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FEATURE ARTICLE

Transcriptome analysis provides insights into a molecular mechanism of histamine response in the cyprid larvae of *Amphibalanus amphitrite*

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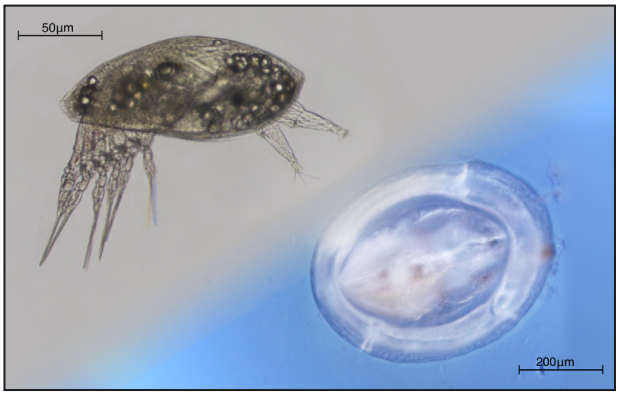
ABSTRACT: Barnacles are notorious marine fouling creatures; their planktonic cyprid larvae attach to material substrates and metamorphose. Histamine has shown great importance in regulating cyprid settlement and metamorphosis. This study aimed to investigate the mechanisms of histamine-induced larval settlement. Cyprids were exposed to histamine or loratadine, an anti-histamine compound. The percent larval settlements of the histamine- and anti-histamine-treated cyprids were significantly higher and lower, respectively, than the control group. Transcriptomic analyses showed that histamine-treated cyprids had 18 498 differentially expressed genes (DEGs, 14 531 up-regulated, 3967 down-regulated) and the anti-histamine group had 18 055 DEGs (17 237 up-regulated, 818 down-regulated) in comparison to untreated controls. In both treatment groups, significant enrichment of DEGs involved in the mitogen-activated protein kinase signaling pathway was observed. Based on the results of larval settlement bioassays, we set 4 filter conditions to perform DEG analyses, and 19 DEGs were selected as functional genes related to cyprid settlement. The functional categories of these genes included structural proteins, spider silk proteins, energy metabolism proteins, cement proteins, glycosyl proteins, and multifunctional proteins. The energy metabolism protein AdipoR was significantly up-regulated in the histamine-treated cyprids but significantly down-regulated in the anti-histamine group. The activity of adenosine monophosphate-activated protein kinase, a downstream signaling protein of AdipoR, increased in the histamine-treated group and decreased in the anti-histamine-treated group. Our results provide new insights into the molecular mechanisms underlying the histamine-induced settlement of barnacle cyprids and identify *AdipoR* as an important gene that can affect the settlement of cyprids, likely through regulating cyprid energy metabolism.



Cyprid larva of the barnacle *Amphibalanus amphitrite* (left) responds to environmental cues, settling onto a suitable substrate and metamorphosing to be a juvenile (right).

Photo: Yang Zhao & Ming Bi

KEY WORDS: Barnacle cyprid · Larval settlement · Histamine · Transcriptome · MAPK signaling pathway

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1. INTRODUCTION

Marine biofouling, which involves attachment to and growth of marine organisms on man-made structures, causes significant damage and cost to marine resource development (Schultz et al. 2011, Vinagre et al. 2020). Barnacles have a global distribution and are notorious fouling organisms. Most barnacles are densely distributed in the intertidal zones of harbors, port terminals, and coastal waters (Connolly et al. 2001). As biofouling frequently appears on vessels after sailing, barnacles have been widely studied as a model organism in antifouling research (Rittschof et al. 1992 a,b, Jin et al. 2014a,b). The barnacle life cycle involves 2 different larval stages: the nauplius and the cyprid (Crisp 1960, Essock-Burns et al. 2017, Dobretsov & Rittschof 2020). During nauplius development, the pelagic larva feeds on phytoplankton (Crisp 1985, Gaonkar & Anil 2009). Cyprids represent a nonfeeding stage dedicated to surface location, settlement, and metamorphosis to achieve the juvenile stage. After permanent attachment by the release of larval cement from a pair of antennae, the larvae metamorphose into juvenile barnacles (Lucas et al. 1979, Kotsiri et al. 2018).

For initiation of the cyprid settlement and metamorphosis processes, stimulation by chemical signals is required (Crisp 1960, Rittschof et al. 1984, Jarrett 1997, Hay 2009). Upon binding to a receptor, the receptor–ligand complex is believed to trigger a signal transduction pathway (or pathways) that leads to a series of physiological changes which eventually induce the cyprid to commit to permanent settlement and metamorphosis (Clare & Matsumura 2000). In 1988, peptides with histidine, lysine, and arginine carboxyl termini were demonstrated to be effective settlement pheromones (Tegtmeyer & Rittschof 1988). Subsequently, in 1998, a glycoprotein, which was later named settlement-inducing protein complex (SIPC), was purified from adult barnacle extracts (Matsumura et al. 1998, Kotsiri et al. 2018). SIPC is also released by cyprids onto the substratum during the exploration period and serves as a pheromone that induces larval settlement and metamorphosis (So et al. 2016). In addition, several neurotransmitters such as histamine (Jin et al. 2014a), adrenaline, and serotonin (Yamamoto et al. 1996, 1998) also regulate cyprid settlement. Among these neurotransmitters, histamine also reportedly functions as a photoreception neurotransmitter in arthropod eyes (Stuart 1999). In barnacles, the cyprid larvae develop with a pair of compound eyes which presumably provide vision perception and direct the larvae to settle close to conspecifics (Barnett et al. 2009). Identification of a histamine-specific transporter in barnacle larvae eyes suggested that histamine indeed exists within the cyprid larval body (Stuart et al. 2002). The study also demonstrated histamine as an inducer of settlement in barnacles. Jin et al. (2014a) found that an anti-histamine compound, loratadine, can prevent barnacle cyprids from settling, while histamine can promote barnacle larval metamorphic competence. However, the mechanism underlying histamine-induced larval settlement and metamorphosis remains unclear.

