology to evaluate the effect of settlement induction by a conspecific cue on *C. gigas* larvae, before and after its recognition, and compare the transcriptomes of the pediveliger and conspecific cue-induced postlarvae. We also carried out gene function annotation, Gene Ontology enrichment, and real-time PCR verification analyses. Moreover, we identified key genes and molecular events involved in this process and proposed the possible involvement of a neuroendocrine-biomineralization crosstalk in *C. gigas* larval settlement.

2. Materials and Methods

2.1. Larval Culture and Shell Extract Preparation

Artificial fertilization and larval culture were conducted as previously described by Sedanza et al. [27], where two male and five female adult oysters were used as parents to create a high genetic diversity. Stripped gametes from a combination of several parents can avoid genetic diversity being too low [5]. In brief, gonads were stripped from all the adult oysters to collect gametes [45]. Eggs and sperm were separately suspended in 2 L glass beakers containing GF/C (Whatman glass fiber filter; pore size: 1.2 mm) filtered seawater (FSW) adjusted to 27 °C. Eggs were washed several times with FSW through repeated decantation and were then fertilized with a small volume of sperm suspension. Thirty minutes after artificial fertilization, fertilized eggs were collected in a 20 µm net, washed four to five times with FSW, and re-suspended in 2 L glass beakers containing FSW. Fertilized eggs were kept at 27 ± 1 °C in an incubator for 24 h. Then, the swimming straight-hinged larvae were collected in a 40 µm mesh, gently washed with FSW, and stocked in four 30 L tanks filled with 10 L FSW, at a density of 5 larvae mL^{-1}. The *C. gigas* larvae were cultured in these tanks under dark conditions with constant aeration. The larvae were fed with *Chaetoceros calcitrans* (10,000–50,000 cells/mL/day) from day 1 to day 5, fed a combination of *C. calcitrans* (25,000 cells/mL/day) and *Chaetoceros gracilis* (25,000 cells/mL/day) from day 6 to day 10, and were then fed *C. gracilis* (50,000 cells/mL/day) from day 11 onward during the culture period. Water quality parameters during the larval culture period were: salinity (30–32 psu), pH (8.02–8.08), and temperature (25–27 °C). The seawater was renewed daily prior to feeding. Small larvae and empty shells that passed through 100 and 200 µm mesh screens in the 1st week and 2nd weeks of culture, respectively, were removed so as to obtain individuals that were of uniform size until they reached a competent stage. Competent larvae reached the eyed pediveliger stage on the 16th to 20th day of culture, when they reached 270 µm [46,47], which was consistent with growth in hatcheries in Japan. Competent larvae were used for settlement experiments 24 days after the start of culture when they reached an average shell height of 322 µm [27]. Competence is defined as the ability to metamorphose following exposure of larvae to a demonstrated natural inducer [48]. All the larvae were actively swimming prior to conducting the settlement assay.

The preparation of the *C. gigas* crude shell EDTA extract (CgSE) was carried out following the method outlined by Sedanza et al. [27]. In brief, clean and dried *C. gigas* shells were crushed with a hammer until shell chips of about 0.5 to 1.0 mm in size were collected. These shell chips (150 g) were decalcified with 1 L of 0.8 M ethylenediaminetetraacetic acid (EDTA, pH 8.0) for 60 h at 4 °C with continuous agitation. The supernatant was collected by centrifugation at 10,000× *g* for 60 min at 4 °C. Then, it was subsequently filtered and dialyzed against distilled water at 4 °C for 3 days. CgSE was lyophilized and the resultant powder was dissolved in as small a volume as possible of distilled water and stored at −20 °C until further use.

2.2. Sample Collection and Research Design

The research design workflow is shown in Figure 1. Our study was framed to evaluate the effect on larval settlement induction when a natural settlement-inducing cue is present,

such as those from the shells of conspecifics (CgSE). Under laboratory conditions, our previous study showed that, in the absence of this conspecific cue, no larvae were induced to settle when immersed in 0.22 µm filtered seawater (FSW) [27]. Whereas when it is present, we observed that more than 80% of the larvae were induced to attach to the substrate and metamorphose after 24 h incubation [27]. Thus, after 24 h, we could observe cement substances surrounding one side of the oyster shell attached to the substrate and new shell growth in the postlarvae [27]. Results in this previous study were consistent with those reported by Coon et al. [48]. They have determined the temporal relationship between the onset of behavioral and morphogenetic competence in *C. gigas* using a neuroactive compound and have demonstrated that, in the absence of chemical stimulation, cultured oyster larvae were able to delay metamorphosis and maintain competence for at least 30 days [48]. In addition, no metamorphosed *C. gigas* larvae were recorded in the absence of chemical inducers in the study of Beiras and Widdows [37]. Hence, in this present study, the transcriptome profiles were compared between the larval batches (a) before exposure to the presence of the conspecific cue, which means they were not exposed to a chemical stimulant and, as such, hereon designated as the control and as pediveliger (Pedi), and (b) settled individuals after exposure to the conspecific cue, CgSE, hereon called postlarvae (PL). Therefore it was necessary for us to have an equal start by using larval batches of the same age. Hence, the Pedi sample group was collected for RNA-seq analysis on the 24th DOC, while the PL sample group was exposed to CgSE at 24 DOC and collected after 24 h. In this study, it was not possible to carry out sampling at subsequent time points, which would have been an interesting approach, as it was hard to collect transitioning samples in real time because oyster settlement processes are quick and usually nonsynchronous. Hence, we only collected PL samples after 24 h exposure.

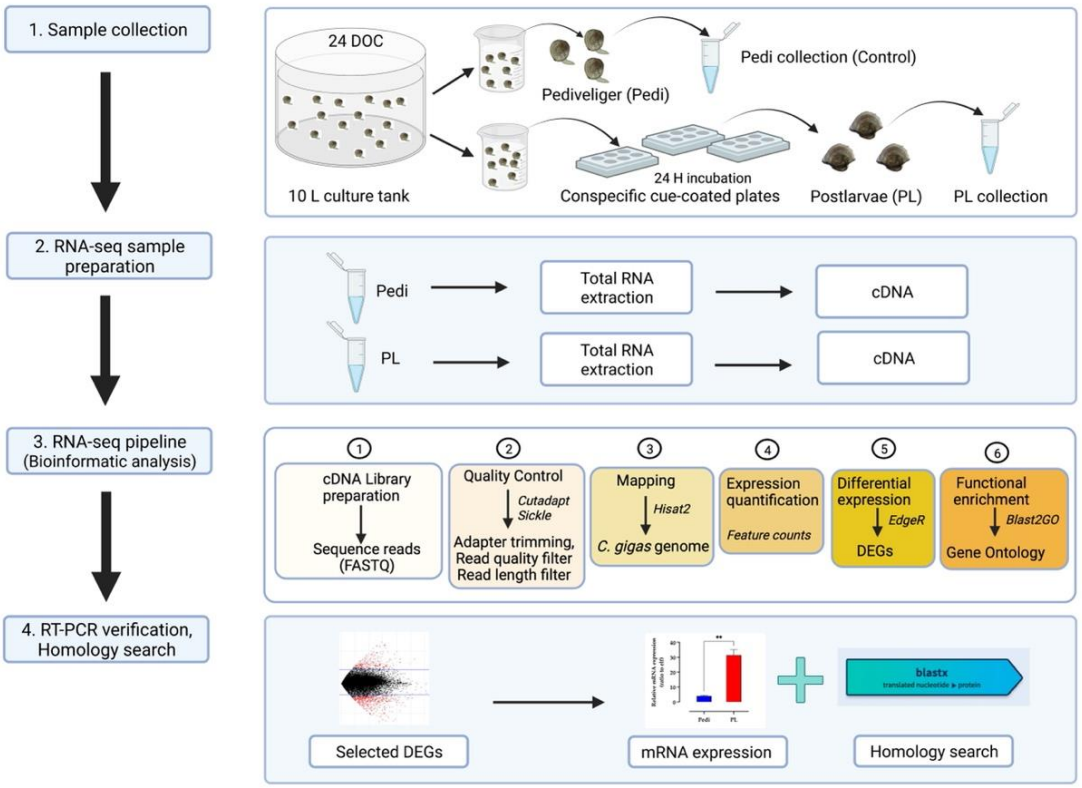

Figure 1. Research design workflow. This figure was created using BioRender.com.

