Quantitative Proteomics Study of Larval Settlement in the Barnacle *Balanus amphitrite*

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Abstract

Barnacles are major sessile components of the intertidal areas worldwide, and also one of the most dominant fouling organisms in fouling communities. Larval settlement has a crucial ecological effect not only on the distribution of the barnacle population but also intertidal community structures. However, the molecular mechanisms involved in the transition process from the larval to the juvenile stage remain largely unclear. In this study, we carried out comparative proteomic profiles of stage II nauplii, stage VI nauplii, cyprids, and juveniles of the barnacle *Balanus amphitrite* using label-free quantitative proteomics, followed by the measurement of the gene expression levels of candidate proteins. More than 700 proteins were identified at each stage; 80 were significantly up-regulated in cyprids and 95 in juveniles vs other stages. Specifically, proteins involved in energy and metabolism, the nervous system and signal transduction were significantly up-regulated in cyprids, whereas proteins involved in cytoskeletal remodeling, transcription and translation, cell proliferation and differentiation, and biomineralization were up-regulated in juveniles, consistent with changes associated with larval metamorphosis and tissue remodeling in juveniles. These findings provided molecular evidence for the morphological, physiological and biological changes that occur during the transition process from the larval to the juvenile stages in *B. amphitrite*.

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Introduction

The life cycles of most sessile marine invertebrates include a microscopic and planktonic larval stage that may last for minutes to months, as well as benthic juvenile and adult stages during which individuals attach to a submerged surface [1]. The transition from pelagic to sessile stages is referred to as larval attachment and metamorphosis (collectively known as larval settlement), which is associated with morphological, physiological and biochemical changes. Larval settlement is crucial not only for recruitment but also for species distribution and community structures [2]. Although details of the signal transduction pathways and mechanisms that regulate larval settlement have been partially reported in some species [3,4], the associated molecular mechanisms remain largely unknown in most marine invertebrate species, due to their high biological diversity.

The barnacle *Balanus amphitrite* is a dominant fouling organism worldwide. *B. amphitrite* larvae released from adults molt 6 times and transit to cyprids, the competent stage for subsequent settlement. The process of settlement can be divided into 3 phases: attainment of competency, attachment to a suitable substratum, and metamorphosis into juveniles [5]. The morphogenetic development associated with metamorphosis includes decortication of the cyprid carapace, formation of a new chitinous layer, migration of the naupliar eye, degeneration of the compound eyes and antenna, and development of the feeding cirri [6]. In addition, physiological, structural and functional changes occur, all of which are regulated by functional genes and proteins [7]. Six cyprid-specific genes were first isolated from a cyprid cDNA library [8], and responded differentially to settlement cues [9]. Recently, we conducted a comparative transcriptomic study and identified several genes with potential roles in the larval settlement process [5].

There is no predictive correlation between mRNA and protein levels. Because proteins directly mediate most biological events, evaluation of changes in their levels could provide comprehensive biological insights [10]. An earlier 2-DE-based proteomic study from our laboratory revealed approximately 400 spots and identified some proteins that were differentially expressed during barnacle larval settlement [10]. Furthermore, a significantly higher number of protein spots were obtained when implementing additional solution-phase IEF sample prefractionation and narrow-pH-range IEF [11]. However, the 2-DE method has a
relatively poor reproducibility, low sensitivity, and narrow linear dynamic ranges [12]. In addition, few proteins exhibiting a relatively lower expression level could be identified using mass spectrometry in a 2-DE-based analysis. In contrast, a gel-free proteomics technique incorporating a combination of multidimensional liquid chromatography (LC) separation, MS analysis and sequence database searches could provide a robust and effective platform for direct analysis of the proteome of the bryozoan Bugula neritina [13]. In the present study, we used a label-free quantitative proteomic platform to profile the proteomes of 4 developmental stages of B. amphitrite, compared 4 proteomes to identify many differentially expressed proteins that might play key roles in the settlement of B. amphitrite, and confirmed the expression patterns of numerous proteins using quantitative real-time polymer chain reaction (qRT-PCR).

Materials and Methods

Sample preparation

The barnacle, Balanus amphitrite, that we used for this study is a common species of marine invertebrates. It is a biofouling species and not endangered or protected. Balanus amphitrite adults were collected from a dock in Pak Sha Wan, Hong Kong (22°21′45″ N, 114°15′35″ E). No specific permits were required for the described field studies. The dock does not belong to any national parks, protected areas, or privately-owned places. The filed studies did not involve any endangered or protected species.

Larvae of different stages were obtained and cultured according to the methods described by Thiyagarajan and Qian [10]. Briefly, newly released larvae were maintained in filtered seawater (FSW) for 2 h and then collected as stage II nauplii. Other larvae were cultured at 27°C and fed with Chaetoceros gracilis Schutt for 3 to 4 d until they had developed into stage VI nauplii with 2 compound eyes. After 18–24 h, a portion of the cyprids undergoing molting from stage VI nauplii was collected; the remaining cyprids attached to polystyrene Petri dishes (Falcon no. 1006) in the dark. Most of the cyprids attached to the dishes within 24 h and completed metamorphosis into juveniles within 48 h. The juveniles were then scraped off the dishes. All of the samples were stored at −80°C until use.

Protein extraction and digestion

The samples were resuspended in 1 mL protein lysis buffer containing 0.1 M Tris-HCl (pH 7.6), 2% SDS, 0.1 M dithiothreitol and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After homogenization, the samples were sonicated 3 times using a Misonix sonicator-XL2020 (Misonix, Farmingdale, NY) and then incubated in boiling water for 5 min. Larvae and debris that were not lysed were pelleted by centrifugation at 15,000 g for 10 min at 4°C. The protein-containing supernatant was transferred into a new tube, and the protein concentration was quantified using the RC/DC protein house protein database developed in our transcriptome study [5]. The 10 most intense ions above a 500-count threshold and carrying a charge from 2+ to 4+, were selected for MS/MS fragmentation. Dynamic exclusion was activated using a repeat count of 2, an exclusion duration of 45 s, and a mass tolerance of ±5 ppm. The CID parameters included a normalized collision energy of 35%, an activation Q of 0.25, an isolation width of 3.0 and an activation time for 10 ms.

Database search and data analysis

The raw MS data were converted into mascot generic files using Proteome Discover (version 2.2, Matrix Sciences Ltd., London, UK) for searching against an in-house protein database developed in our transcriptome study [5]. The mass tolerances were set at 10 ppm for the peptide precursors and 0.5 Da for the fragment ions. A decoy option was included. Carbamidomethylation at cysteine residues was set as a fixed modification, and oxidation at methionine residues was set as a variable modification. Up to 1 missed trypsin cleavage was permitted.

The resulting .dat files from the Mascot search were processed using Scaffold (version 4.0, Proteome Software Inc., OR, USA) to validate the MS/MS identification. Peptide identification was accepted if the result could established at >95.0% probability by the Scaffold Local FDR, whereas protein identification was accepted if the result had a probability of >99.0% and contained at least 1 identified peptide. Peptide and protein probabilities assigned by the PeptideProphet [16] and ProteinProphet [17]. Protein XML files were then exported from Scaffold to calculate the protein abundances were calculated using APEX quantitation proteomics tools [18,19]. A 1.5-fold change was set as the cutoff, and only proteins with >5,000 molecules per cell during at least one stage were considered for significantly up-regulated proteins and proteins with >5,000 molecules per cell during at least 3 stages were considered for significantly down-regulated proteins [20].

Phylogenetic analysis of vitellogenin

Amino acid sequences of vitellogenin from various species were downloaded from GenBank and aligned using MUSCLE [21] via the CIPRES Portal v2.2 [22]. Neighbor-joining analysis was performed with MEGA4. All of the positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion option). The topological stability was evaluated based on 1,000 bootstrapping (BS) replications.
Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from each stage as well as from adults was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The quantity and quality of the RNA were assessed using agarose gel electrophoresis and a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), respectively. Trace genomic DNA in the RNA solution was removed using the Turbo DNAfree Kit (Ambion Inc, Austin, TX). First-strand cDNA was synthesized from RNA using MMLV reverse transcriptase (USB, Cleveland, OH) with oligo d(T)18 primer. Gene-specific primers were designed with Primer3 software [23], and the sequences of all primers used are listed in Table S1. According to the standard protocol, the qRT-PCR assays were conducted using SYBR Green Supermix (BioRad) on an ABI 7500 fast real-time PCR machine (Applied Biosystems, Foster City, CA). Cytochrome b (Cyb) was employed as an internal control for normalization [24]; the relative expression patterns were calculated based on the 2^-ΔΔCt method [25,26]. Significant differences in expression patterns were analyzed by one-way ANOVA followed by the Tukey’s post-hoc test.