Transcriptome analysis has been widely applied to explore the molecular mechanisms underlying larval settlement and metamorphosis in various marine invertebrate taxa (Jain 2012, Wong et al. 2014, Lowe et al. 2017). For instance, Chen and colleagues performed *Amphibalanus amphitrite* transcriptome sequencing at different developmental stages and highlighted important functional genes relating to cyprid settlement and metamorphosis (Chen et al. 2011). Subsequently, Yan and colleagues identified 14 neuropeptides that may be involved in barnacle larval settlement (Yan et al. 2012). Several cement proteins and adhesion-related genes have been identified and found to be highly expressed in the cyprid of the acorn barnacle *Tetraclita formosana* (Lin et al. 2014). These reports highlight the robustness of transcriptomic analysis in identifying important genes in larval settlement studies.

In this research, we sought to explore the mechanisms underpinning histamine-induced larval settlement by performing transcriptomic analyses and probing new candidate genes that are crucial to cyprid settlement and metamorphosis. We sequenced the transcriptomes of histamine and anti-histamine-exposed barnacle larvae. Expression of selected differentially expressed genes (DEGs) was validated by real-time quantitative PCR (qRT-PCR).

2. MATERIALS AND METHODS

2.1. Sample preparation and testing

Adult barnacles were collected from the rocky shore of the East China Sea (34° 88′ N, 119° 19′ E). Barnacles were kept in an aquarium (2000 ml) filled with 26°C filtered seawater (FSW, 0.45 µm, salinity, S = 32) and fed with nauplii of *Artemia* sp. and the diatom *Chaetoceros gracilis* Schutt 1895 in the labo-

ratory of the Institute of Marine Science and Technology at Yangzhou University, PR China (Rittschof et al. 1984, 1992a). Release of nauplius larvae was triggered by white light illumination. The nauplius larvae were collected using a mesh and were cultured in the laboratory as described in our previous studies (Jin et al. 2014a,b). In detail, the nauplii were incubated in FSW (0.45 µm, S = 32) at a density of approximately 5 to 10 larvae ml^{-1} and a temperature of 26°C with a 16 h light:8 h dark cycle and fed with *C. gracilis*. An aeration device was used to increase the dissolved oxygen content in the culture chamber. Antibiotic solutions of penicillin (1 mg ml^{-1}) and streptomycin (1.2 mg ml^{-1}) were added to the culture at a ratio of 1:1000 (v/v) to inhibit bacterial growth (Thiyagarajan et al. 2002, Jin et al. 2014a,b). After ~5 d, the nauplii developed into cyprids. Cyprid larvae were stored in 100 × 20 mm Petri dishes (BD Falcon) in a refrigerator at 4°C for less than 12 h before using and were designated newly metamorphosed cyprid larvae.

Newly metamorphosed cyprid larvae were divided into 3 groups: a histamine (His) group, an anti-histamine (anti-His) cohort, and a control (Con) group. The His, anti-His, and Con groups were treated with 5×10^{-8} mol l^{-1} histamine solution, 5×10^{-7} mol l^{-1} loratadine solution, and FSW, respectively. After 4 h of treatment in darkness in beakers, 300 cyprid larvae (1 sample) from each group were collected in a centrifuge tube and temporarily stored in liquid nitrogen for future RNA isolation. Each group included 3 biological replicates (total no. of samples = 9). The remaining larvae in the His, anti-His, and Con groups were separately subjected to larval settlement bioassays. The percent larval settlement after 48 h of incubation was recorded as described in Jin et al. (2014b).

2.2. Settlement analysis

Histamine and loratadine solutions were prepared as described above and were added to 24-well polystyrene plates (1.5 ml $well^{-1}$). To each well, 10 to 15 cyprid larvae were added. With FSW as a blank control, there were 3 treatments in total, with at least 4 parallel treatments in each group. The 24-well plates were covered with aluminum foil and kept in darkness at room temperature. Larval settlement in each well was recorded after 48 h post treatment and the percent larval settlement calculated. The calculation formula for the percent larval settlement was as follows: percent larval settlement = total number of settled larvae / total larvae × 100%.

2.3. RNA extraction and sequencing

Total RNA extraction was performed using TRIzol Reagent (TaKaRa) following the manufacturer's protocol. The quality of the total RNA of each sample was examined by 1% agarose gel electrophoresis. Sequencing libraries were processed using 1.5 µg of total RNA, following the protocols provided by the Ultra™ RNA Library Prep Kit for Illumina® (NEB). Poly-dT oligo-conjugated magnetic beads were used for each sample to purify the mRNA. As a result, paired-end reads with length 250 to 300 bp were selected for the fragment library and purified with the AMPure XP system (Beckman Coulter).

2.4. Functional annotation and gene expression analysis

Clean data (clean reads) were obtained after removing reads with adaptors and those of low quality from the raw reads; these reads were assembled using the de novo assembler Trinity with default parameters (Grabherr et al. 2011). All unigenes were annotated using 7 public databases: Nr (National Center for Biotechnology Information [NCBI] non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam (protein families), KOG/COG (clusters of orthologous groups of proteins), Swiss-Prot (a manually annotated and reviewed protein sequence database), KO (Kyoto Encyclopedia of Genes and Genomes [KEGG] Orthology database), and GO (Gene Ontology database). RSEM (Li & Dewey 2011) was used to calculate relative gene expression by determining the read count of the mapped clean reads to the assembled unigenes. Relative expression levels were normalized to fragments per kilobase of unigene sequence per million prior to differential expression analysis. Differential expression analysis of samples was performed using the R package DEGseq. p-values were adjusted using q-values (Storey & Tibshirani 2003). $p < 0.05$ and $|\log_2(\text{foldchange})| > 1$ was set as the threshold for statistically significant differential expression. The software KOBAS was used to test the statistical enrichment of DEGs in KEGG pathways (Mao et al. 2005). GO enrichment analysis of the DEGs was implemented using the GOseq R package (Young et al. 2010).