Our method design was as follows: On the day of the assay, all larvae were not fed. These larvae were at 24 days post-fertilization (or 24 days of culture, DOC) when approximately 70–80% have developed an eyespot and started to crawl. To ensure that

all the larvae were at a competent stage, larvae from multiple 10-L FSW-filled tanks were sieved over a 300 µm mesh-covered tip nestled on a glass bowl filled with FSW. They were carefully washed in Millipore FSW (0.22 µm) and transferred to several 3-L beaker containers newly filled with FSW. After filtration, more than 95% of the larvae were verified under the microscope to have reached the pediveliger stage. The larval samples were then divided into two batches. Beakers containing the control batch of competent pediveliger larvae used for RNA-seq analysis were siphoned over a 300 µm mesh-covered tip nestled on a glass bowl filled with FSW, carefully washed with distilled water, and collected as a pooled Pedi sample. Approximately 100,000 individuals of pooled pediveliger larvae (183 mg wet weight) in a microcentrifuge tube were treated immediately with RNAlater Stabilization Solution (Invitrogen, Vilnius, Lithuania) for 24 h at 4 °C following the manufacturer's protocol. After which, RNAlater was removed and the pooled Pedi batch was stored at −80 °C prior to the subsequent experiments. For the PL batch, the method previously described by Sedanza et al. [27] was adopted for the induction of oyster larvae with CgSE. Briefly, 6-well plates were pre-coated with 50 µg CgSE and subsequently filled with 10 mL of filtered seawater per well. Twenty larvae were released in each well and induced to settle on the same day as the collection of the Pedi larvae, i.e., at 24 DOC. In this study, we define settlement as the sequential transition of a competent pediveliger larva to a postlarva, which includes the attachment to a substrate and then eventual metamorphosis.

Postlarvae were confirmed under the microscope as individuals that secreted cement substances or those with postlarval shell growth. We observed about 80% settlement success after induction. The remaining larvae that were not induced to settle were found actively swimming after 24 h. No mortality was observed. All postlarvae that metamorphosed after 24 h were carefully removed from the 6-well plate bases. After removal of the postlarvae from each well, they were pipetted out to a 300 µm mesh-covered tip nestled on a glass bowl filled with FSW, washed gently several times with distilled water to remove any traces of the protein extract, and were transferred to a clean microcentrifuge tube. After the removal of the excess liquid on the tube, the postlarvae were immediately treated with RNAlater. This whole process was repeated several times until all the samples were collected. After 24 h, a similar procedure to that of the Pedi batch was carried out, wherein all the postlarvae were pooled and RNAlater was removed. Approximately 3000 individuals of postlarvae, equivalent to about 100 mg of pooled sample, were stored at −80 °C until further use.

2.3. RNA Extraction, cDNA Library Preparation, Sequencing, and Bioinformatic Analysis

Total RNA extraction was performed by treating one hundred milligrams of each pediveliger (Pedi) and postlarvae (PL) samples with ISOGEN II (Nippongene, Tokyo, Japan) following the manufacturer's instructions. A 100 mg sample of each pooled Pedi and PL batches contained approximately 50,000 and 3000 individuals, respectively. Total RNA concentration was measured using a Quantus Fluorometer and QuantiFluor RNA system (Promega, Madison, WI, USA). The quality was then confirmed using the 5200 Fragment Analyzer System and the Agilent HS RNA kit (Agilent Technologies, Santa Clara, CA, USA).

The cDNA library was prepared using the MGIEasy RNA Directional Library Prep Set (MGI Tech Co., Ltd., Shenzhen, China) following the instruction manual. The Qubit 3.0 Fluorometer and dsDNA HS Assay kit (Thermo Scientific, Waltham, MA, USA) were used to measure the concentration of the prepared library solution. The Fragment Analyzer and dsDNA Reagent 915 kit (Advanced Analytical Technologies, Ames, IA, USA) were used to check the quality of the prepared libraries. The DNA-depleted and purified RNA was used to construct the single-stranded circular DNA library using the MGIEasy Circulation kit (MGI Tech Co., Ltd., Shenzhen, China) to generate the DNA nanoball (DNB)-based libraries. DNB libraries were sequenced and analyzed using the DNBSEQ-G400RS High-throughput sequencing kit and platform (MGI Tech Co., Ltd., Shenzhen, China), with a set 2 × 100 bp condition.

Adapter sequences were removed using cutadapt (version 1.9.1) (Martin, M. [49], Dortmund, Germany); then, sickle (ver 1.33) [50], was used to remove bases with a quality

score of less than 20 and paired reads with less than 40 bases. Cleaned and high-quality sequencing reads were mapped to the *C. gigas* genome (https://www.ncbi.nlm.nih.gov/assembly/GCF_902806645.1/, accessed on 25 February 2022) with Hisat2 (version 2.2.1, Pertea et al. [51], Maryland, MD, USA). Featurecounts (version 2.0.0, Liao et al. [52], Melbourne, Australia) was used to count the reads that mapped to the gene regions of the reference sequence. EdgeR [53] was used to perform differential analysis of sequence read count data using the R software [54]. EdgeR uses the quantile-adjusted conditional maximum likelihood (qCML) method for experiments with a single factor [53]; qCML is the most reliable in terms of bias on a wide range of conditions and, specifically, performs best in the situation of many small samples with a common dispersion, the model which is applicable to the Next-Gen sequencing data [53]. Transcripts with more than 2 M count-per-million (CPM) were considered expressed and were used for further analysis. A trimmed mean of M-values (TMM) was used to determine the scale factors in normalizing the library sizes and produce an effective library size that minimizes the log-fold changes between the samples. To test for differentially expressed genes (DEGs), a dispersion value for the biological coefficient of variation (BCV or square-root dispersion) was set at 0.25 [53]. Likewise, the threshold values of the false discovery rate (FDR) < 0.01, the absolute value of the log-fold of change $|\log2\ \text{Ratio}| \geq 2$ for DEGs analysis, and $|\log2\ \text{Ratio}| > 1.5$ for Gene Ontology enrichment analysis were set as conditions to determine the upregulated and downregulated transcripts. These sets of selected transcripts from each batch, i.e., Pedi and PL, were then subjected to Gene Ontology (GO) enrichment analysis using Blast2GO [55]. Fischer's exact test was used to evaluate the significance of enrichment levels of DEGs under each GO term. Multiple testing p-values were adjusted based on the Benjamini and Hochberg method (adjusted $p < 0.01$) [56]. In this study, DEGs were identified at the transcript level [57].

The top thirty transcripts, and those that were significantly enriched after GO analysis and were annotated as uncharacterized were further subjected to a homology search and used as a query to search the non-redundant protein databases available at the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi/, accessed on 17 April 2022) using the BlastX algorithm with an E-value $\leq 10^{-3}$ [58].

2.4. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The total RNA used in this experiment was reverse-transcribed following the method of Gao et al. [59]. In brief, all RNA samples were measured for quantity and quality by a NanoDrop ND-1000 spectrometer (Thermo Fisher Scientific, Wilmington, DE, USA). The cDNA was synthesized by treating the total RNA sample (500 ng) with oligo-dT primer CDS-BR (5-GGCCACGCGTCGACTAGTAC(T)16–3′), random primer (5-NNNNNNNNN-3′), and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Six candidate transcripts were chosen for real-time PCR verification to evaluate the transcriptome sequence integrity and expression trends indicated by RNA-seq. Selected differentially expressed transcripts were those that showed a log-fold of change (logFC) ≥ 2 and FDR < 0.01 and those that were significantly associated with GO-enriched categories. We designed gene-specific primers for real-time PCR by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 17 March 2022) with default settings, except for PCR product size (100–150 bp). The list of the genes and their respective primers is found in Table 1.