Results

Four developmental stages of B. amphitrite were collected for our proteomic profiling. After MS profiling, 2,121 proteins were isolated from stage II nauplii, 785 from stage VI nauplii, 1,036 from cyprids, and 1,176 from juveniles (Figure 1A, Table S2). Among the total 2,520 proteins, 360 proteins were common to all 4 developmental stages. Using gene ontology (GO), 1,793 of 2,520 proteins (accounting for 71.15% of the total proteins) were categorized into several functional groups, including 1,555 of 2,118 (73.42%) in stage II nauplii, 570 of 783 (72.80%) in stage VI nauplii, 790 of 1,034 (76.40%) in cyprids and 895 of 1,174 (76.24%) in juveniles (GO categories are shown in Figure 2). The majority of the proteins displayed binding and catalytic activity (37.47%–44.14%), and the remainder were associated with structural molecule activity and transporter functions (4.19%–7.90%). Unlike the other 3 developmental stages, stage VI nauplii had more proteins that displayed catalytic (44.09%) rather than with binding activity (37.47%). The remaining categories included electron carrier activity, enzyme regulator activity, receptor activity, antioxidant activity, nucleic acid binding, and transcription factor activity, among others.

Comparative proteomic analysis among the 4 developmental stages of B. amphitrite revealed that 398 proteins met the established criteria, displaying a 1.5-fold change and >5,000 molecules per cell in at least one stage (Figure 1B). Among them, 143 in stage II nauplii, 80 in stage VI nauplii, 80 in cyprids and 95 in juveniles were significantly up-regulated (listed in Tables 1, 2, S3 and S4). Interestingly, 118 proteins were uniquely expressed in stage II nauplii, accounting for 82.51% of the total 143 proteins. In contrast, 36, 22 and 27 proteins were uniquely expressed in stage VI nauplii, cyprids and juveniles, respectively. In addition, 129 proteins were significantly down-regulated in the 4 developmental stages, which is less than the up-regulated ones (Figure 1B). Among them, 19 were in stage II nauplii, 82 in stage VI nauplii, 9 in cyprids and 19 in juveniles (listed in Table S5). To identify the important proteins involved in the larval settlement process of barnacles, proteins that were significantly up-regulated in cyprids (listed in Table 1) were categorized into a diverse set of functional groups using GO and eukaryotic orthologous groups (KOG). Approximately 30% proteins were related to energy and metabolism; others were associated with structural molecules, nervous system-related molecules, and signaling molecules, among others. Additionally, proteins that were significantly up-regulated in juveniles (Table 2) might be crucial for tissue reorganization and development postlarval settlement. The top 3 categories detected were structural, transcription and translation, and energy and metabolism proteins. The other proteins were categorized into various functional groups such as cell differentiation-related, cell proliferation-related, shell calcification-related, and stress-induced proteins.

To analyze the gene expression of the selected proteins, qRT-PCR was conducted. As shown in Figure 3, a positive correlation between transcription and translation expression patterns was detected in several proteins, such as neuroglian, failed axon connection protein and lipoprotein receptor. In contrast, the expression trends observed for some proteins, including fructose-bisphosphate aldolase, histone cluster 1 H2a and glutathione S-transferase Mu 3, did not display any similarity with their gene expression profiles. Interestingly, some transcripts accumulated prior to the peak expression of the protein products, as observed for fructose 1,6-bisphosphatase, arginine kinase, histone H1, nmyc downstream gene protein, cyclolin B and peptidyl-prolyl cis-trans isomerase.

Discussion

Barnacle larval settlement includes the attainment of competency by swimming cyprids, attachment to the substratum, and the metamorphosis of attached cyprids into juveniles, which involve energy-related, neurotransmission-related, and cell proliferation and differentiation-related molecules are involved [5]. With a special focus on larval settlement and subsequent juvenile development, we identified 80 and 95 proteins that were significantly up-regulated in the cyprid and the juvenile proteome, respectively.

Proteins that were significantly up-regulated in cyprids

Energy and metabolism proteins. Twenty-four energy-related proteins were significantly up-regulated in cyprids, accounting for approximately 30% of the up-regulated proteins in the larval stage (shown in Table 1). Fructose-bisphosphate aldolase, fructose 1,6-bisphosphatase, glucose-6-phosphate isomerase, gluconic-acid-6-phosphate isomerase 1 and mannose-6-phosphate isomerase are involved in the metabolism of fructose 6-phosphate, which is one of the key products in glycolysis and its reverse process, gluconeogenesis, for converting a non-carbohydrate to glucose [27]. Because cyprids do not feed, they rely on stored lipids and proteins as primary energy sources to swim, search for a suitable surface for attachment and metamorphosis [28]. The up-regulation of fructose 1,6-bisphosphatase in cyprids suggested that lipids and proteins were converted to glucose via active gluconeogenesis to support cyprid energy consumption. Interestingly, the transcript displayed a higher expression level in stage VI nauplii than in other stages (Figure 3B). Furthermore, because two of the enzymes catalyzed reactions in both glycolysis and gluconeogenesis, fructose-bisphosphate aldolase and glucose-6-phosphate isomerase might play important roles in maintaining the balance between these two metabolic pathways in cyprids.

In the present study, acetyl-CoA acetyltransferase was significantly up-regulated during the cyprid stage, which is consistent to the results of an early study demonstrating that the acetyl-CoA acetyltransferase gene was significantly up-regulated during this stage [29]. Acetyl-CoA carboxylase 2 and acyl-CoA oxidase participate in the catabolism of lipids through fatty acid oxidation [30,31]. The product of acetyl-CoA enters citric acid cycle to...
produce energy. Acetyl-CoA acetyltransferase is involved in several metabolic pathways, including ketone body synthesis and degradation, fatty acid metabolism and pyruvate metabolism. Cyprids become less active and their settlement rate decreases in response to treatment with butenolid [32], as acetyl-CoA acetyltransferase is one of the binding targets of butenolid in barnacle cyprids and is involved in the inhibition of larval settlement triggered by butenolid [29]. Taken together, these results indicated that acetyl-CoA acetyltransferase is a crucial enzyme for maintaining barnacle cyprid activity and their subsequent successful settlement.

In the barnacle proteome, two vitellogenins were identified. Vitellogenin 1α was only detected in the stage II nauplii (Table S2); however, its gene expression level was 1,000 times higher in adults (Figure 3D), suggesting that this maternal vitellogenin 1α might function as a storage protein for embryonic and early larval development, similar to the general vitellogenin in other species. Interestingly, unlike vitellogenin 1α, vitellogenin 1β was highly expressed in stage VI nauplii and cyprids (Table S2), and its
The expression pattern was similar to that of the cyprid major protein. The cyprid major protein is utilized as an energy resource during cyprid settlement and metamorphosis [33]. It was identified as a vitellogenin 1β-like protein based on the results of SDS-PAGE and LC-MS analysis [34]. To better explore the relationship between the two vitellogenins detected in B. amphitrite, we conducted phylogenetic reconstruction (Figure 4). Our results revealed the monophyly of copepod Vitellogenin 1 (BS = 100), copepod Vitellogenin 2 (BS = 100) and the sister-relationship between Vitellogenin 1α and 1β (BS = 81). Furthermore, the last two genes also formed the sister taxon of copepod Vitellogenin 1 (BS = 58). These relationships suggest that barnacle Vitellogenins have functions similar to other vitellogenins; however, Vitellogenin 1β has evolved a novel and related function after gene duplication such that it is highly expressed in stage VI nauplii and cyprids rather than embryos or adults.

Nervous system-related molecules. Cyprids possess a more complicated nervous system compared with nauplii and adults [35]. Extensive studies have been conducted to investigate neurotransmitters such as serotonin and prostanoid [36,37]. In the current study, we detected one neurotransmitter receptor, i.e., acetylcholine receptor, with a high expression level in cyprids. This receptor binds to the neurotransmitter acetylcholine, which is involved in barnacle cyprid muscular contraction and cement gland exocytosis [38]. Elevated levels of acetylcholine lead to a higher settlement rate of cyprids [38]. The up-regulation of this receptor in cyprids herein confirmed that acetylcholine has a key role in barnacle larval settlement.