2.5. Identification of histamine- and anti-histamine-responsive genes

To further select functional DEGs under the histamine and loratadine treatments, we set 4 filter conditions: (1) the gene read count of the His group was higher than that of the Con group; (2) the gene read count of the anti-His group was less than that of the Con group; (3) the gene read count of the His group was >300; (4) the gene read count of the His group was 20 times that of the anti-His group.

2.6. Validation of DEGs by qRT-PCR

All primers used for qRT-PCR are listed in Table 1, and *mt-cyb* (cytochrome *b*) was chosen as an internal control (Bacchetti De Gregoris et al. 2009). qRT-PCR was conducted on a CFX96 Touch™ Real-Time PCR System with SYBR Premix Ex *Taq* Kit following the manufacturer's protocol. qRT-PCR was performed in 20 µl volumes containing 10 µl 2× SYBR® Green Realtime PCR Master Mix (TaKaRa), 1 µl cDNA (100 ng μl^{-1}), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), and 7 µl of H_2O. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. AMPK enzyme activity assay

Triplicate pooled samples (150 larvae) from the 3 experimental groups were taken for the biochemical determination of AMP-activated protein kinase (AMPK) enzyme activity. Collected samples were washed with phosphate-buffered saline 3 times, homogenized in a tissue grinder for 10 min to create homogenates, and then centrifuged for 10 min at 3200 × *g* to obtain supernatants. Assays were conducted in triplicate for each replicate sample and were performed by diluting with 5 volumes of assay buffer. Samples were incubated with 100 µl of horseradish peroxidase-labeled detection antibody for 60 min (37°C) and washed with 50 µl 1× detergent buffer solution 5 times. After samples had been incubated with 50 ml substrate in darkness for 15 min, 50 µl stop solution was added to terminate the reactions. Finally, the optical density of samples was measured at 450 nm using a microplate reader.

2.8. Statistical analysis

Statistical results (expressed as means ± SD) were analyzed by 1-way ANOVA, followed by Dunnett's test for multiple comparisons using IBM SPSS Statistics v.22 software. In all cases, $p < 0.01$ and $p < 0.05$ were considered to be statistically significant. All experiments were repeated at least 3 times.

3. RESULTS

3.1. Larval settlement bioassay

The percent larval settlement of histamine- and loratadine-treated cyprid larvae varied significantly from the control group ($p < 0.05$, Fig. 1). Compared to percent larval settlement of 40.85% in the Con group, 62.94 and 25.19% larval settlements were observed in the His and the anti-His groups, respectively. His promoted larval settlement, whereas the anti-histamine compound loratadine led to the inhibition of larval settlement.

Table 1. Primers used in the present study

Primer ID	Sequence of primers (5′-3′)	Gene
F1	CAA GCG GAT TAC GGT GGA G	*MAPK3* (mitogen-activated protein kinase 3)
R1	CCC GTT TTG AAC TTC TCC GT	
F2	AAC GAC CAC TAT TCG GGA GC	*Resilin* (pro-resilin)
R2	CAC TTC AGC GTT GTA GCC TC	
F3	TCC ATC GCG TCG TCA GCT TC	*RYR2* (ryanodine receptor 2)
R3	TCG TCC TCG TCT CCG TCC TC	
F4	CGT GTC TGG CAC CGA TGT	*SLC8A* (solute carrier family 8)
R4	TCG CCA TTC TGG GCA TAG	
F5	TAC GGC AGT GAG CCC AGC TA	*AdipoR* (adiponectin receptor protein)
R5	GTT GGC GTC GGC AGT GTA GA	
F6	GGA CAC TGC ATG CTA ATG GA	*mt-cyb* (cytochrome *b*)
R6	AGG CAG CAG CCA TAG TCA AG	

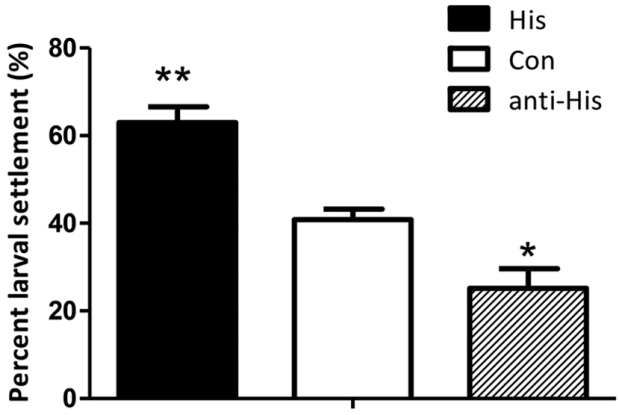

Fig. 1. Comparison of the percent cyprid settlement of histamine (His), control (Con), and anti-histamine (anti-His) groups. The His group was treated with 5×10^{-8} mol l^{-1} histamine solution and the anti-His group with 5×10^{-7} mol l^{-1} loratadine solution; the Con group was untreated (filtered seawater only). All data represent the mean ± SD of at least 3 replicates. *$p < 0.05$ (compared with control group); **$p < 0.01$ (compared with control group)

3.2. Transcriptome data analysis

The Illumina sequencing generated 68.77 Gbp of raw data. The raw reads, clean reads, clean bases, Phred quality score (Q) 20, Q30, and mapped percent for each library are presented in Table 2. Q20 ≥ 96.44 % and Q30 ≥ 87 % indicated the high quality of the sequencing data and its suitability for further analysis. After assembling, 124 617 unigenes with an N50 length of 879 bp were generated (Table 3); 50 443 unigenes were assigned to at least 1 GO term, and 7303 unigenes were assigned to 123 KEGG pathways. The sequencing data of this study have been uploaded to NCBI (accession number: PRJNA728053).