The qRT-PCR assay was conducted using the Power SYBR Green PCR Master Mix and a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Rockford, IL, USA) following the manufacturer's instructions. Thermocycling was performed as follows: enzyme activation for 10 min at 95 °C, denature step for 15 s at 95 °C, and annealing and extension for 1 min at 60 °C for 40 cycles. All data were normalized with the *C. gigas elf* gene as an internal reference. The mRNA expression levels were quantified by the standard curve method, and the relative mRNA expression levels were calculated by the ratio to the *elf* reference gene. All data were presented as the mean ± standard deviation (SD). This experiment was conducted in triplicate. The comparison of the relative

mRNA expression levels between pediveliger and postlarvae samples of each DEG was analyzed using a Student's t-test and was determined using the GraphPad 9 software program (GraphPad Prism, Software Inc., San Diego, CA, USA). The figure generated in the qPCR assay was also performed using the same software program.

Table 1. Primers of selected genes used for qPCR verification.

Accession No.	Annotation	Primer Sequence (5′−3′)	Product Size	Reference
LOC105332577	Chit1-SP	AGGAAATGGGAACAGAAGTTAG	139	-
	Chit1-AP	AGGACGGTTTGTCAATGGGAGG		
LOC105327998	Calm A-SP	GAGTATTCCGTGTTTCCAATG	150	-
	Calm A-AP	TCTGTTAGCTGTTCTGCCATGT		
LOC105335791	A-A4-SP	ATTATACAGGTCTTGGGAAAC	155	-
	A-A4-AP	GCAGGGGGTCTCATACAA		
LOC105331104	CA1-XII-SP	GACGAAATACGGGCACTCAAAG	145	-
	CA1-XII-AP	TTGTCTCCAATGTGACTC		
LOC105327922	VWC2-SP	TTGCTGTCCAGTGTGTCCTAAC	114	-
	VWC2-AP	CCGAAATGTGAACAATGAC		
LOC105335286	Wif-1-SP	CGAATGAACGACCACTGCAACAAG'	133	Huan et al. [5]
	Wif-1-AP	TGTCTGTCTGTCCAGGCTGTAGGC		
LOC105338957	Elf-SP	CGAGAAGGAAGCTGCTGAGATGG	208	Huan et al. [5]
	Elf-AP	ACAGTCAGCCTGTGAAGTTCCTGTA		

Abbreviations: Chit1—putative chitinase 1; Calm A—calmodulin A; A-A4—Annexin A4, CA1-XII—Collagen alpha 1 (XII) chain; VWC2L—von Willebrand factor C domain-containing protein 2-like; Wif-1—Wnt inhibitory factor 1/EGF-like domain-containing protein 2 (primers used were as reported by Huan et al. [5]); *elf*—elongation factor 1 (as reference gene, primers used were as reported by Huan et al. [5]).

3. Results

3.1. Transcriptome Sequencing and Mapping

In this study, the cDNA pool of *C. gigas* pediveliger (Pedi) and postlarvae (PL) samples were subjected to RNA-seq analysis and were compared to obtain a comprehensive view of the transcriptome profile upon larval settlement induction. The statistical information of the sequencing and mapping results is given in Table 2. The average number of pair reads from each library was 22,987,478 and the average number of high-quality pair reads was 22,495,268. The average total mapping rate of the libraries at Q20 and Q30 values were 97.1% and 89.4%, respectively. There was also a high percentage of the cleaned and filtered number of reads in both samples that were successfully mapped to the *C. gigas* reference genome (Table 2). The results showed that the RNA-seq data could be used for further analysis.

Table 2. Statistics summary of the Pacific oyster, *Crassostrea gigas*, transcriptome.

Sample	Sequencing Results			Quality Filter Results	Summary of Mapping Results		
	No. of Pair Reads	>=Q20 (%)	>=Q30 (%)	High-Quality Pair Reads	No. of Reads Not Mapped to the Reference Array	No. of Reads Mapped to the Reference Array (One Site)	No. of Reads Mapped to the Reference Array (Multiple Sites)
Pediveliger (Pedi)	23,141,856	97.0	89.0	22,628,121	8,184,886 (36.17%)	13,473,074 (59.54%)	970,161 (4.29%)
Post larvae (PL)	22,833,100	97.2	89.7	22,362,414	8,126,324 (36.34%)	13,440,437 (60.10%)	795,653 (3.56%)
Average	22,987,478	97.1	89.4	22,495,268	8,155,605 (36.25%)	13,456,756 (59.82%)	882,907 (3.93%)

Different normalization models were also used to find the effective library size in analyzing the differentially expressed transcripts in the transcriptome data. The supplemental Figure S1 file shows in the histogram profile that the average log CPM of each transcript was four CPM. The trimmed mean of M-values (TMM) in this figure shows the best normalization model fit for the downstream analysis of differentially expressed

transcripts in this experiment. The raw reads of the RNA-seq data are openly available in the public domain NCBI Sequence Read Archive, BioProject ID: PRJNA841258 (https://www.ncbi.nlm.nih.gov/sra/PRJNA841258, accessed on 22 May 2022).

3.2. Identification and Analysis of the Differentially Expressed Genes (DEGs) in the Pediveliger (Pedi) and Postlarvae (PL)

Post quality control analysis yielded 39,108 transcripts. We identified the transcripts with differential expression in the Pedi and PL batches by setting a threshold filtering value of $|\log2\ \text{Ratio}| \geq 2$ and Benjamini–Hochberg false discovery rate correction (FDR) < 0.01 after TMM normalization. Our transcriptome results showed a total of 2383 transcripts were differentially expressed between pre-settlement pediveliger larvae (Pedi) and postlarvae (PL). There were 740 upregulated and 1643 downregulated transcripts after settlement, while the rest of the 36,725 transcripts were found common in Pedi and PL batches. The full list of transcripts tested for differential expression is attached in Supplemental Table S1, which we expect to provide support for related research. The MA plot and heat map profiles of the transcripts with differential expression are found in Figure 2. A partial list showing the top thirty transcripts with differential expression in the Pedi and PL batches is shown in Table 3. A blast homology search was performed on some of the uncharacterized genes in this list and its full annotation is provided in Supplemental Table S2.

3.2.1. Pre-Settlement Pediveliger Larvae

Some transcripts found in the top thirty list were upregulated in the pediveliger larvae but were found downregulated after settlement code for a variety of functions: immunity and stress response (ADAM-1, CD283, Acanthoscurrin-2, 2 IgGFc-binding genes, LRRC4C); nervous system remodeling (1 SCO-spondin, 3 SCO-spondin-like genes); structural protein/cell adhesion (Coadhesin, Stereocilin); signal regulation (CPK7, LGI2), and an uncharacterized LOC105322623 gene with unknown function. Another interesting set of differentially expressed transcripts that were downregulated after settlement, which were not within the top thirty list, code for the regulation of ciliary motility (dynein regulatory complex subunit 5 (LOC105344378)), ion transport (transient receptor potential cation channel subfamily M member-like 2 (LOC105348393)), as well as cell signaling related receptors: octopamine receptor b-2R (LC105343310), leucine-rich repeat-containing G-protein coupled receptor 4 (LOC105325936), nuclear receptor subfamily 2 stage E member 1 (LOC105336095), neuronal acetylcholine receptors (LOC105331790, LOC105117683432, LOC105335119), FMRFamide receptor (LOC105331946), and ecdysone receptor (LOC105320474).