N-acetylated alpha-linked acidic dipeptidase is responsible for the cleavage of the neuropeptide N-acetyl-L-aspartate-L-glutamate (NAAG), which is abundant in the central nervous system and functions as a neurotransmitter [39]. A previous study revealed the effects of NAAG on the reduction of cAMP levels and the release of GABA [40,41]. In B. amphitrite, the intracellular cAMP level affects the patterns of cyprid settlement [42]. As a neurotransmitter, GABA controls thoracic muscle contraction and...
Table 1. Significantly Up-regulated Proteins in Cyprids.

| Accession No. | Protein Description                                      | Protein abundance (<1000 molecules/cell) | Protein ratios |
|---------------|---------------------------------------------------------|------------------------------------------|----------------|
|               |                                                         | nau2          | nau6          | cyp           | juv           | nau2/cyp | nau6/cyp | juv/cyp |
| CL5556.Contig1| Chromosome 14 open reading frame 149                   | N.D.          | 8.78          | 2.01          | 29.75         | 0.93      | 0.00      | 0.00     |
| Unigene12197  | Serine hydroxymethyl-transferase, mitochondria         | 4.03          | 2.33          | N.D.          | 16.26         | 2.86      | 0.25      | 0.14     |
| CL13389.Contig1| prolyl endopeptidase                                    | 2.59          | 0.90          | 1.54          | 0.07          | 21.40     | 1.52      | 0.12     |
| CL6434.Contig1| Cytosolic non-specific dipeptidase                      | 16.25         | 1.39          | 23.57         | 6.75          | 47.51     | 3.40      | 0.34     |
| Unigene15104  | CG3999 CG3999-PA                                        | N.D.          | N.D.          | 8.78          | 2.01          | 29.75     | 0.93      | 0.00     |
| Unigene6569   | dimethylglycine dehydrogenase, mitochondrial-like       | N.D.          | N.D.          | N.D.          | 8.65          | 2.53      | 0.00      | 0.00     |
| CL346.Contig1  | 4-aminobutyrate aminotransferase                       | N.D.          | N.D.          | N.D.          | 6.14          | 0.29      | 0.54      | 0.02     |
| CL10509.Contig1| betaine homocysteine methyltransferase                 | N.D.          | N.D.          | N.D.          | 8.65          | 2.53      | 0.00      | 0.00     |
| CL3803.Contig1 | Acetyl-CoA acetyltransferase, mitochondrial             | 3.33          | 1.28          | 2.06          | 0.02          | 19.44     | 2.63      | 0.17     |
| CL1120.Contig2 | pyrroline-5-carboxylate dehydrogenase                  | 20.82         | 0.76          | 26.34         | 6.95          | 69.73     | 3.99      | 0.30     |
| CL6520.Contig1 | hydroxyacid-o xoacid transhydrogenase, mitochondrial  | 5.39          | 2.42          | N.D.          | 20.87         | 1.39      | 0.26      | 0.12     |
| Accession No. | Protein Description | Protein abundance (<1000 molecules/cell) | Protein ratios |
|--------------|---------------------|----------------------------------------|----------------|
|              |                     | nau2 | nau6 | cyp | juv | nau2/cyp | nau6/cyp | juv/cyp |
|              |                     | ave  | SD   | ave  | SD  | ave  | SD  | p    | ave  | SD   | p    | ave  | SD   | p    |
| CL1939.Contig1 | malate synthase, glyoxysomal | N.D. | N.D. | N.D. | 14.20 | 3.58 | 4.67 | 1.18 | 0.00 | 0.00 | 0.005 | 0.00 | 0.005 | 0.33 | 0.08 | 0.007 |
| CL5668.Contig1 | CG7280-PA            | 1.62 | 0.69 | N.D. | 17.34 | 4.89 | 4.95 | 4.05 | 0.09 | 0.04 | 0.001 | 0.07 | 0.00 | 0.006 | 0.29 | 0.23 | 0.028 |
| CL271.Contig3 | mitochondrial aldehyde dehydrogenase precursor | 27.10 | 1.68 | N.D. | 63.61 | 5.66 | 32.13 | 5.27 | 0.43 | 0.03 | 0.001 | 0.00 | 0.001 | 0.51 | 0.08 | 0.003 |
|              |                     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
|              | Structural Molecules |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Unigene13912 | projectin | 6.34 | 0.39 | 8.77 | 0.11 | 23.62 | 1.19 | 6.36 | 0.53 | 0.27 | 0.02 | 0.001 | 0.37 | 0.00 | 0.001 | 0.27 | 0.02 | 0.00 |
| Unigene28824 | Titin              | 6.83 | 0.69 | N.D. | 14.87 | 0.34 | 32.13 | 5.27 | 0.43 | 0.03 | 0.001 | 0.00 | 0.001 | 0.51 | 0.08 | 0.003 |
| CL682.Contig1 | Muscle M-line assembly protein unc-89 | 1.11 | 0.39 | N.D. | 6.42 | 1.26 | N.D. | N.D. | 0.17 | 0.06 | 0.001 | 0.00 | 0.001 | 0.00 | 0.00 | 0.00 |
| Unigene10292 | translocase of outer membrane protein 34 | N.D. | N.D. | N.D. | 5.96 | 0.34 | N.D. | N.D. | 0.28 | 0.05 | 0.001 | 0.00 | 0.001 | 0.00 | 0.00 | 0.00 |
| Unigene2708  | BMKETTIN           | 3.60 | 3.14 | N.D. | 21.33 | 6.94 | N.D. | N.D. | 0.17 | 0.05 | 0.001 | 0.00 | 0.001 | 0.00 | 0.00 | 0.00 |
| CL1129.Contig1 | Echinoderm microtubule-associated protein | 4.68 | 0.86 | N.D. | 11.25 | 2.12 | 2.50 | 2.20 | 0.42 | 0.08 | 0.001 | 0.00 | 0.001 | 0.03 | 0.22 | 0.20 |
|              | Transcription and Translation |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| CL128.Contig1 | ribosomal protein L10 | 16.26 | 4.07 | 5.93 | 1.62 | 29.64 | 1.00 | 18.58 | 1.00 | 0.55 | 0.14 | 0.009 | 0.20 | 0.05 | 0.001 | 0.63 | 0.03 | 0.00 |
| CL136.Contig1 | ribosomal protein L18A family member | 5.59 | 1.02 | 11.50 | 1.33 | 20.06 | 1.63 | N.D. | N.D. | 0.28 | 0.05 | 0.001 | 0.57 | 0.07 | 0.001 | 0.00 | 0.00 | 0.00 |
| CL814.Contig1 | 40S ribosomal protein S2 | 9.17 | 1.01 | 7.44 | 1.78 | 25.55 | 2.99 | 15.14 | 1.26 | 0.36 | 0.04 | 0.001 | 0.29 | 0.07 | 0.004 | 0.59 | 0.05 | 0.004 |
| Unigene1657  | ribosomal protein L9e | 15.02 | 3.74 | 16.52 | 1.52 | 42.90 | 2.09 | 23.35 | 7.07 | 0.35 | 0.09 | 0.001 | 0.39 | 0.04 | 0.001 | 0.54 | 0.16 | 0.014 |
| CL6677.Contig1 | translational activator GCN1 | 12.85 | 1.02 | N.D. | 21.49 | 1.69 | 11.36 | 3.60 | 0.60 | 0.05 | 0.001 | 0.00 | 0.001 | 0.00 | 0.00 | 0.00 |
| CL2773.Contig1 | 10-formyltetrahydrofolate dehydrogenase | 1.59 | 0.13 | N.D. | 11.22 | 2.23 | N.D. | N.D. | 0.14 | 0.01 | 0.008 | 0.00 | 0.008 | 0.00 | 0.00 | 0.00 |
|              | Stressed-Induced Protein |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| CL1841.Contig1 | catalase             | 10.20 | 1.92 | N.D. | 27.14 | 2.84 | 5.48 | 1.22 | 0.38 | 0.07 | 0.001 | 0.00 | 0.001 | 0.00 | 0.02 | 0.05 |
| CL7722.Contig1 | glutathione S-transferase mu | 3.85 | 1.84 | N.D. | 12.42 | 2.48 | N.D. | N.D. | 0.31 | 0.15 | 0.011 | 0.00 | 0.001 | 0.00 | 0.00 | 0.206 |
| CL1159.Contig1 | glutathione S-transferase Mu 3 | 3.40 | 0.95 | N.D. | 20.55 | 3.13 | N.D. | N.D. | 0.17 | 0.05 | 0.001 | 0.00 | 0.001 | 0.00 | 0.00 | 0.002 |
| Unigene5253  | glutathione peroxidase | 6.94 | 1.21 | N.D. | 10.88 | 1.95 | N.D. | N.D. | 0.64 | 0.11 | 0.036 | 0.00 | 0.003 | 0.00 | 0.00 | 0.003 |
|              | Nervous System-Related Molecules |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Unigene6214  | N-acetylated alpha-linked acidic dipeptidase 2 | 5.39 | 4.28 | 11.05 | 0.74 | 22.38 | 1.63 | 6.42 | 0.38 | 0.24 | 0.19 | 0.008 | 0.49 | 0.03 | 0.002 | 0.29 | 0.02 | 0.00 |
| CL1083.Contig2 | neuroglan | 3.30 | 0.63 | N.D. | 20.66 | 1.85 | 8.32 | 0.41 | 0.16 | 0.03 | 0.001 | 0.00 | 0.001 | 0.00 | 0.00 | 0.40 | 0.02 |
| Unigene14124 | Cysteine string protein | N.D. | N.D. | N.D. | 6.50 | 0.63 | N.D. | N.D. | N.D. | only in cyp | 0.001 | only in cyp | 0.001 | only in cyp | 0.001 |
| CL745.Contig1 | Fasciclin-2 | 4.75 | 0.73 | N.D. | 17.03 | 3.42 | 3.67 | 0.12 | 0.28 | 0.04 | 0.002 | 0.00 | 0.002 | 0.00 | 0.00 | 0.04 | 0.22 |