Table 3. Summary of the barnacle cyprid transcriptome assembly and its functional annotations. NCBI: National Center for Biotechnology Information; KEGG: Kyoto Encyclopedia of Genes and Genomes; KOG: clusters of orthologous groups of proteins database

De novo assembly by Trinity	
Total base (bp)	68.77G
Total number of transcripts	307 857
Number of genes	124 617
Mean length of genes (bp)	879
N50 (bp)	1145
Transcripts size range (bp)	301–33 494
Functional annotation	
Total number of transcripts annotated by public databases	69 708
NCBI non-redundant database	54 420
KEGG	7303
Gene Ontology	50 443
KOG	23 252

3.3. Identification of DEGs

A total of 23 147 DEGs were identified in the 3 pairwise comparisons (His vs. Con [His-Con], anti-His vs. Con [anti-His-Con], and His vs. anti-His [His-anti-His]) ($p < 0.05$, $|\log_2(\text{foldchange})| > 1$). As shown in Fig. 2, there were 14 531 up-regulated and 3967 down-regulated genes in the His-Con comparison, whereas 17 237 up-regulated and 818 down-regulated genes were identified in the anti-His-Con comparison. All of the DEGs identified in these 2 groups are listed in File S1 in the Supplement at www.int-res.com/articles/suppl/m681p001_supp.xlsx. In addition, 12 847 up-regulated and 559 down-regulated DEGs were found to be differentially expressed in the His-Con and anti-His-Con comparisons.

Table 2. Summary of sequencing data in the study. Q: Phred quality score; GC: guanine–cytosine content

Sample	Raw read	Clean read	Clean base	Error (%)	Q20 (%)	Q30 (%)	GC (%)
Con1	42545756	41167270	6.18G	0.03	96.44	90.90	48.82
Con2	46766900	45581846	6.84G	0.03	96.66	91.56	56.39
Con3	58681592	56980654	8.55G	0.03	96.97	92.17	56.36
His1	47400702	46240882	6.94G	0.03	97.39	92.78	48.26
His2	67973688	65998958	9.9G	0.03	96.77	91.62	52.99
His3	49066660	47326328	7.1G	0.03	96.83	91.63	48.97
Anti-His1	61869748	59559658	8.93G	0.03	96.99	92.08	53.06
Anti-His2	51315070	49355448	7.4G	0.03	96.77	91.59	51.19
Anti-His3	48356728	46215946	6.93G	0.03	96.90	91.89	52.93

3.4. GO and KEGG enrichment of DEGs

A total of 10 828 DEGs were annotated with at least 1 GO term involving 56 functional groups. The most significantly enriched GO term among the list of DEGs from His-Con and anti-His-Con comparisons was protein binding (Fig. 3). Other significantly enriched GO terms mainly belonged to the cellular component category. Three GO terms (intracellular membrane-bound organelle, membrane-

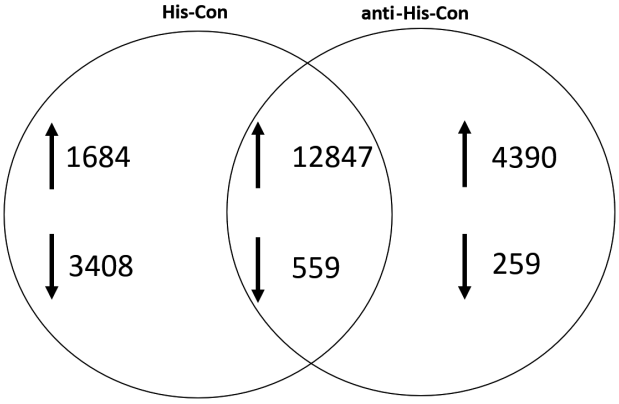

Fig. 2. Venn diagram of differentially expressed genes (up- and down-regulated) between the His-Con (histamine vs. control) and anti-His-Con (anti-histamine vs. control) groups

bound organelle, and nucleus) were significantly enriched in both His-Con and anti-His-Con comparisons. The full list of enriched GO terms in the DEG extracted from His-Con and anti-His-Con comparisons are listed in Files S2 and S3.

Through KEGG analysis, 1530 DEGs from His-Con comparisons were identified as being significantly enriched in 284 pathways (File S4), while 1459 DEGs from anti-His-Con comparisons were enriched in 282 pathways (File S5). KEGG terms involved in the progress of cell proliferation and apoptosis such as extracellular matrix–receptor interactions, mitogen-activated protein kinase (MAPK) signaling pathway, protein digestion and absorption, and RNA degradation were significantly enriched. KEGG pathway enrichment of the top 20 DEGs from both His-Con and anti-His-Con comparisons showed that the MAPK, PI3K–Akt, and focal adhesion signaling pathways were significantly enriched (Fig. 4).

3.5. Verification of DEGs by qRT-PCR

Three genes (*MAPK3*, ryanodine receptor 2 [*RYR2*], and solute carrier family 8 [*SLC8A*]) from each of the 3 significantly enriched pathways marked in Fig. 4 in red and 1 randomly chosen gene (*resilin* [pro-resilin]) were used to verify the cyprid transcriptome data. The relative expression levels of 4 DEGs (*MAPK3*, *RYR2*, *SLC8A,* and *resilin*) were validated by qRT-PCR. These 4 genes exhibited similar trends in terms of mRNA expression in the His, anti-His, and Con groups and showed strong correlation to the expression levels observed in RNA-seq experiments (Fig. 5).