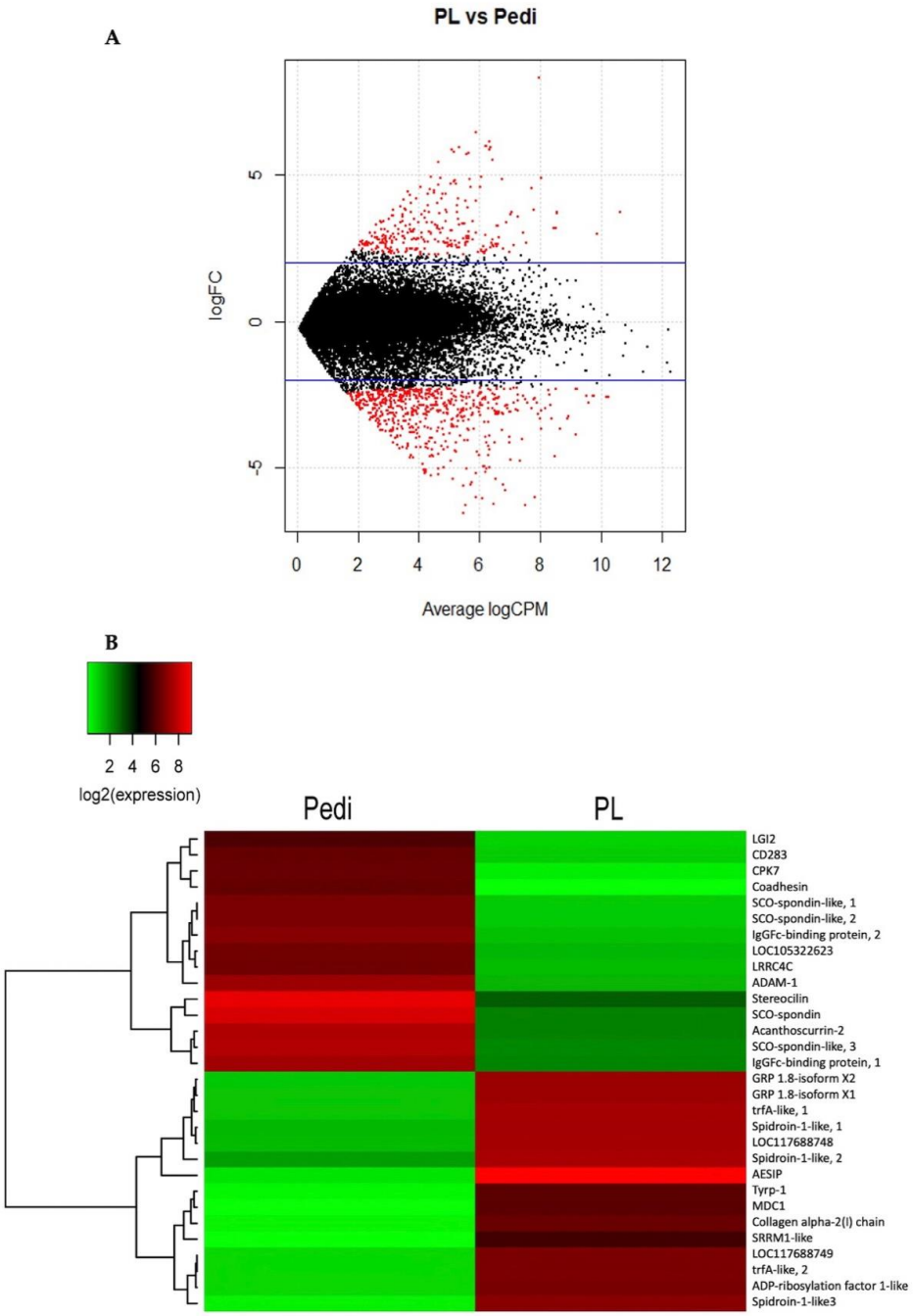

Figure 2. MA plot of transcripts with differential expression in *Crassostrea gigas* after settlement induction by a conspecific cue (**A**) and heat map expression pattern of the top thirty transcripts with differential expression (**B**). (**A**) Blue lines indicate transcripts that were filtered with a threshold value set at FDR < 0.01. The red dots indicate those transcripts that were considered as differentially expressed and met the two filtration threshold criteria: log-fold of change (logFC) $\geq$ 2 and FDR < 0.01. Red dots shown with a positive and negative logFC denote transcripts that were upregulated and downregulated, respectively, after settlement induction. Abbreviation: Average log CPM = log count-per-million; (**B**) The list of the top thirty transcripts with differential expressions were those that showed the highest logFC and lowest FDR values. The color intensity from green to red indicates low to highest logFC of expression in Pedi and PL batches. Transcripts with similar expression levels were clustered as shown in the tree on the left of Figure 2. The unabbreviated names of the transcripts in this heat map are provided in Table 3.

Table 3. Top thirty transcripts with differential expression between pre-settlement pediveliger larvae (Pedi) and postlarvae (PL) in Pacific oyster, *C. gigas*.

Accession No. [a]	Annotation	Reference Species	Evalue [b]	logFC [c]	FDR [d]
XP_011437564.2	Abscisic acid and environmental stress-inducible protein (AESIP)	*Crassostrea gigas*	0	9	1.05×10^{-26}
XP_034329440.1	Coadhesin	*Crassostrea gigas*	0	−6.56	3.83×10^{-18}
XP_034330544.1	SCO-spondin	*Crassostrea gigas*	0	−6.29	3.83×10^{-18}
XP_045201952.1	General transcriptional corepressor trfA-like, 1 (trfA-like, 1)	*Mercenaria mercenaria*	3×10^{-29}	6.44	3.83×10^{-18}
XP_034329445.1	A disintegrin and metalloproteinase with thrombospondin motifs adt-1 (ADAM-1)	*Crassostrea gigas*	0	−6.23	1.07×10^{-17}
XP_011427257.2	Calcium-dependent protein kinase 7 (CPK7)	*Crassostrea gigas*	2×10^{-37}	−6.28	1.44×10^{-17}
XP_034323067.1	Spidroin-1-like, 1	*Crassostrea gigas*	2×10^{-08}	6.12	2.88×10^{-17}
OWF40357.1	Stereocilin	*Mizuhopecten yessoensis*	0	−6.02	2.90×10^{-17}
XP_011422608.2	IgGFc-binding protein	*Crassostrea gigas*	6×10^{-135}	−6.05	4.55×10^{-17}
XP_022339848.1	SCO-spondin-like, 1	*Crassostrea virginica*	0	−5.99	7.63×10^{-17}
XP_022339848.1	SCO-spondin-like, 2	*Crassostrea virginica*	0	−5.99	7.63×10^{-17}
XP_045201952.1	General transcriptional corepressor trfA-like, 2 (trfA-like, 2)	*Mercenaria mercenaria*	9×10^{-31}	5.96	7.63×10^{-17}
XP_034323070.1	Spidroin-1-like, 2	*Crassostrea gigas*	7×10^{-13}	5.97	7.63×10^{-17}
XP_034322956.1	uncharacterized LOC117688748	*Crassostrea gigas*	0	5.95	7.71×10^{-17}
XP_034322955.1	Uncharacterized LOC117688749	*Crassostrea gigas*	0	5.85	1.81×10^{-16}
XP_022339848.1	SCO-spondin-like, 3	*Crassostrea virginica*	0	−5.79	2.11×10^{-16}
XP_034313686.1	ADP-ribosylation factor 1-like	*Crassostrea gigas*	3×10^{-136}	5.93	2.28×10^{-16}
XP_011426386.2	Mediator of DNA damage checkpoint protein 1 (MDC1)	*Crassostrea gigas*	0	5.87	5.08×10^{-16}
XP_034323479.1	Glycine-rich cell wall structural protein 1.8 transcript isoform X2 (GRP 1.8- isoform X2)	*Crassostrea gigas*	0	5.74	7.33×10^{-16}
XP_011450501.3	Glycine-rich cell wall structural protein 1.8 transcript isoform X1 (GRP 1.8-isoform X1)	*Crassostrea gigas*	0	5.74	7.33×10^{-16}
APC92582.1	Tyrp-1	*Hyriopsis cumingii*	2×10^{-157}	5.74	7.66×10^{-16}
XP_011436430.2	Collagen alpha-2(I) chain	*Crassostrea gigas*	6×10^{-37}	5.70	1.03×10^{-15}
XP_011422608.2	IgGFc-binding protein	*Crassostrea gigas*	0	−5.58	1.40×10^{-15}
XP_011452538.2	Leucine-rich repeat-containing protein 4C (LRRC4C)	*Crassostrea gigas*	0	−5.63	2.00×10^{-15}
XP_011419747.2	Uncharacterized LOC105322623	*Crassostrea gigas*	0	−5.56	2.75×10^{-15}
XP_034323071.1	Spidroin-1-like, 3	*Crassostrea gigas*	3×10^{-08}	5.51	2.83×10^{-15}
CAG2230452.1	CD283	*Mytilus edulis*	3×10^{-59}	−5.49	5.61×10^{-15}
XP_011415271.2	Acanthoscurrin-2	*Crassostrea gigas*	0	−5.40	7.66×10^{-15}
XP_011452536.2	Leucine-rich repeat LGI family member 2 (LGI2)	*Crassostrea gigas*	0	−5.38	2.24×10^{-14}
XP_021357253.1	Serine/arginine repetitive matrix protein 1-like (SRRM1-like)	*Mizuhopecten yessoensis*	9×10^{-62}	5.44	2.88×10^{-14}