**Table 1.** Proteins with significant changes in abundance and ratios between various stages of barnacle development.
| Accession No. | Protein Description | Protein abundance (<1000 molecules/cell) | Protein ratios |
|---------------|---------------------|------------------------------------------|----------------|
|               |                     | nau2 | nau6 | cyp | juv | nau2/cyp | nau6/cyp | juv/cyp |
| CL716.Contig1 | Fasciclin-2          | 7.65 | 1.22 | 1.40| 0.01| 28.50    | 5.46     | 6.16    | 2.20 | 0.27 | 0.04 | 0.001 | 0.05 | 0.00 | 0.005 | 0.22 | 0.08 | 0.002 |
| CL6416.Contig1| Acetylcholine receptor subunit alpha-type acr-16 | N.D. | N.D. | 4.82| 0.36| 7.27     | 0.53     | N.D.    | N.D. | 0.00 | 0.00 | 0.000 | 0.66 | 0.04 | 0.008 | 0.00 | 0.00 | 0.000 |
| Unigene11480 | ELAV-like protein 2  | N.D. | N.D. | N.D.| N.D.| 9.65     | 3.46     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.011 | only in cyp | 0.011 | only in cyp | 0.011 |
|               |                     | ave  | SD  | ave | SD | ave     | SD | ave | SD | p | ave | SD | p | ave | SD | p | ave | SD | p |
| CL78.Contig2  | cyclophilin type peptidyl-prolyl cis-trans isomeras | 43.10 | 6.70 | 19.85 | 3.57 | 86.99 | 9.51 | 26.99 | 4.82 | 0.50 | 0.08 | 0.003 | 0.23 | 0.04 | 0.002 | 0.31 | 0.06 | 0.001 |
| CL3896.Contig1| malectin-A           | 5.27 | 1.15 | N.D.| N.D.| 13.15    | 3.71     | N.D.    | N.D. | 0.40 | 0.09 | 0.015 | 0.00 | 0.00 | 0.006 | 0.00 | 0.00 | 0.006 |
| Unigene7031  | Putative protein disulfide-isomerase A4 | 12.76 | 1.02 | N.D.| N.D.| 43.82    | 15.45    | N.D.    | N.D. | 0.29 | 0.02 | 0.043 | 0.00 | 0.00 | 0.010 | 0.00 | 0.00 | 0.001 |
| CL18.Contig2  | Peptidyl-prolyl cis-trans isomerase | 12.15 | 0.56 | 6.79| 0.22| 53.30    | 15.90    | 1.71    | 0.07 | 0.02 | 0.000 | 0.13 | 0.00 | 0.000 | 0.30 | 0.03 | 0.000 |
| CL7127.Contig1| cyclophilin B        | 3.47 | 1.41 | N.D.| N.D.| 6.50     | 0.47     | N.D.    | N.D. | 0.53 | 0.22 | 0.031 | 0.00 | 0.00 | 0.000 | 0.00 | 0.00 | 0.000 |
| CL14028.Contig1| receptor accessory protein 5 | N.D. | N.D. | N.D.| N.D.| 6.15     | 0.49     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.001 | only in cyp | 0.001 | only in cyp | 0.001 |
| CL4480.Contig1| Replication protein A 32 kDa subunit | N.D. | N.D. | N.D.| N.D.| 12.43    | 4.10     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.010 | only in cyp | 0.010 | only in cyp | 0.010 |
| CL4060.Contig1| Probable G-protein coupled receptor 15B | 5.55 | 0.80 | N.D.| N.D.| 11.39    | 0.58     | 3.51    | 1.65 | 0.49 | 0.07 | 0.001 | 0.00 | 0.00 | 0.000 | 0.31 | 0.14 | 0.004 |
| CL188.Contig2 | Protein phosphatase 2 | 2.42 | 1.15 | N.D.| N.D.| 17.05    | 4.88     | 7.71    | 0.67 | 0.14 | 0.07 | 0.003 | 0.00 | 0.00 | 0.007 | 0.45 | 0.04 | 0.018 |
| CL7 Contig1  | CUB-serine protease | N.D. | N.D. | N.D.| N.D.| 7.69     | 2.34     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.009 | only in cyp | 0.009 | only in cyp | 0.009 |
| CL8242.Contig1| Dipetidyl peptidease 4 | N.D. | N.D. | N.D.| N.D.| 5.77     | 1.80     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.009 | only in cyp | 0.009 | only in cyp | 0.009 |
| CL62.Contig1  | intracellular fatty acid-binding protein | 10.22 | 2.47 | 5.56| 0.21| 34.45    | 5.10     | N.D.    | N.D. | 0.30 | 0.07 | 0.001 | 0.16 | 0.01 | 0.002 | 0.00 | 0.00 | 0.000 |
| CL3340.Contig1| chorion peroxidase | N.D. | N.D. | N.D.| N.D.| 7.71     | 0.54     | 1.70    | 0.23 | 0.00 | 0.00 | 0.000 | 0.00 | 0.00 | 0.000 | 0.22 | 0.03 | 0.000 |
| Unigene8333  | Sterile alpha and TIR motif-containing protein | N.D. | N.D. | N.D.| N.D.| 11.14    | 0.41     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.000 | only in cyp | 0.000 | only in cyp | 0.000 |
| CL1705.Contig1| serine-threonine kinase receptor-associated protein | 12.27 | 0.33 | N.D.| N.D.| 27.71    | 3.93     | 3.79    | 0.57 | 0.46 | 0.01 | 0.016 | 0.00 | 0.00 | 0.002 | 0.14 | 0.02 | 0.000 |
| Unigene25709| mannosidase alpha class 2a | N.D. | N.D. | N.D.| N.D.| 13.00    | 0.65     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.000 | only in cyp | 0.000 | only in cyp | 0.000 |
| CL6388.Contig1| BCS-2 | 2.61 | 0.18 | N.D.| N.D.| 48.27    | 4.50     | 10.27   | 2.65 | 0.05 | 0.00 | 0.000 | 0.00 | 0.00 | 0.001 | 0.21 | 0.05 | 0.000 |
| Unigene6188  | CG11190 CG11190-PA | N.D. | N.D. | N.D.| N.D.| 17.22    | 1.06     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.000 | only in cyp | 0.000 | only in cyp | 0.000 |
| CL3280.Contig1| proprotein convertase subtilisin/ kexin type 9 preproprotein | N.D. | N.D. | N.D.| N.D.| 9.09     | 0.68     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.000 | only in cyp | 0.000 | only in cyp | 0.000 |
| CL6163.Contig1| AGAP003142-PA | N.D. | N.D. | N.D.| N.D.| 4.94     | 1.30     | N.D.    | N.D. | only in cyp | only in cyp | only in cyp | 0.006 | only in cyp | 0.006 | only in cyp | 0.006 |
modulates eye vision and antennule activity [43]. Thus, in cyprids, N-acetylated alpha-linked acidic dipeptidase may be involved in regulating cAMP and GABA levels by catabolizing NAAG.

In addition, we identified neuroglian, which belongs to the Ig superfamily and is essential for the development of neuroglia and the formation of synapses [44]. Neuroglian gene expression increased during larval development and peaked in cyprids, but no significant difference was observed after settlement (Figure 3E). The expression of its protein product displayed similar trends during all larval stages but decreased after settlement (Table 1). Barnacle neuroglian was found to contain 6 Ig superfamily domains and possess region homologies to cell adhesion molecules or a neuroglian-like isoform in insects. It has been reported that neuroglian regulates glial morphogenesis and antennal lobe development in Drosophila larvae undergoing metamorphosis [45].

**Signaling molecules.** In the present study, the CUB-serine protease was found to be uniquely expressed in cyprids, the antennules of which contain an olfactory receptor neuron-like structure. The CUB-serine protease was first located in the olfactory organ and eyestalk of the spiny lobster Panulirus argus, which suggested that it functions in the olfactory system [46]. When barnacle cyprids were treated with the anti-settlement compound meleagrin, the protein expression of the CUB-serine protease was modulated [34]. Up-regulation of the CUB-serine protease in barnacle cyprids suggested that this protein might be involved in the cyprid olfactory chemoreception system during the search for an ideal settlement spot.