3.6. Identification of histamine- and anti-histamine-responsive genes

We anticipated that genes directly related to histamine stimulus might be differentially expressed in both histamine- and anti-histamine-treated cyprids. To identify any such histamine-responsive genes, we filtered the DEGs using 4 criteria (see Section 2.5), and as a result, 19 histamine- and anti-histamine-responsive genes were identified (Table 4). These 19 genes could be divided into 6 categories: structural proteins, spider silk proteins, energy metabolism proteins, cement proteins, glycosyl proteins, and multifunctional proteins. Among them, elongation factor 1-alpha (*eEF1a1*), ATP-cAMP (*cAMP*), and adiponectin receptor protein (*AdipoR*) are related to energy metabolism. The expression of *AdipoR* was significantly up-regulated in the histamine-treated cyprids but significantly down-regulated in the anti-histamine-treated cyprids compared to the control group, which indicates the importance of *AdipoR* in regulating cyprid settlement under both histamine induction and loratadine inhibition (Fig. 6).

3.7. Verification of AMPK activities

After identifying *AdipoR* as a crucial gene, we compared AMPK activity, one of the downstream signaling pathways of *AdipoR* activation, in all treatment and control groups. As shown in Fig. 7, corresponding to the up-regulation of *AdipoR*, higher AMPK activity was observed in the histamine-treated cyprids. Meanwhile, AMPK activity was decreased in anti-histamine-treated cyprids.

4. DISCUSSION

Histamine is one of the neurotransmitters that mediate synaptic signal transduction in the optical system of barnacles (Stuart 1999). It also promotes the settlement and metamorphosis of cyprid larvae (Jin et al. 2014b). Histamine receptor antagonists are considered to be a new class of environmentally friendly antifouling candidates (Jin et al. 2014a). This research used RNA-seq to reveal the molecular mechanisms behind the settlement of cyprid larvae in response to exposure to histamine and the anti-histamine compound loratadine.

Our transcriptome data showed that MAPK, PI3K–Akt, and focal adhesion signaling pathways were involved in the settlement of cyprid larvae in

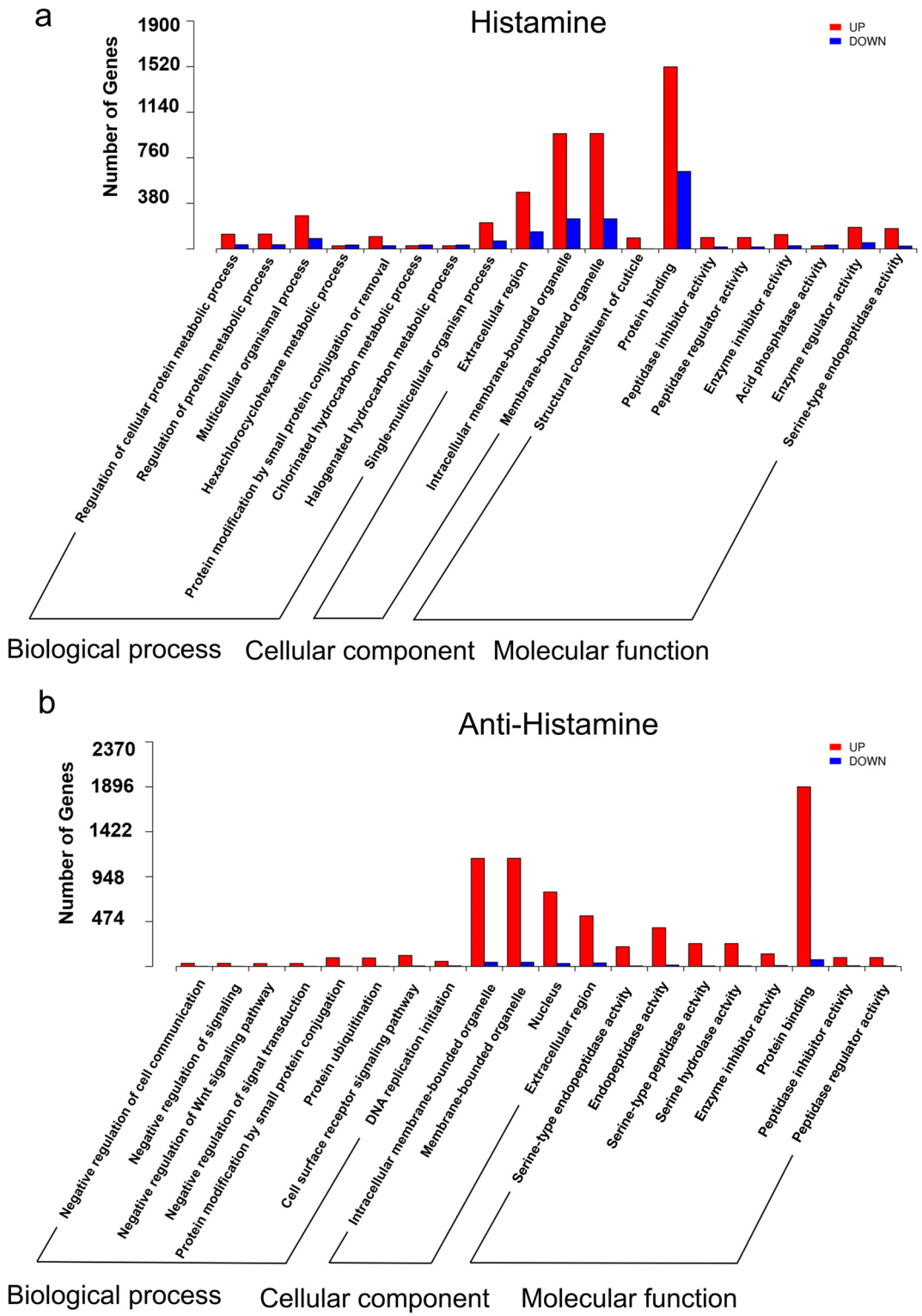

Fig. 3. Kyoto Encyclopedia of Genes and Genomes distributions of differentially expressed genes in the (a) histamine and (b) anti-histamine groups. Wnt signaling: wingless/integrated signaling

response to histamine and loratadine treatments. MAPK signaling participates in cell proliferation and apoptosis (Zhang & Liu 2002). Among the 3 classic MAPKs, previous studies have highlighted the involvement of p38 MAPK in barnacle larval settlement (Clare 1996, Pearson et al. 2001, He et al. 2012,