a: protein name according to NCBI database; b: Blastx Expect value; c: log fold change of transcripts; d: false discovery rate, FDR.

3.2.2. Postlarvae

Among those in the top thirty list, some transcripts which were upregulated after settlement were found to hold a variety of functional roles: protein synthesis and degradation (2 trfA-like transcription factor genes), structural proteins (3 Spidroin-1-like and 2 GRP1.8 variant genes), immune and stress response (AESIP), ion transporter (ADP-ribosylation factor 1-like), oxidoreductase activity (Tyrp-1), cytoskeleton/cell adhesion (Collagen alpha-2(I) chain), and two uncharacterized LOC117688748 and LOC117688749 transcripts with unknown function. Notably, other upregulated transcripts after settlement that were not shown in this list include transcription factors (ATP-dependent RNA helicase *elf* 4A (LOC105324495), ETS-related transcription factor Elf3-like (LOC105325143), activating transcription factor 3 (LOC105337015), cyclic AMP-dependent transcription factor ATF-3 (LOC105337016)), an enzyme for hormone synthesis and breakdown (cytochrome

P450 (LOC105325143)), and structural proteins (highly expressed transcripts: gigasin-6 isoforms X1 and X2 (LOC105343850), dentin sialophosphoprotein-like (LOC105323395), and clumping factor A (LOC105346538)). Interestingly, gigasin-6 isoforms X1 and X2, as well as dentin sialophosphoprotein-like, were upregulated in the conspecific cue-induced PL batch. Gigasin-6 and its isoforms have been reported as a component in the adult shell matrix in *C. gigas* [60,61], while our sequence homology search on dentin sialophosphoprotein-like showed close homology to folian-cv1 in *C. viriginica* [62]. Others were related to signal regulation processes such as calmodulin A (LOC105327998), Wnt inhibitory factor 1/EGF-like domain containing protein 2 (Wif-1) (LOC105335286), tyrosine-protein kinase receptor Tie (LOC105338560), beta-2 adrenergic receptor (LOC105336903), nuclear receptors (LOC105338842, LOC105345246), and FMFRamide receptor (LOC105343238). The differential expression of beta-2 adrenergic receptor in the conspecific cue-induced PL batch is of particular interest. This is in contrast with the findings of Coon et al. [63] wherein the endogenous alpha-1 adrenergic receptor was implicated in *C. gigas* larval settlement induction processes through pharmacological evidence. In addition, as observed by Wang et al. [64], the larval metamorphosis in *Meretrix meretrix* competent clam was successfully induced or inhibited by a beta-adrenoreceptor agonist and antagonist, respectively. The possible role of beta-2 adrenergic receptors in *C. gigas* larval settlement warrants further investigation. It is also noteworthy that we were able to identify some differentially expressed transcripts that manifested some form of isoform switching [57], wherein the FMFRamide receptor (LOC105331946) was downregulated, while the FMFRamide receptor (LOC105343238) was upregulated after settlement. An isoform of Calmodulin A (LOC105328000) was downregulated, but the precursor form of Calmodulin A (LOC105327998) was upregulated after settlement (RNA-seq) and was supported by qRT-PCR. This may also imply that some genes play unique functions during oyster larval settlement, similar to those reported in other invertebrates [65,66].

3.3. Gene Ontology (GO) Enrichment Analysis

To understand how the presence of a conspecific cue, CgSE, impacts larval settlement responses and to interpret the differential expression (DE) results in terms of higher-order biological processes or molecular pathways, we counted the number of DE transcripts that were annotated with each possible GO term [67]. GO terms that occurred frequently in the list of DE genes are said to be over-represented or enriched [67]. The filtering threshold values in this analysis were set at FDR < 0.01 and $|\log2\ \text{Ratio}| > 1.5$. Table 4 presents the results of the GO enrichment analysis while the Supplemental Table S3 displays the full annotation on the sequence homology search for the uncharacterized transcripts associated with each of the GO terms.

A total of 43 transcripts had a GO functional annotation associated with them. They were assigned into two major functional categories: molecular functions and cellular components. Overall, 26 transcripts were classified into molecular function processes, that is, chitin-binding (9) and calcium ion binding (17), while 17 transcripts were assigned to the extracellular region, under the cellular component processes. Seven transcripts were also found in two categories in both molecular function and cellular components. These results reflect the difficulty in associating a possible function and an appropriate match [2] with existing GO term annotations. One possible explanation for why it was difficult to parse the differentially expressed transcripts into discrete GO terms could be explained by the findings of Zhang et al. [2], wherein they found that the *C. gigas* genome exhibited a high degree of polymorphism and an abundance of repetitive sequences, of Ih over 62% could not be assigned to known categories. This reflects not only the presence of numerous isoforms of many genes, but also the paucity of genomic information from this species [2]. Our present findings are also in agreement with a previous study by Huan et al. [5], where they examined the proteomic profiles between the pediveliger larvae and biofilm-induced postlarvae in *C. gigas*. They found no significant result in the GO analysis that could associate the DE proteins with particular biological processes owing to

their functional diversity and the relatively small number of proteins [5]. They resorted to manually dividing the proteins into different groups according to their potential functions based on sequence homology [5], and which we also applied in this present study for those uncharacterized transcripts so as to know their possible function.

Table 4. Gene Ontology analysis of transcripts with differential expression in Pedi and PL batches before and after conspecific cue settlement induction. Underlined gene transcripts are those found in two GO categories.