Furthermore, in the present study, we identified a putative G protein-coupled receptor in the barnacle proteome that was significantly up-regulated during the cyprid stage. In the red abalone Haliotis rufescens, a G protein-coupled receptor and downstream PKA-dependent cyclic AMP (cAMP) pathway are crucial participants in the pathway controlling larval settlement and metamorphosis [47]. In B. amphitrite, it has been suggested that a G protein-coupled receptor binds to the exogenous metamorphic cue while the settlement signal is conducted via a PKA independent pathway [48,49]. Therefore, the G protein-coupled receptor identified in the current study may play a crucial role in recognizing the settlement cue. Interestingly, we did not find any other cAMP-pathway-related proteins that were differentially expressed in cyprids, yet 1 cAMP-dependent protein kinase (PKA) was up-regulated in juveniles. Signal transduction regulated by cAMP is required for the development of a metazoan [50]. Down-regulation of PKA in cyprids and up-regulation in juveniles indicates that this PKA may be important for juvenile development rather than for larval settlement.

**Structural proteins.** 7 structural proteins were differentially expressed during the cyprid stage. Among them, 5 belonged to the connectin/titin family, including projectin, twitchin and 3 titins (Table 1). Titins are giant proteins that contribute to muscle assembly and contraction, especially in striated muscle [51]. In barnacle cyprids, striated muscle is distributed in antennules, thoracopods and their related muscles [52]. Antennules and thoracopods function differently in cyprids: antennules are the prime locomotor and sensory apparatus, which helps these organisms contact with the substratum during the attachment process. In contrast, thoracopods enable cyprids to move or stay in the water column [53]. The up-regulation of the proteins involved in the assembly and contraction of striated muscle supported the active searching and swimming behavior displayed during barnacle larval settlement.

**Functionally ungrouped proteins.** In addition to the functional groups mentioned above, we also identified many proteins that belonged to some other functional groups or which
### Table 2: Significantly Up-regulated Proteins in Juveniles

| Accession No. | Protein Description | Nau2 | SD | Nau6 | SD | CyP | SD | Juv | SD | Nau2/juv | SD | Nau6/juv | SD | CyP/juv | SD | Nau2/juv | SD | Nau6/juv | SD | CyP/juv | SD |
|---------------|---------------------|------|----|------|----|-----|----|-----|----|---------|----|----------|----|--------|----|---------|----|---------|----|--------|----|---------|----|
| CL8091.Contig1 | calponin/transgelin | 3.90 | 1.58 | N.D. | N.D. | 16.36 | 3.24 | 0.23 | 0.09 | 0.003 | 0.00 | 0.003 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL2061.Contig1 | GAPDH/7532-PA isoform 2 | 6.38 | 2.53 | N.D. | N.D. | 16.55 | 2.37 | 0.39 | 0.15 | 0.010 | 0.00 | 0.002 | 0.00 | 0.001 | 0.00 | 0.002 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL3600.Contig1 | beta chain spectrin isoform 2 | 10.33 | 1.36 | 2.55 | 1.32 | 3.39 | 1.03 | 0.51 | 0.11 | 0.007 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL4402.Contig1 | lamina beta chain | 10.59 | 1.84 | 2.55 | 1.32 | 3.39 | 1.03 | 0.51 | 0.11 | 0.007 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL3753.Contig1 | myosin heavy chain, non-muscle | 10.33 | 1.36 | 2.55 | 1.32 | 3.39 | 1.03 | 0.51 | 0.11 | 0.007 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL3442.Contig1 | laminin beta chain | 10.33 | 1.36 | 2.55 | 1.32 | 3.39 | 1.03 | 0.51 | 0.11 | 0.007 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL352.Contig1 | Talin-1 | 9.02 | 0.24 | N.D. | N.D. | 3.55 | 1.68 | 0.69 | 0.18 | 0.02 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL8008.Contig1 | myosin regulatory light chain | 13.53 | 3.34 | N.D. | N.D. | 3.26 | 1.10 | 1.03 | 0.11 | 0.004 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL1498.Contig1 | Beta-parvin | 0.68 | 0.09 | N.D. | N.D. | 3.55 | 1.68 | 0.69 | 0.18 | 0.02 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL6818.Contig1 | heterogeneous nuclear ribonucleoprotein F | 10.35 | 1.77 | N.D. | N.D. | 3.55 | 1.68 | 0.69 | 0.18 | 0.02 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL6551.Contig1 | heterogeneous nuclear ribonucleoprotein Q | 14.95 | 1.68 | N.D. | N.D. | 3.55 | 1.68 | 0.69 | 0.18 | 0.02 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 |
| CL268.Contig1 | ribosomal protein S3 | 27.26 | 3.26 | 2.175 | 0.44 | 15.26 | 3.23 | 10.19 | 1.68 | 0.74 | 0.08 | 0.008 | 0.003 | 0.008 | 0.003 | 0.008 | 0.003 | 0.008 | 0.003 | 0.008 |
| CL129.Contig1 | Filamin-like | 2.98 | 0.32 | N.D. | N.D. | 2.55 | 1.68 | 16.90 | 0.99 | 0.18 | 0.02 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 |
| CL7751.Contig1 | vacuolar protein sorting 35 | 16.07 | 3.96 | N.D. | N.D. | 9.60 | 5.63 | 30.35 | 7.95 | 0.43 | 0.11 | 0.004 | 0.00 | 0.004 | 0.00 | 0.004 | 0.00 | 0.004 | 0.00 | 0.004 |
| CL6555.Contig1 | histone H1 | 27.72 | 3.90 | 3.13 | 0.60 | 29.21 | 0.85 | 51.52 | 4.68 | 0.54 | 0.14 | 0.016 | 0.00 | 0.016 | 0.00 | 0.016 | 0.00 | 0.016 | 0.00 | 0.016 |
| CL268.Contig1 | ribosomal protein S6 | 8.2 | 0.16 | N.D. | N.D. | 8.17 | 1.61 | 24.93 | 3.27 | 0.32 | 0.01 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 |
| CL9127.Contig1 | histone cluster 1, H2ad | 24.18 | 2.70 | 3.28 | 0.71 | 20.69 | 0.71 | 93.18 | 2.48 | 0.64 | 0.07 | 0.000 | 0.00 | 0.000 | 0.00 | 0.000 | 0.00 | 0.000 | 0.00 | 0.000 |
| CL6298.Contig1 | Elongation factor 2 | 27.72 | 3.90 | 3.13 | 0.60 | 29.21 | 0.85 | 51.52 | 4.68 | 0.54 | 0.14 | 0.016 | 0.00 | 0.016 | 0.00 | 0.016 | 0.00 | 0.016 | 0.00 | 0.016 |
| CL3892.Contig1 | DNA topoisomerase 2 | 8.2 | 0.16 | N.D. | N.D. | 8.17 | 1.61 | 24.93 | 3.27 | 0.32 | 0.01 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 | 0.00 | 0.001 |