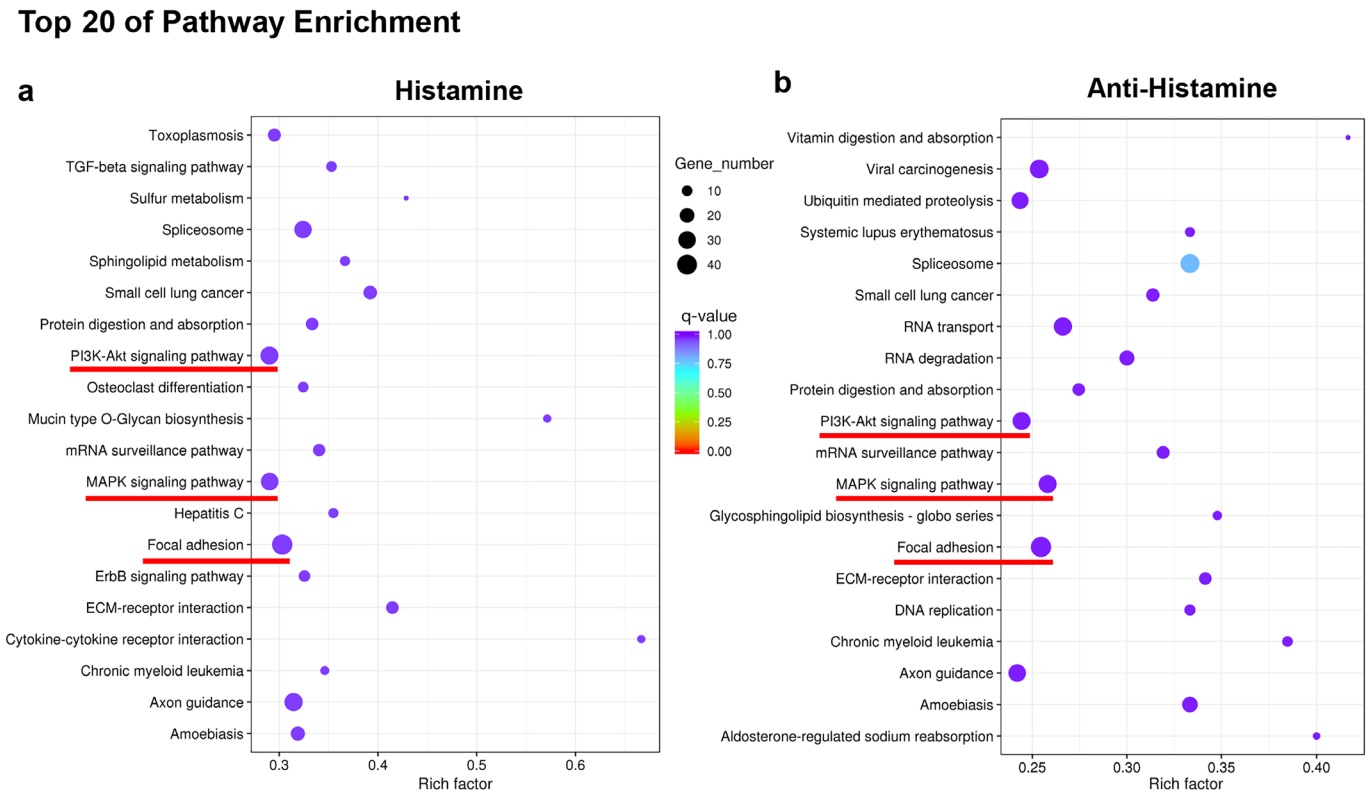

Fig. 4. Top 20 enriched Kyoto Encyclopedia of Genes and Genomes pathways in the (a) histamine and (b) anti-histamine groups. Red underlining indicates the critical enriched pathways related to the larval settlement which may need more attention