Description	Chitin-Binding	Calcium Ion Binding	Extracellular Region
GO ID	GO:0008061	GO:0005509	GO:0005576
FDR value	8.13×10^{-5}	0.0024	5.67×10^{-6}
Ontology	Molecular function (MF)	Molecular function (MF)	Cellular component (CC)
Count	9	17	17
Gene	cgPif97 (↑), Peritrophin-44 (↓), Lactahedrin (↓), Collagen alpha 1 (XII) chain(↓), Peritrophin-1 -like,1 (↓), Peritrophin-like,2 (↓), Cleavage and polyadenylation factor 1 subunit 1 (Clip1) (↑), Putative chitinase 1 precursor (↑), Putative chitinase (↑)	DNA ligase 1 (↓), EF-hand domain-containing protein 1 (↓), Calmodulin-4 (↓), Calcium-binding protein E-63-1-like (↓), Calmodulin-A isoforms (↓), Leucine-rich repeat-containing protein 74B (↓), Sarcoplasmic calcium-binding protein (↑), Myosin (↑), EGF-like repeat and discoidin-I domain-containing protein 3 (↓), Mammalian ependymin-related protein 1 (↓), EF-hand domain-containing family member B (↓), Sarcoplasmic calcium-binding protein (↑), Neurocalcin homolog,1,(↓), Neurocalcin homolog,2 (↑), Regucalcin,1 (↓), Regucalcin,2 (↓), Annexin A4 (↓)	Poly(3-hydroxyalkanoate) depolymerase C (↓), cgPif97 (↑), Trithorax group protein osa-like (↑), NPC intracellular cholesterol transporter 2 (↓), Mammalian ependymin-related protein 1 (↓), Peritrophin-44 (↓), Lactadherin (↓), Golgi-associated plant pathogenesis-related protein 1 (↓), Collagen alpha 1 (XII) chain (↓), Dermatopontin (↓), Metalloproteinase inhibitor 3 (↓), Peritrophin-1-like, 1 (↓), Peritrophin-1-like, 2 (↓), inactive pancreatic lipase-related protein 1 (↓), pancreatic lipase-related protein 2 (↓), adenosine deaminase AGSA (↓), von Willebrand factor C domain-containing protein 2-like (VWC2L) (↓)

Under the chitin-binding pathway (GO:0008061; Q = 8.13×10^{-5}), we found four significantly enriched transcripts were upregulated while five transcripts were downregulated after settlement. The potential functions of these upregulated transcripts were as structural proteins (cgPif97) and enzymes (Clip1, 2 Putative chitinases), while those of the downregulated transcripts indicate roles in the cytoskeleton and cell adhesion (peritrophin-44, 2 lactadherin, collagen alpha 1 (XII) chain, peritrophin-1-like).

Three upregulated and fourteen downregulated transcripts were significantly enriched in the calcium ion binding pathway (GO:0005509; Q = 0.0024) after settlement. The associated transcripts under this category highlight upregulated transcripts with functional roles such as those related to the cytoskeleton and cell adhesion (sarcoplasmic calcium-binding protein), ion transport (neurocalcin homolog), and tissue development (myosin), while the rest of the downregulated transcripts may have potential functions involved in protein synthesis and degradation (DNA ligase 1), signal regulation (EF-hand domain-containing protein 1, calmodulin-4, calcium-binding protein E63-1-like, calmodulin-A, EGF-like repeat and discoidin I-like domain-containing protein 3, EF-hand domain-containing family member B, Annexin A4), immunity and stress response (LRR74), tissue development (mammalian ependymin-related protein), and ion transport (neurocalcin homolog, regucalcin).

Lastly, two upregulated transcripts and fifteen downregulated transcripts after settlement were significantly enriched under the cellular component processes associated with the extracellular region (G0:0005576; Q = 5.67×10^{-6}). The upregulated transcripts associated with this category include shell matrix protein (cgPif) and another transcript related to protein synthesis (Trithorax stage protein osa-like). However, the fifteen downregulated transcripts code for a variety of functions such as enzymes (poly(3-hydroxyalkanoate) depolymerase C, inactive pancreatic lipase-related protein 1, pancreatic lipase-related protein 2, adenosine deaminase AGSA), ion transport (NPC intracellular cholesterol transporter 2), particular tissue development (mammalian ependymin-related protein 1), cytoskeleton and cell adhesion (peritrophin-44, lactadherin, collagen alpha 1 (XII) chain,

dermatopontin, 2 peritrophin-1-like genes), a protease inhibitor (metalloproteinase inhibitor 3), and signal regulation (Golgi-associated plant pathogenesis-related protein 1, von Willebrand factor C domain-containing protein 2-like).

3.4. Validation of Transcriptomic Data via qRT-PCR

The qRT-PCR validation experiments were performed on six selected transcripts that were differentially expressed before and after settlement, as well as those found in the GO-enriched categories in the transcriptome dataset. Figure 3 illustrates their relative mRNA expression levels. Three upregulated transcripts before settlement but downregulated after settlement such as VWC2L (GO-extracellular region), collagen alpha 1 (XII) (GO-extracellular region), and annexin A4 (GO-calcium ion binding) were consistent in the qRT-PCR results, showing higher relative mRNA expression levels before settlement but lower expression levels after settlement. Likewise, three downregulated transcripts before settlement but upregulated after settlement such as putative chitinase 1 (GO-Chitin binding), calmodulin A, and Wnt inhibitory factor 1 were supported by qRT-PCR, showing lower relative mRNA expression levels before settlement but with higher expression after settlement.

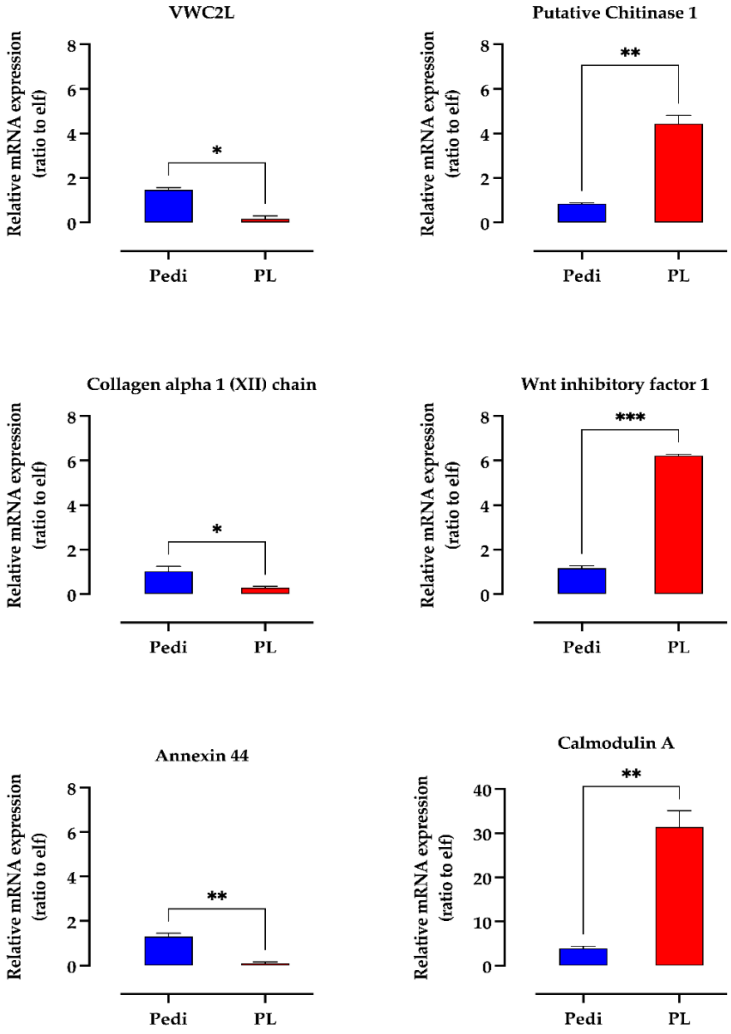

Figure 3. Relative expression levels of selected genes before (pediveliger, Pedi) and after (postlarvae, PL) settlement induction by a conspecific cue from shells in *C. gigas* using quantitative real-time PCR.

Data are expressed as the mean + SD (n = 3). Values with "*, **, ***" are significantly different ($p < 0.05$, <0.01, <0.001, respectively). Abbreviation: VWC2L = von Willebrand factor C domain-containing protein 2-like.