**Gel-Free Proteomics for Barnacle Larval Settlement**

PLOS ONE | www.plosone.org | 10 February 2014 | Volume 9 | Issue 2 | e88744
| Accession No. | Protein Description                                                                 | Protein abundance (>1000 molecules/cell) | Protein ratios |
|--------------|-------------------------------------------------------------------------------------|------------------------------------------|----------------|
|              |                                                                                     | nau2 | nau6 | cyp | juv | nau2/juv | nau6/juv | cyp/juv |
|              |                                                                                     | ave  | SD   | ave  | SD  | ave  | SD  | p     | ave  | SD  | p    | ave  | SD  | p    |
| CL1214.Contig3 | DNA-directed RNA polymerase II largest subunit isoform 12                           | N.D. | N.D. | N.D. | N.D. | 15.44 | 7.46 | only in juv | 0.018 | only in juv | 0.018 | only in juv | 0.018 |
| Unigene23156  | mannose-binding protein                                                              | N.D. | N.D. | N.D. | N.D. | 5.42  | 0.24 | only in juv | 0.000 | only in juv | 0.000 | only in juv | 0.000 |
| CL7413.Contig1 | trans-sialidase                                                                      | N.D. | N.D. | N.D. | N.D. | 6.41  | 1.90 | only in juv | 0.008 | only in juv | 0.008 | only in juv | 0.008 |
|              | **Energy and Metabolism**                                                            |      |      |      |      |      |      |       |      |      |      |      |      |      |
| CL2238.Contig1 | phosphate carrier protein, mitochondrial-like isoform 1                             | 24.12| 4.27 | 33.15| 2.76 | 27.08 | 2.17 | 56.52  | 4.46  | 0.43  | 0.08  | 0.001 | 0.59  | 0.05  | 0.006 |
| CL36616.Contig1| 6-phosphogluconate dehydrogenase, decarboxylating                                   | 16.24| 5.81 | 9.02 | 2.76 | 9.25  | 3.00 | 30.72  | 6.34  | 0.53  | 0.19  | 0.047 | 0.29  | 0.09  | 0.015 |
| CL6215.Contig1 | phosphoacetylglucosamine mutase                                                     | 5.90 | 0.45 | N.D. | N.D. | N.D.  | N.D. | 10.72  | 3.36  | 0.55  | 0.04  | 0.048 | 0.00  | 0.00  | 0.008 |
| CL1940.Contig2 | galactokinase 2                                                                      | 2.45 | 0.15 | N.D. | N.D. | N.D.  | N.D. | 10.98  | 0.82  | 0.22  | 0.01  | 0.000 | 0.00  | 0.00  | 0.000 |
| CL46611.Contig3| MOXD1-like protein 2                                                                 | 4.95 | 0.70 | N.D. | N.D. | N.D.  | N.D. | 8.12   | 0.68  | 0.61  | 0.09  | 0.006 | 0.00  | 0.00  | 0.001 |
| Unigene32056  | Putative glycerol [starch] synthase                                                  | N.D. | N.D. | N.D. | N.D. | N.D.  | N.D. | 16.80  | 1.84  | only in juv | 0.001 | only in juv | 0.001 | only in juv | 0.001 |
| CL10045.Contig1| dithiopyrpylyene-residue acetyltransferase component 1 of pyruvate dehydrogenase complex, mitochondrial-like | 3.62 | 1.36 | N.D. | N.D. | N.D.  | N.D. | 10.61  | 4.21  | 0.34  | 0.13  | 0.037 | 0.00  | 0.00  | 0.014 |
|              | **Extracellular Matrix and Adhesion Molecules**                                      |      |      |      |      |      |      |       |      |      |      |      |      |      |
| CL339.Contig5  | ATP citrate lyase                                                                    | N.D. | N.D. | N.D. | N.D. | N.D.  | N.D. | 17.56  | 10.48 | only in juv | 0.040 | only in juv | 0.040 | only in juv | 0.040 |
| CL1080.Contig3 | Acetyl-coenzyme A synthetase, cytoplasmic                                            | N.D. | N.D. | N.D. | N.D. | N.D.  | N.D. | 7.68   | 3.65  | only in juv | 0.003 | only in juv | 0.022 | only in juv | 0.022 |
| Unigene26389  | probable 4-coumarate–CoA ligase                                                     | 14.27| 2.19 | N.D. | N.D. | N.D.  | N.D. | 11.56  | 3.12  | 0.37  | 0.19  | 0.034 | 0.00  | 0.00  | 0.006 |
| CL1.Contig245  | arginine kinase                                                                      | 19.32| 3.92 | 26.89| 8.83 | 4.00  | 4.00 | 46.72  | 6.30  | 0.41  | 0.08  | 0.002 | 0.58  | 0.02  | 0.11  |
| Unigene11220  | Estradiol 17-beta-dehydrogenase                                                      | 12N.D.| N.D. | N.D. | N.D. | 6.14  | 2.87 | 12.61  | 1.17  | 0.00  | 0.00  | 0.000 | 0.00  | 0.00  | 0.49  |
|              | **Cell Signaling**                                                                   |      |      |      |      |      |      |       |      |      |      |      |      |      |
| CL14344.Contig1| integrin beta-PS                                                                     | N.D. | N.D. | N.D. | N.D. | N.D.  | N.D. | 15.10  | 6.78  | only in juv | 0.020 | only in juv | 0.020 | only in juv | 0.020 |
| CL475.Contig2  | neuroblast differentiation-associated protein AHAHAN                                 | 13.63| 2.17 | N.D. | N.D. | 3.18  | 1.48 | 35.33  | 0.44  | 0.39  | 0.06  | 0.005 | 0.00  | 0.00  | 0.003 |
| Unigene6163   | Basement membrane-specific heparan sulphate proteoglycan core protein                | 8.58 | 3.68 | N.D. | N.D. | 15.42 | 0.96 | 40.67  | 7.04  | 0.21  | 0.09  | 0.002 | 0.00  | 0.00  | 0.003 |
| Unigene32829  | chorion peroxidase                                                                   | N.D. | N.D. | N.D. | N.D. | N.D.  | N.D. | 8.17   | 3.05  | only in juv | 0.011 | only in juv | 0.011 | only in juv | 0.011 |
| Unigene9044   | probable chitinase 3                                                                 | N.D. | N.D. | N.D. | N.D. | N.D.  | N.D. | 7.82   | 3.57  | only in juv | 0.016 | only in juv | 0.016 | only in juv | 0.016 |

Table 2. Cont.
| Accession No. | Protein Description                              | nau2 | SD  | nau6 | SD  | cyp | SD  | juv | SD  | nau2/juv | SD  | p     | nau6/juv | SD  | p     | cyp/juv | SD  | p     |
|--------------|-------------------------------------------------|------|-----|------|-----|-----|-----|-----|-----|----------|-----|-------|----------|-----|-------|----------|-----|-------|
| Shell Calcification | Carbonic anhydrase 2 | 13.78 | 1.84 | 5.09 | 2.44 | N.D. | N.D. | 24.43 | 1.76 | 0.56 | 0.08 | 0.002 | 0.21 | 0.10 | 0.003 | 0.00 | 0.00 |
| Protein Degradation | alpha-carbonic anhydrase | N.D. | N.D. | N.D. | N.D. | 31.20 | 3.76 | only in juv | 0.001 | only in juv | 0.001 | only in juv | 0.001 |
|             | Ubiquitin-like modifier-activating enzyme 1     | 14.57 | 4.98 | N.D. | N.D. | 30.21 | 4.63 | 0.48 | 0.16 | 0.021 | 0.00 | 0.002 | 0.00 | 0.002 | 0.00 | 0.00 |
|             | 26S proteasome non-ATPase regulatory subunit 2  | 11.37 | 2.49 | N.D. | N.D. | 9.92 | 0.80 | 18.94 | 2.15 | 0.60 | 0.13 | 0.019 | 0.00 | 0.00 | 0.01 | 0.52 | 0.04 |
| Protein Modification | AGAR009694-PA | 14.51 | 0.20 | 5.10 | 1.23 | 6.33 | 2.24 | 22.59 | 1.70 | 0.64 | 0.01 | 0.011 | 0.23 | 0.05 | 0.001 | 0.28 | 0.10 |
|             | AGAR007393-PB | 21.04 | 0.59 | 5.71 | 3.41 | 12.50 | 2.86 | 37.85 | 3.43 | 0.56 | 0.02 | 0.001 | 0.15 | 0.09 | 0.003 | 0.33 | 0.08 |
| Cell Differentiation | n-myc downstream regulated | 29.52 | 5.94 | N.D. | N.D. | 27.01 | 4.12 | 59.86 | 1.69 | 0.49 | 0.10 | 0.002 | 0.00 | 0.00 | 0.000 | 0.45 | 0.07 |
|             | kakapo | 4.36 | 1.36 | 0.33 | 0.15 | 1.18 | 0.18 | 18.20 | 0.40 | 0.24 | 0.07 | 0.007 | 0.02 | 0.01 | 0.000 | 0.07 | 0.01 |
|             | 70kDa heat shock protein | 6.09 | 0.48 | N.D. | N.D. | 1.07 | 0.07 | 12.33 | 0.36 | 0.49 | 0.04 | 0.000 | 0.00 | 0.00 | 0.000 | 0.09 | 0.01 |
|             | kakapo | 1.46 | 0.16 | N.D. | N.D. | N.D. | N.D. | 10.26 | 2.18 | 0.14 | 0.02 | 0.001 | 0.00 | 0.00 | 0.004 | 0.00 | 0.00 |
| Cell Proliferation | annexin 1 | N.D. | N.D. | N.D. | N.D. | 2.21 | 1.90 | 10.63 | 1.41 | 0.00 | 0.00 | 0.001 | 0.00 | 0.00 | 0.001 | 0.21 | 0.18 |
|             | ovulatory protein-2 precursor | N.D. | N.D. | N.D. | N.D. | 23.76 | 7.63 | only in juv | 0.009 | only in juv | 0.009 | only in juv | 0.009 |
| Stress-induced Protein | Glycoprotein 93 CG5520-PA | 8.11 | 1.68 | 12.89 | 0.41 | 6.12 | 0.64 | 31.49 | 2.17 | 0.26 | 0.05 | 0.000 | 0.41 | 0.01 | 0.001 | 0.19 | 0.02 |
|             | 70kDa heat shock protein | 20.10 | 2.71 | 21.29 | 0.08 | 19.17 | 2.38 | 40.46 | 4.34 | 0.50 | 0.07 | 0.007 | 0.53 | 0.00 | 0.012 | 0.47 | 0.06 |
|             | Cct5-prov protein | 6.48 | 0.94 | 2.52 | 0.10 | 3.05 | 0.20 | 13.78 | 1.22 | 0.47 | 0.07 | 0.001 | 0.18 | 0.01 | 0.000 | 0.22 | 0.01 |
| Inorganic Ion Transportaion | Nav+K+ ATPase alpha subunit | 35.37 | 1.81 | 10.17 | 0.91 | 22.21 | 0.32 | 61.04 | 2.01 | 0.58 | 0.03 | 0.000 | 0.17 | 0.01 | 0.000 | 0.36 | 0.01 |
|             | Nav+K+ ATPase beta subunit | 22.21 | 1.72 | 11.59 | 2.01 | N.D. | N.D. | 35.44 | 3.73 | 0.63 | 0.05 | 0.004 | 0.33 | 0.06 | 0.003 | 0.00 | 0.00 |
|             | nitric oxide synthase | N.D. | N.D. | N.D. | N.D. | 7.09 | 2.68 | only in juv | 0.001 | only in juv | 0.014 | only in juv | 0.014 |
| Cell Wall/Membrane/Envelope Biogenesis | ribophorin I | 9.08 | 0.45 | N.D. | N.D. | N.D. | N.D. | 32.37 | 6.47 | 0.28 | 0.01 | 0.013 | 0.00 | 0.00 | 0.003 | 0.00 | 0.00 |
| Cell Cycle Control | uncharacterized protein C6orf68 | 16.46 | 1.09 | 8.23 | 0.01 | 10.49 | 2.86 | 32.76 | 2.27 | 0.50 | 0.03 | 0.000 | 0.25 | 0.00 | 0.002 | 0.32 | 0.09 |