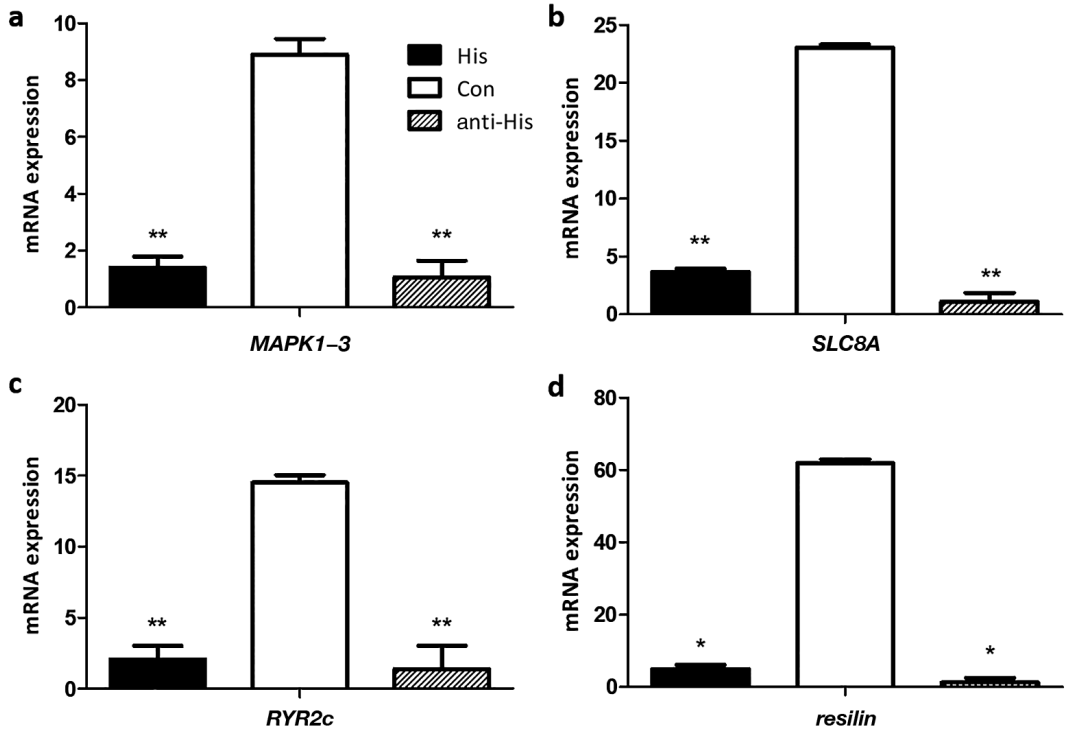

Fig. 5. Confirmation of the transcriptome sequencing data by real-time quantitative PCR (qRT-PCR) in the histamine (His), control (Con), and anti-histamine (anti-His) groups. Four differentially expressed genes participating in calcium regulation and cell proliferation were selected for qRT-PCR: (a) *MAPK1–3*; (b) *SLC8A*; (c) *RYR2c*; (d) *resilin*. All data represent the mean ± SD of 3 replicates. *$p < 0.05$ (compared with control group); **$p < 0.01$ (compared with control group)

Table 4. Summary of functional genes. Nr: National Center for Biotechnology Information non-redundant database

Classification	Gene ID	Nr ID	Nr description
Structural proteins	Cluster-15697.62226	XP_018332688.1	Pupal cuticle protein
	Cluster-15697.1975	XP_021190301.1	Endocuticle structural glycoprotein
	Cluster-15697.2036	AQT26399.1	Chitin-binding protein
	Cluster-15697.62225	XP_014363698.1	Larval cuticle protein LCP-22-like
Spider silk proteins	Cluster-13681.1	PRD30395.1	*astD* (*Nephila clavipes*)
	Cluster-49897.0	PRD32697.1	*oprD* (*Nephila clavipes*)
	Cluster-33700.0	PRD39122.1	*ppnN* (*Nephila clavipes*)
	Cluster-28953.0	PRD34671.1	*dmoA* (*Nephila clavipes*)
Energy metabolism proteins	Cluster-15697.121	AOR07112.1	Elongation factor 1-alpha
	Cluster-36431.0	–	ATP-cAMP, G protein
	Cluster-15697.44848	XP_023711278.1	Adiponectin receptor protein
Cement proteins	Cluster-43911.0	AQA26372.1	CP19-like cement protein 3, partial
Glycosyl proteins	Cluster-27156.3	XP_018022545.1	N-acetylglucosaminyl-diphospho-decaprenol
Multifunctional proteins	Cluster-15697.5254	KMQ83307.1	Agmatine deiminase
	Cluster-15697.49958	XP_017011291.1	Anthranilate 1,2-dioxygenase large subunit-like
	Cluster-15697.47121	AAN10061.1	Kunitz-like protease inhibitor precursor
	Cluster-15697.62346	BAU68125.1	Carbonic anhydrase
	Cluster-15697.53273	XP_019627849.1	Fibrillin-1-like
	Cluster-39363.0	ABU41029.1	Hypothetical protein

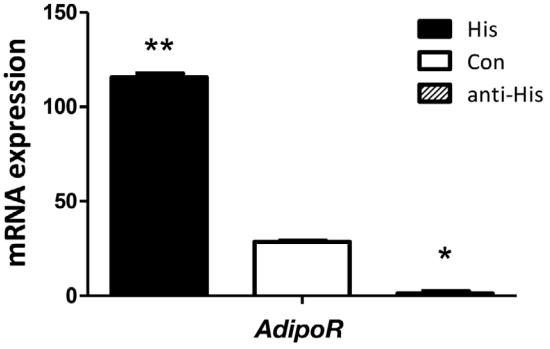

Fig. 6. Comparison of *AdipoR* gene expression in histamine (His), control (Con), and anti-histamine (anti-His) groups by real-time quantitative PCR. All data represent the mean ± SD of 3 replicates. $^{*}p < 0.05$ (compared with control group); $^{**}p < 0.01$ (compared with control group)

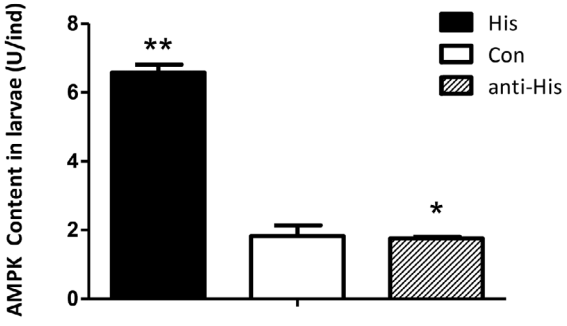

Fig. 7. AMP-activated protein kinase (AMPK) activities of cyprid larvae in the histamine (His), control (Con), and anti-histamine (anti-His) groups. The His group was treated with 5×10^{-8} mol l^{-1} histamine solution and the anti-His group with 5×10^{-7}mol l^{-1} loratadine solution; the Con group was untreated (filtered seawater). All experiments were replicated at least 3 times. All data represent the mean ± SD of 3 replicates. $^{*}p < 0.05$ (compared with control group); $^{**}p < 0.01$ (compared with control group)