4. Discussion

Oysters have a complex life cycle in which they exhibit an active, pelagic phase and an attached, benthic phase. The transition from one phase to the next is one of the most life-changing events common to many mollusks, such as oysters. Physiological mechanisms associated with the induction of molluscan larval settlement, including oysters, are hypothesized to involve the nervous system as a result of stimulation of different neural circuits by external cues [48,68–73]. Previous studies have shown that irreversible larval attachment to a substrate and metamorphosis, which comprise the entire settlement process in oyster larvae, can be induced and experimentally separated using pharmacological agents [48]. Coon et al. [3] demonstrated that L-3,4-dihydroxyphenylalanine (L-DOPA) induced settlement behavior that led to the cementation of the *C. gigas* larvae to a substrate followed by metamorphosis, while the use of epinephrine led to the induction of metamorphosis bypassing the attachment of the larvae on a substrate [74] by interacting with endogenous α1-adrenergic receptors [63]. In this present study, we provide new insights into the possible involvement of a hormonal signaling system associated with the onset of metamorphic processes upon settlement induction by a natural settlement inducer from shells of conspecifics. Our understanding of the neuroendocrine-biomineralization gene activities that accompany oyster larval settlement could provide insights into pathways and mechanisms, and help to understand how the larvae respond to external cues to initiate these processes [8,75]. To the best of our knowledge, this is the first time that omics technologies as a tool, such as transcriptomics, have been applied to understand the response of oyster larvae to a natural settlement-inducing cue from the shells of conspecifics. We hope that this data provides a new direction to studies aimed at understanding the gregarious phenomenon among oysters.

This present study aims to provide transcriptomics insights on differential expression patterns that happen within an oyster larva as it encounters the presence of a conspecific cue from shell protein extracts and is induced to settle. Hence, transcriptome profile comparisons were analyzed between the larval batches before and after conspecific cue exposure which led to their transition from being a pediveliger larva into a postlarva. Our discussion here will elaborate inferences on a snapshot of one group of pediveliger larvae that settled first. The biological relevance of this fraction of the total population is worth considering and introducing as future research topics.

In this study, we found a total of 2383 transcripts that were differentially expressed between the pre-settlement pediveliger larvae (Pedi) and postlarvae (PL). There were 740 upregulated and 1643 downregulated transcripts after settlement. These identified transcripts may play important roles during the conspecific cue-mediated induction of *C. gigas* larval settlement. It would also be essential to elucidate their individual functions for future studies.

A graphical representation of the key molecular events in conspecific cue-mediated *C. gigas* larval settlement induction is shown in Figure 4. Comparison of the transcriptomes before and after exposure of the competent larvae to a conspecific cue represents a wide range of functions and indicates the complex regulatory mechanisms during the *C. gigas* larval settlement processes. Before the onset of metamorphosis, several transcripts related to the cytoskeleton, cell adhesion, and ECM were differentially expressed and may implicate muscular tissue development and structural reorganization of body patterns [76]. Furthermore, transcripts associated with the cytoskeleton may facilitate cell and tissue assembly and the movement and disassembly of muscle filaments during the metamorphosis [76].

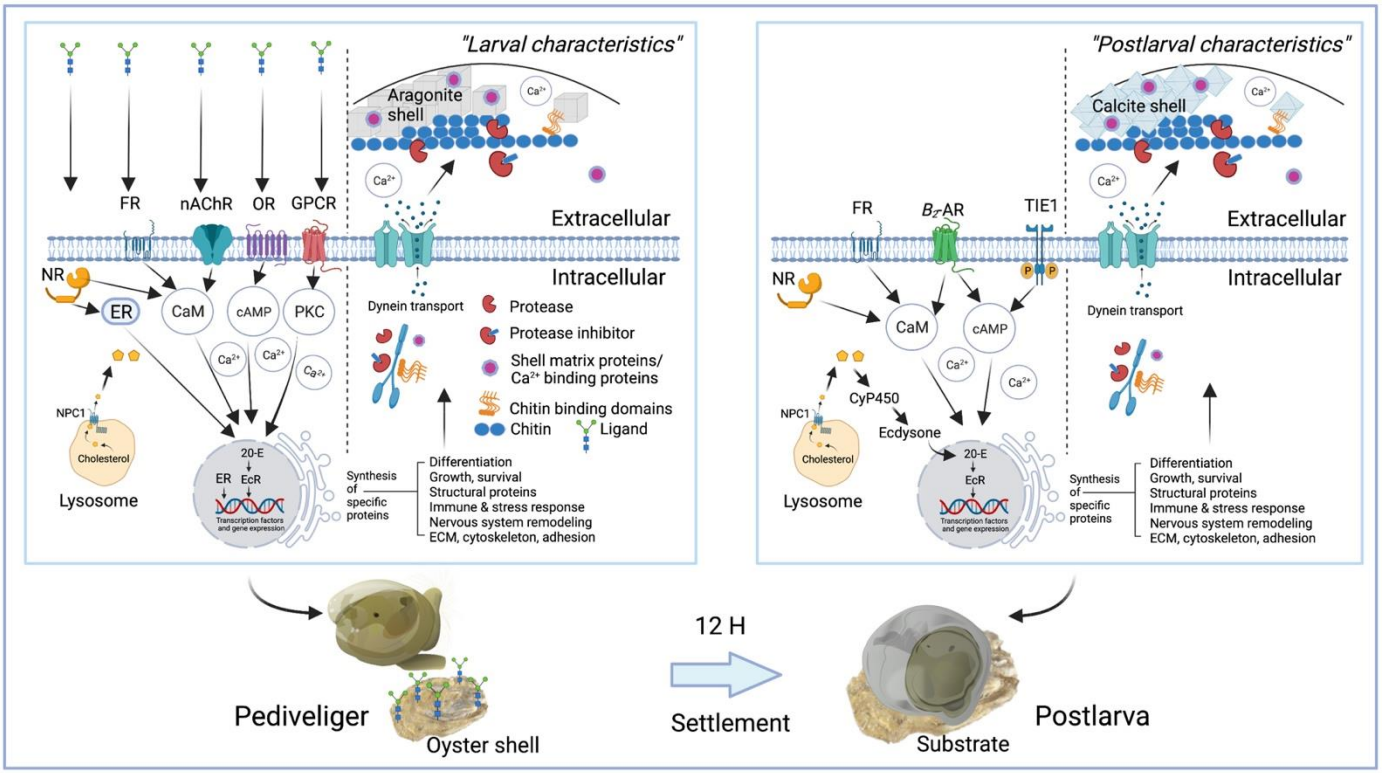

Figure 4. Schematic of the neuroendocrine-biomineralization crosstalk in *C. gigas* conspecific cue-induced larval settlement. This indicates the biological and molecular processes identified through the differential transcript expression in the competent pediveliger as it transitioned into postlarvae by conspecific cue-mediated settlement induction. As the pediveliger commences its searching behavior, it makes use of its external chemoreceptors to detect signals for a suitable location for settlement. Once it detects a ligand and other signaling cues coming from shells of conspecifics, various intracellular signaling receptors act in a concerted way and a molecular switch is turned on for the initiation of downstream signal cascades into the nucleus with the possible involvement of the ecdysone signaling pathway. Then, various transcription factors and gene expressions for the synthesis of specific proteins are activated. Subsequently, rapid morphological, physiological, structural, and functional changes occur as the competent pediveliger undergoes its metamorphosis into a postlarva within 24 h. Abbreviations: NR, nuclear receptor; FR, FRMF (Phe-Met-Arg-Phe) amide receptor; nAChR, neuronal acetylcholine receptor; OR, octopamine receptor; GPCR, leucine-repeat-containing GPCR 4; ER, estrogen receptor; CaM, calmodulin groups; cAMP, cAMP-regulated D2 protein; PKC, protein kinase C; Ca^{2+}, calcium ion; NPC1, NPC cholesterol transporter 1; 20-E, hydroxyecdysone; EcR, ecdysone receptor; B_2AR, β_2 adrenoreceptor; TIE 1, tyrosine-protein kinase receptor TIE; CyP450, cytochrome P450; and P, phosphate. Some concept models were adapted and modified to illustrate processes of shell formation [77], chitin-mediated biomineralization [78], and dynein transport [79] that reflect the transcriptome larval settlement dynamics in our study. This figure was created using BioRender.com.