**Intracellular Trafficking, Secretion, and Vesicular Transport**
| Accession No. | Protein Description | Protein abundance (>1000 molecules/cell) | Protein ratios |
|--------------|---------------------|------------------------------------------|---------------|
|              | nau2                | nau6                                      | cyp           | juv |
|              | ave | SD | ave | SD | ave | SD | ave | SD | ave | SD |
|              |     |    |     |    |     |    |     |    |     |    |
| CL1315.Contig1 | clathrin heavy chain-like isoform 1 | 18.28 | 0.30 | 3.21 | 1.39 | 13.58 | 0.22 | 33.17 | 1.29 |
| CL1268.Contig2 | signal recognition particle receptor subunit alpha homolog isoform | 1.70 | 0.51 | N.D. | N.D. | N.D. | N.D. |
| Unclassified and Function Unknown | endothelin-converting enzyme 1 | 3.99 | 0.31 | N.D. | N.D. | N.D. | N.D. |
| Unigene24129 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| CL3782.Contig1 | sporozoite surface protein | 3.16 | 0.65 | 1.15 | 0.01 | 7.27 | 2.70 |
| CL6558.Contig1 | anion exchange protein 2-like isoform 2 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| CL3782.Contig1 | settlement inducing protein complex | 6.16 | 1.60 | 2.24 | 0.56 | 12.88 | 4.05 |
| CL6595.Contig1 | settlement inducing protein complex | N.D. | N.D. | 0.87 | 0.03 | N.D. | N.D. |
| CL217.Contig3 | cross-beta structure silk protein 1 | N.D. | N.D. | N.D. | N.D. | 8.58 | 0.80 |
| CL7617.Contig1 | sodium-driven chloride bicarbonate exchanger-like isoform 3 | N.D. | N.D. | N.D. | N.D. | 5.97 | 1.04 |
| CL608.Contig2 | trans-sialidase | 2.21 | 1.24 | N.D. | N.D. | N.D. | N.D. |
| CL4385.Contig1 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| CL5942.Contig1 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Unigene4943 | cystathionine-beta-synthase | N.D. | N.D. | N.D. | N.D. | 5.63 | 0.55 |
| Unigene24129 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| CL2143.Contig2 | cement protein-100k | N.D. | N.D. | N.D. | N.D. | 9.77 | 3.13 |
| CL1152.Contig1 | BCS-4 | N.D. | N.D. | N.D. | N.D. | 35.37 | 4.58 |
| Unigene7077 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |

nau2, the stage II nauplii; nau6, the stage VI nauplii; cyp, the cyprids; juv, the juveniles; Ave, average; SD, standard deviation; N.D., not detected, p value was obtained by Student t-test. doi:10.1371/journal.pone.0088744.t002
unknown functions. Opsin 5 showed the most similarity to *Laminus* opsin 5, the protein component of visual pigment that is sensitive to visible light (400–700 nm) [54]. Barnacle cyprids, which have a pair of stage-specific compound eyes and 1 naupliar eye, display a phototactic response to light flux. The photoreception in cyprids has been suggested to be associated with a visual sensory system needed for larval settlement and distribution [55].

A previous study described the cloning of 6 barnacle cyprids larva-specific genes (*bcs*) by screening cDNA libraries [8]. As an ‘early’ gene, *bcs-2* was highly expressed in cyprids, and then decreased dramatically when larval settlement occurred [8]. In the present study, the protein product of this gene displayed the same abundance in cyprids but could not be detected in juveniles, which suggested a specific role for *Bcs-2* in barnacle cyprids.

Proteins that were significantly up-regulated in juveniles

**Energy and metabolism proteins.** In juveniles, we identified 12 proteins that were related to energy and metabolism. Among these 12 proteins, arginine kinase was significantly up-regulated during the juvenile stage, whereas its gene expression was 2-fold higher in stage VI nauplii and cyprids than in the other 3 stages (Figure 3C). These results indicated that the arginine kinase transcript might be accumulated prior to that of fructose 1,6-bisphosphatase. Arginine kinase often serves as a temporal energy buffer system, increasing the efficiency of the reversible phosphorylation of arginine by ATP and of fluctuating energy requirements in invertebrates [56,57]. In addition, both pyruvate dehydrogenase and ATP citrate lyase were up-regulated in juveniles. This up-regulation might ensure the production of acetyl-CoA for subsequent energy production and fatty acid synthesis, as the pyruvate dehydrogenase complex catalyzes the transformation from pyruvate to acetyl-CoA via pyruvate decarboxylation. Alternately, this phenomenon might stimulate the generation of acetyl-CoA from acetate and CoA via the catalysis of acetyl-CoA synthetase [58]. In contrast, ATP citrate lyase is involved in the generation of acetyl CoA together with the ATP hydrolysis [59].

**Structural proteins.** In the juvenile proteome, 17 structural proteins were significantly up-regulated. Among them, several proteins were found to be involved in muscle assembly and contraction, including 4 connectin/titin family proteins, 2 troponin isoforms, myosin light chain and transgelin (Table 2). In addition, several actin-binding proteins were detected, such as vinculin, lammin, talin, β-parvin, and thymosin β. Spectrin is another actin scaffold protein that helps maintain the shape of the cell and plays a crucial role in the survival and development of *Drosophila* larval [60]. The up-regulation of these cytoskeletal proteins was not unexpected, as barnacles must undergo drastic tissue degeneration and organ remodeling during metamorphosis, such as the reduction of larval muscles, the formation of juvenile muscles, rotation of the thorax, the development of cirri, and raising of the body, among others [61].

**Proteins related to transcription and translation.** Thirteen proteins related to transcription and translation were significantly up-regulated during the juvenile stage. By comparison, only 6 proteins were up-regulated during the cyprid stage. We identified two heterogeneous nuclear ribonucleoproteins (F and Q) and 1 polyadenylate-binding protein, all of which were found to be involved in gene transcription and post-transcriptional modification by regulating mRNA metabolism [62,63]. A similar expression pattern was observed for histone H2ad and histone H1, which are coupled to DNA replication, and elongation factor 2, which facilitates translation elongation. Interestingly, the gene expression of histone H2ad did not show much fluctuation during the larval stage or in juveniles but increased dramatically in adults. However, the histone H1 gene was expressed at least 2.4-times higher in cyprids than during any other stage (Figures 3F & 3G). These results suggested that histone H1 might be important for juvenile development only and that histone H2ad might function in both juveniles and adults. In short, the up-regulation of these proteins indicated that active transcription and translation might occur in barnacle juveniles.