Zhang et al. 2013). Our research identified that the percent larval settlement in anti-histamine-treated animals (25.19%) was significantly lower than that in the histamine-treated group (62.94%). In addition, a remarkable number of components in the MAPK pathway were differentially expressed upon histamine and anti-histamine treatment. These results suggested that the down- and up-regulation of the MAPK pathway were consistent with the induction and inhibition of cyprid larvae settlement. Although it is difficult to clarify the role of each specific component in the MAPK pathway in regulating larval settlement and metamorphosis, the key enzyme p38 MAPK was found to be differentially expressed upon histamine and anti-histamine treatment, and the His group was significantly higher than the anti-His group. Our results therefore represent an addition to the existing evidence that p38 MAPK plays a critical role in regulating larval settlement in various fouling organisms, including tubeworms, bryozoans, and barnacles (Wang & Qian 2010, He et al. 2012, Yang et al. 2018). Another 2 pathways involving PI3K–Akt (Kim & Chung 2002) and focal adhesion (Gan et al. 2020) have also been found to play critical roles in larval survival and settlement in sessile organisms. These 2

pathways are also implicated in environmental perception and physiological responses and are suggested to be part of the physiological adaptation by deep-sea barnacles in response to complex deep-sea environments (Gan et al. 2020). Since histamine and anti-histamine play major roles in larval physiology, differential expression of components involved in the PI3K–Akt pathway and focal adhesion suggested linkages between histamine and anti-histamine, the PI3K–Akt pathway, focal adhesion, and larval physiology. We speculate that these factors may exhibit complex interactions and function as an intact regulatory system in regulating larval settlement in barnacles. In addition to signal transduction pathway genes, we performed in-depth analysis to identify highly expressed genes that showed distinct patterns and were clearly responsive to histamine treatment.

The 4 filtering criteria for histamine-responsive genes enabled us to identify genes that are upregulated in response to histamine treatment, downregulated by anti-histamine treatment, abundantly expressed in cyprids, and statistically significant in the triplicated experimental design. Although we expected to retrieve mostly structural genes, we were nevertheless surprised to identify 4 cuticle proteins, 4 spider silk proteins, and an adult cement protein CP-19k homologue, which have all been implicated in barnacle cement or cuticle formation. During cyprid settlement, the larva undergoes drastic tissue degeneration and organ remodeling (Maruzzo et al. 2012). These changes might be reflected in the differential expression of some structural proteins. Once the larva is committed to permanent attachment, cyprid cement from the larval cement gland is released from the antennae, and this is a highly energy-consuming process (Lucas et al. 1979, Thiyagarajan et al. 2002). Hence, it is interesting to have recovered a CP-19k homologue as well as silk-like proteins from the list of DEGs, as both categories are implicated in attachment. More intriguingly, CP-19k has always been reported as the adult cement in barnacles, and this is perhaps the first report that this adult cement protein homologue gene is expressed in cyprids. Similarly, silk-like proteins were recovered from *Amphibalanus amphitrite* adult cement but have never been implicated in larval cement (So et al. 2016), probably owing to a lack of studies concerning barnacle larval cement. While it was not the objective of the present study to determine if CP19k or silk-like proteins are involved in larval permanent attachment, the result that histamine and anti-histamine treatment led to differential expression of CP19k, silk proteins, and cuticle proteins suggests the neurotransmitter could exert a major effect on the preparation or production of structural proteins that are important for cyprid settlement. These results further emphasize the master regulatory role of histamine and the importance of MAPK and PI3K–Akt pathways in cyprid settlement and metamorphosis.

In addition to structural components, 3 energy metabolism-related proteins, eEF1a1, cAMP, and AdipoR, were also found to be histamine responsive in cyprids. Energy metabolic genes play crucial roles in the development of cyprid larvae (Zhang et al. 2016). Interestingly, histamine is one of the neurotransmitters that can regulate numerous aspects of energy balance. Histamine receptor 1 (HR1) mediates excitatory actions on central neurons by recruiting Gq/11 and PLC, which causes the release of Ca^{2+} ions from intracellular stores (Tabarean 2016), and calcium channels have been found to be involved in barnacle settlement. In contrast, histamine antagonists (anti-histamine) decrease energy expenditure (Clare 1996).

Adiponectin (AdipoQ) and its receptor (AdipoR) are closely associated with skeletal muscle growth and development, along with glucose and lipid metabolism (Kim et al. 2016). Decreased *AdipoR* expression results in diminished fatty acid metabolism. In contrast, increased *AdipoR* expression facilitates fatty acid oxidation (Matsunami et al. 2011). In our research, *AdipoR* expression was elevated in the His group but was inhibited in the anti-His cohort, which indicated that histamine treatment led to differential expression of *AdipoR*. We anticipate that the differential expression of *AdipoR* will signal consumption of lipids, thereby enhancing cyprid settlement. In contrast, the anti-histamine compound loratadine can impair such settlement by inhibiting *AdipoR* expression. Furthermore, *AdipoQ* can induce Ca^{2+} influx via *AdipoR*, which is critical for the activation of the calmodulin-dependent protein kinase beta, AMPK, and NAD-dependent protein deacetylase sirtuin-1 (Iwabu et al. 2010).

AMPK plays a critical role in regulating the whole-body energy metabolism of the cell (Carling 2004). For the settlement of barnacles, energy metabolism plays a key role in supporting a series of cellular processes (Zhang et al. 2016). The AMPK downstream pathway, Adenosine kinase-AMPK-Forkhead box O (FoxO), can induce larval settlement and metamorphosis of marine invertebrates by activating the downstream AMPK–FoxO signaling pathway (He et al. 2021). To further validate the effects of histamine and anti-histamine treatment on cyprid energy metabolism, we performed analyses of biochemical com-

ponents and measurements of oxygen consumption rates by cyprids. The results showed that AMPK activity decreased with diminishing *AdipoR* expression, suggesting that *AdipoR* activates the AMPK pathway to regulate the settlement of barnacle cyprids.

5. CONCLUSIONS

This study improves our understanding of the complex process of histamine-induced settlement of *Amphibalanus amphitrite* cyprid larvae. Importantly, up-regulation of cement protein components in response to histamine treatment provides novel insights into the development of environmentally friendly anti-fouling compounds. Our transcriptome data also indicate the critical role of the MAPK signaling pathway in the settlement of larval barnacles. Our data suggest the energy metabolism gene *AdipoR* could affect cyprid larvae settlement by regulating fatty acid oxidation. Further studies should focus on specific molecular regulatory mechanisms involved in histamine-induced larval settlement.

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