On the other hand, immune- and stress-related genes may play a role in the regulation of apoptosis, protein degradation, cell proliferation, and cell processes [76,80,81], and may be required to counter the damage caused by oxidative stress [76]. Genes related to nervous system remodeling may allow the oyster larvae greater sensitivity to external cues as it reaches a metamorphic competent stage. Thus, taking all these data together implies that the competent pediveliger larvae become equipped to transduce external cues from the environment into internal processes with neural and/or hormonal elements as it tries to find a suitable substrate for settlement [8,11,75,82]. Once the larvae have accumulated a significant amount of cue from a suitable settlement site, the onset of settlement is triggered.

In *C. gigas*, just like other marine invertebrates, this occurs in a rapid process. In our case, within 24 h, the induced larvae acquired postlarval characteristics such as permanent attachment to a substrate and new shell growth, to name a few. The fast transition from pediveliger to postlarvae is necessary as these organisms are particularly vulnerable to predation during this event [83]. Hadfield et al. [84] proposed that marine invertebrates appear to require little or no *de novo* gene action during the metamorphic induction process which makes them capable of responding rapidly to environmental cues to settlement and undergoing very rapid metamorphic morphogenesis [84]. They then put in contrast, the slow, hormonally regulated metamorphic transitions of vertebrates and insects. Our present data show, however, that several elements related to various hormonal signaling pathways were also differentially expressed in the pediveliger larvae. This may indicate their involvement in oyster metamorphosis. Recent molecular studies have shown that Lophotrochozoans, such as *C. gigas*, may contain integrated hormone systems [33,85] such as ecdysone [8,33,85] and thyroid hormone [86] (for the hormonal system); octopamine [34] and epinephrine [63] (for the nervous system) were reported in the development of *C. gigas*. We then hypothesized that the differential expression of some elements related to various hormonal signaling pathways in *C. gigas* may be necessary for preparation for the onset of metamorphosis. This variety of hormones may act in a concerted manner when activated by their respective receptors. They can then act as transcription factors that unleash cascades of *de novo* transcription and synthesis of new proteins, which may be similar to the mode of action reported in insects and amphibians [84,87]. Simultaneous to the ongoing loss of larva-specific structures at the onset of metamorphosis, postlarval structures are also being formed [84,87]. Our data also shows that several differential transcript expressions were found related to transcription factors, protein synthesis, structural proteins including shell matrix proteins often found in adult shells, biomineralization-related enzymes, ion transport, chitin, and calcium ion binding pathways in the postlarval stage. After 24 h of exposure of the oyster larvae to the settlement-inducing conspecific cue, shell matrix proteins such as cgPif97 indicate the start of the restructuring of the shell composition from aragonite (dominant in the pediveliger stage) to calcite (dominant in postlarvae to adults) [2]. As the postlarvae develop to the adult stage, this calcified shell becomes thicker, and sturdy enough to protect it from harsh and dynamically changing environments, predation, and desiccation [2]. Moreover, our transcriptomics analysis also shows several similar genes to those reported in epinephrine-induced *C. gigas* larvae, especially on the possible involvement of ecdysteroid signaling pathways [33]. Furthermore, in this study, several elements involved in the ecdysone signal pathway, including the ecdysone receptor, NPC cholesterol transporter, cytochrome P450, tyrosine-protein kinase receptor Tie, tyrosine (Tryp-1), and other nuclear receptors, were differentially expressed. Likewise, we also found receptors of neuronal acetylcholine, octopamine b-2R like, estrogen, FMRFamide, and GPCRs that were differentially expressed. A similar observation was made by Zheng et al. [30], having compared *P. fucata* and *C. gigas* proteomes. Broadly, our results provide support to the hypothesis proposed by Zheng et al. [30], that a gene regulatory network may have closely linked dynamics with shell formation and may involve the interplay of various hormone receptors, neurotransmitters, and neuropeptide receptors working together in a concerted way during larval settlement-induction processes. That conspecific cue-mediated larval settlement induction may involve a neuroendocrine-biomineralization crosstalk during the settlement processes in *C. gigas*. In comparison to our findings, the presence of elements related to the ecdysone signal pathway in *C. gigas*, was further evidenced by the study of Xu and Zhang [33] on the transcriptomic and proteomic dynamics profile of larvae exposed to epinephrine stimulation. Likewise, a recent study by Xiong et al. [44], demonstrated that the ecdysone signal pathway participates in the shell formation of the Pearl oyster *P. fucata martensii*. They have reported that ecdysone could activate the ecdysone receptor complex to regulate the expression of biomineralization-related genes. Hence, on the basis of the current findings and previous reports, we propose the pos-

sibility of a neuroendocrine-biomineralization crosstalk in *C. gigas* conspecific cue-induced larval settlement.

Our present findings demonstrate the mode of action of a naturally occurring inducer such as the chemical cue from shells of conspecifics, which complements and show some common differential transcript expression that was reported in neuroactive compound-induced and biofilm-induced *C. gigas* larvae and in other marine invertebrate larvae, which may have potential roles in larval settlement. However, our results are based on the comparison of two pooled populations in two RNA-seq samples representing Pedi and PL batches. This finding may warrant further study using multiple samples. Nonetheless, our present findings showed that expression trends of selected representatives from the most significantly expressed genes were consistent between the real time-PCR and transcriptome data. Hence, the lack of replicates did not affect the results and the model of edgeR could compensate for it. On the other hand, conditions for RNA-seq larval sample preparation may also impact gene expression trends, and this warrants future investigation [79].

This data can be a useful baseline in further identifying the natural settlement-inducing compound from shells of conspecifics and elucidating their genetic mechanisms. In addition, a more in-depth examination of the various pathways is essential by cloning their essential components in such a way that the receptors may be utilized as targets to define pathways. Knowledge of receptor characterization and the downstream signaling cascade for each pathway might lead to significant advances in our knowledge of larval settlement as well as the neuroendocrine functions in oysters. Further identification and characterization of this settlement-inducing cue from shells in conspecifics can enable us to utilize its properties as a possible attractant for wild and hatchery-grown oyster larvae to attach to artificial substrates for aquaculture. Development in our knowledge and techniques in this field may be useful in increasing hatchery production of potential mollusk species, developing new antifouling compounds capable of deliberately inhibiting bivalve attachment, and helping other related industries.

5. Conclusions

We report, for the first time, a transcriptome profile of an oyster larval response to a conspecific cue-mediated settlement induction in *Crassostrea gigas*. Our transcriptomics analysis has identified key transcripts that were differentially expressed in the pediveliger and conspecific cue-induced postlarvae in *C. gigas*. Quantitative analysis revealed 2383 candidate transcripts that may play important roles during larval settlement processes. Several transcripts with differential expression were significantly enriched in GO analysis associated with chitin-binding, calcium-ion binding, and extracellular region-related categories. Differential expressions of six candidate transcripts representing Pedi and PL batches were validated by qRT-PCR, which showed relative expression trends were consistent between qRT-PCR and transcriptome data. By comparing the expression patterns of the Pedi and conspecific cue-induced PL batches, we hypothesized that shell formation shows closely linked dynamics with a gene regulatory network in *C. gigas* that may involve the interplay of various hormone receptors, neurotransmitters, and neuropeptide receptors working together in a concerted way in the induction of conspecific cue-mediated larval settlement. Our results highlight the transcriptome dynamics underlying the gregarious settlement of oysters on conspecific adult shells and show the potential use of this settlement-inducing cue as an attractant for wild and hatchery-grown oyster larvae to attach to artificial substrates for aquaculture. Furthermore, we hope that this study could provide hints and support for future studies related to the involvement of the ecdysone signal pathway and the linkages within the neuroendocrine-biomineralization crosstalk in *C. gigas* settlement.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14070559/s1, Figure S1: Histogram profile of the average log CPM of the mapped gene sequences and the Venn diagram of DEGs using various normalization models. Table S1: Full list of transcripts used for differential expression analysis in the *C. gigas* transcriptome. Table S2: Results of the Blastx homology search for the thirty differential transcript expression in the

C. gigas transcriptome. Table S3: Results of the Blastx homology search for the differential transcript expression involved in the Gene Ontology enriched processes of the *C. gigas* transcriptome.

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