**Proteins related to cell proliferation and differentiation.** Proteins involved in cell proliferation and differentiation were significantly up-regulated in juveniles only. In total, we identified 4 kakapos, one of which was detected only in juveniles. Kakapo, a cytoskeletal-associated protein expressed in *Drosophila* tendon cells, may be important for muscle-dependent tendon cell differentiation [64]. In barnacles, tendon cells, which are specially organized epithelial cells, function as a link between muscles and the overlying chitinous exoskeleton [65]. Up-regulation of these 4 kakapo proteins indicated that they have crucial functions in cell differentiation, mainly in the barnacle integument. In addition, N-myec downstream regulated gene (NDRG) protein was highly abundant in the juvenile proteome. This protein was down-regulated during the larval stage and increased in juveniles (Table 2); however, its gene expression peaked in cyprids (Figure 3H). The NDRG protein family contributes to cell differentiation and proliferation in diverse tissues of various animals [66]. Our results suggested that NDRG functions in cell differentiation processes associated with tissue reorganization in barnacle juveniles.

In the current study, we identified one annexin that was differentially expressed in juveniles. The expression of this protein was first detected in cyprids and then peaked in juveniles (4.9-fold higher than in cyprids) (Table 2). Annexins comprise a group of proteins that bind to phospholipids in a calcium-dependent manner. Previous studies have shown that the expression patterns of annexins change significantly when cells undergo proliferation or differentiation [67]. During silkworm metamorphosis of *Bombyx mori*, 20-hydroxyecdysone (20-HE) triggers programmed cell death to remove larval-specific tissues. Annexin, identified as a 20-HE inducible gene, was shown to be involved in this process [68]. The 20-HE gene has also been detected in barnacle cyprids, and its regulation of barnacle larval metamorphosis has been verified [69]. Although the detailed mechanism by which 20-HE affects barnacle larval metamorphosis remains unclear, the expression levels of annexin might be modulated by the tissue degeneration reorganization that occurs during barnacle larval development and metamorphosis, which in turn might be regulated by 20-HE.

**Shell calcification proteins.** Two carbonic anhydrases related to shell calcification were significantly up-regulated in juveniles and might be involved in biomineralization in *B. amphitrite*, especially β CA, which was uniquely expressed in juveniles. Carbonic anhydrase (CA) participates in a variety of metabolic pathways and is widely distributed in the tissues that are responsible for the formation of calcium carbonate [70]. It is also important for the molting cycle in crustaceans [71]. Inhibition of CA activity prevents shell development and growth of the barnacle *Balanus improvisus*, resulting in a failure in the initiation of normal development [70].

**Functionally ungrouped proteins.** In the present study, we discovered that the protein product of the *hcs-4* gene was uniquely expressed in juveniles. Compared with *hcs-2*, *hcs-4* is a ‘late’ gene showing weak expression levels in young cyprids that have recently molted from nauplii, increasing gradually as cyprids age, and beginning to decline in settled larvae [8]. This time lag between the expression of gene and protein supports the possibility that accumulation of the gene is required prior to protein function in juveniles.
Figure 3. Quantitative real-time PCR (qRT-PCR) results of the levels of 13 genes with protein products that were significantly up-regulated in either cyprids or juveniles (white bars). The corresponding protein expression levels are presented as line charts. The detected developmental stages included stage II nauplii (Nau2), stage VI nauplii (Nau6), cyprids (Cyp), young juveniles (Juv) and adults (Adu). Values are expressed as the mean ± SD of 3 different experimental replicates. In the figures, a, b, c, d and e in the figures showed significantly different expression patterns among the samples detected, as determined by one-way ANOVA followed by Tukey’s post-hoc test (P < 0.05).

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In addition to Bsc-4, one cement protein-100k was found to be uniquely expressed during the juvenile stage. Its gene transcript was detected in the adult but not in the larval transcriptome [5]. In fact, several proteins have been identified, such as two 20 kDa-cement proteins characterized in the cyprids of *B. amphitrite* and large cement proteins fractionated from the adult cement of *Megabalanus rosa* (100 kDa, 68 kDa & 52 kDa) [72,73].

Three proteins belonging to the heat shock protein (HSP) family were significantly up-regulated in juveniles. HSPs were initially known as stress-responsive proteins and later found to interface with various developmental pathways, as molecular chaperones associated with protein folding, assembly and transport [74,75]. HSP70 is developmentally regulated in a diverse range of organisms, and HSP90 functions as a regulator during metamorphosis and the molt cycle of invertebrates [76–78]. Additionally, the expression levels of HSP70 and HSP90 increase in response to cell differentiation and tissue morphogenesis occur in the vetigastropod *Haliotis asinina* [79].

**Comparison of proteome profiles with other marine invertebrates**

In addition to the present study, proteomic approaches have been applied to study larval attachment and metamorphosis in several marine invertebrates. Although the species were from several evolutionarily distant phyla, common changes in the proteome were observed. First, during attachment and metamorphosis, a larva goes through serial body reconstructions, including the degeneration of larval structures and the subsequent emergence of juvenile tissues. The larvae of the bryozoan *Bugula neritina*...
and the polychaete *Hydroides elegans*, swim via beating cilia, and all of these ciliated tissues degenerate during metamorphosis, resulting in drastic changes in structural proteins, such as actin and tubulin, during larval settlement [20,80]. Barnacle larvae swim by beating thoracopods driven by striated muscles. Actin and tubulin were not differentially expressed in the bimaculate proteomes, but several proteins related to muscle structures and muscle contraction were up-regulated. In addition to structural proteins, in the present study, proteins involved in transcription and translation, such as ribosomal proteins, histones and elongation factors, were highly expressed during larval settlement in the bryozoan *B. neritina* [20], the polychaete *Pseudopolydora vexillosa* [81] and so as in *B. amphitrite*. These findings indicated that transcription and translation level were active when larvae underwent metamorphosis.

Larval settlement is an extremely energy-consuming process, especially for the whole larval settlement process. Proteins involved in the citric acid cycle, glycolysis, and fatty acid metabolism have been found to be up-regulated during the competent larval stage of *B. neritina, P. vexillosa* and the polychaete *Capitella* sp. I [20,80,81]. Similarly, these proteins were found to be up-regulated in barnacle cyprids herein. In addition, some proteins related to energy and metabolism were also up-regulated in barnacle juveniles, as described for *P. vexillosa* [81]. These findings indicated that these organisms might require different energy metabolism pathways for larval competency and metamorphosis. Interestingly, vitellogenins have been described in competent larvae of *B. neritina, B. amphitrite* and *Capitella* [20,82], indicating that the non-feeding larvae of different phyla all consume vitellogenins as an energy source.

Carbonic anhydrase was detected in juveniles of both *B. neritina* and *B. amphitrite*, which require a calcified body wall or a calcareous shell [83]. This result indicated that carbonic anhydrase is important for the calcification of marine invertebrates.

Conclusions

This study investigated the regulation of protein expression patterns in response to larval development and settlement of the barnacle *B. amphitrite*. The utilization of label-free quantitative proteomics allowed us to conduct a comparative proteomics analysis among different developmental stages and to identify protein candidates that might be involved in barnacle larval settlement. Functional analysis of 4 proteins revealed their significant up-regulation of proteins involved in energy and metabolism, the nervous system, and signaling transduction in the cypris stage. In addition, proteins related to cytoskeletal remodeling, translation and transcription, cell proliferation and differentiation, and biomineralization were up-regulated in the juvenile proteome. Notably, the expression patterns of some proteins, such as neuroglian, failed axon connection protein, and lipoprotein receptor, displayed the same trends as their transcripts; In contrast, the expression of fructose-1, 6-bisphosphatase, cyclophilin B, NDRG, and histone H1 transcripts occurred 1 developmental stage earlier than their protein products. The expression levels of some other proteins, such as fructose-biphosphate aldolase, were not associated with their mRNA expression. These results provide information about the molecular activities related to the changes in morphology, physiology, structure, and function that occur during barnacle larval settlement and juvenile development. Additional functional assays and characterization of protein candidates, for instance, nervous system-related proteins, signaling molecules, shell calcification proteins and heat shock proteins, could help identify more detailed molecular mechanisms underlying barnacle larval settlement.

Supporting Information

Table S1 Primers for genes under real-time PCR assay.

Table S2 Total proteins identified and quantified from each LC-MS experiments.

Table S3 Significantly up-regulated proteins in stage II nauplii.

Table S4 Significantly up-regulated proteins in stage VI nauplii.

Table S5 Significantly down-regulated proteins in stage II nauplii.

Table S6 Significantly down-regulated proteins in stage VI nauplii.

Table S7 Significantly down-regulated proteins in cyprids.

Table S8 Significantly down-regulated proteins in juveniles.

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Author Contributions

Conceived and designed the experiments: Z-FC HZ. Performed the experiments: Z-FC HZ HW. Analyzed the data: Z-FC HZ KM. Contributed reagents/materials/analysis tools: Z-FC HZ TR P-YQ. Wrote the paper: Z-FC HZ HW KM YHW P-YQ.

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