

Transcriptomic Analysis of Neuropeptides and Peptide Hormones in the Barnacle *Balanus amphitrite*: Evidence of Roles in Larval Settlement

Xing-Cheng Yan¹, Zhang-Fan Chen¹, Jin Sun², Kiyotaka Matsumura¹, Rudolf S. S. Wu³, Pei-Yuan Qian^{1*}

1 Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China, **2** Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China, **3** School of Biological Sciences, University of Hong Kong, Hong Kong SAR, China

Abstract

The barnacle *Balanus amphitrite* is a globally distributed marine crustacean and has been used as a model species for intertidal ecology and biofouling studies. Its life cycle consists of seven planktonic larval stages followed by a sessile juvenile/adult stage. The transitional processes between larval stages and juveniles are crucial for barnacle development and recruitment. Although some studies have been conducted on the neuroanatomy and neuroactive substances of the barnacle, a comprehensive understanding of neuropeptides and peptide hormones remains lacking. To better characterize barnacle neuropeptidome and its potential roles in larval settlement, an *in silico* identification of putative transcripts encoding neuropeptides/peptide hormones was performed, based on transcriptome of the barnacle *B. amphitrite* that has been recently sequenced. Potential cleavage sites and structure of mature peptides were predicted through homology search of known arthropod peptides. In total, 16 neuropeptide families/subfamilies were predicted from the barnacle transcriptome, and 14 of them were confirmed as genuine neuropeptides by Rapid Amplification of cDNA Ends. Analysis of peptide precursor structures and mature sequences showed that some neuropeptides of *B. amphitrite* are novel isoforms and shared similar characteristics with their homologs from insects. The expression profiling of predicted neuropeptide genes revealed that pigment dispersing hormone, SIFamide, calcitonin, and B-type allatostatin had the highest expression level in cypris stage, while tachykinin-related peptide was down regulated in both cyprids and juveniles. Furthermore, an inhibitor of proprotein convertase related to peptide maturation effectively delayed larval metamorphosis. Combination of real-time PCR results and bioassay indicated that certain neuropeptides may play an important role in cypris settlement. Overall, new insight into neuropeptides/peptide hormones characterized in this study shall provide a platform for unraveling peptidergic control of barnacle larval behavior and settlement process.

Citation: Yan X-C, Chen Z-F, Sun J, Matsumura K, Wu RSS, et al. (2012) Transcriptomic Analysis of Neuropeptides and Peptide Hormones in the Barnacle *Balanus amphitrite*: Evidence of Roles in Larval Settlement. PLoS ONE 7(10): e46513. doi:10.1371/journal.pone.0046513

Editor: Sam Dupont, University of Gothenburg, Sweden

Received: May 28, 2012; **Accepted:** September 1, 2012; **Published:** October 2, 2012

Copyright: © 2012 Yan et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by a research grant (DY125-15-T-02) from China Ocean Mineral Resources Research and Development Association and an Award (SA-C0040/UK-C001) from King Abdullah University of Science and Technology (KAUST) to PY Qian. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: boqianpy@ust.hk

Introduction

Neuropeptides constitute the largest class of intercellular messenger molecules and play key roles in many physiological processes, e.g. reproduction, homeostasis and locomotion [1]. In general, they are produced from endocrine cells or neurons as precursors (preprohormones) and become biologically active after post-translational modifications. Secreted neuropeptides can have autocrine, paracrine and hormonal effects, by binding to membrane receptors of organ systems [2]. The earliest traceable ancestral neuropeptides date back to primitive metazoans, i.e. cnidarians [3,4]. In arthropods, neuropeptide studies have so far been limited to insects and decapods. For instance, eclosion hormone and ecdysis triggering hormone are the most well-known neuropeptides extensively studied in the moth *Manduca sexta* and the fruit fly *Drosophila melanogaster* [5]. Prothoracicotropic hormone has been characterized in various insects and proposed to initiate larval metamorphosis through stimulating prothoracic glands via G protein-coupled receptor/cAMP [6]. The crustacean hypergly-

cemic hormone family, originally isolated from X-organ-sinus gland (XO-SG) complex of decapods, was involved in regulating energy and ionic metabolism, or inhibiting molting and reproduction [7]. Besides insects and decapods, only very limited information is available on arthropod neuropeptides.

Barnacles are common in intertidal communities worldwide, and often cause biofouling problems. The life-history of barnacles consists of six naupliar stages and one cypris stage when larvae become competent to attach to substratum and then metamorphose into sessile juveniles (collectively referred to as "settlement"). Cypris is non-feeding stage and has evolved highly specialized features and behavior for settlement [8]. Besides the various exogenous inducers such as conspecific biogenic cues [9,10], various biogenic amines and hormones such as serotonin [11,12], dopamine, methyl farnesoate and 20-Hydroxyecdysone were also reported to regulate larval attachment and metamorphosis [13–16]. While extensive studies have been carried out on neurotransmitters/hormones in barnacle, only limited information is available on barnacle settlement.

Table 1. Neuropeptide/peptide hormones predicted based on transcriptome mining of *Balanus amphitrite*.

Peptide family	Barnacle accession No.	Reference accession No. ^{&}	RACE confirmed	E-value	Score
A-type allatostatin	JQ864191	BAF64528.1	+	1E-22	1122
B-type allatostatin	JQ864192	NP_001036890.1	+	7E-22	249
C-type allatostatin	JQ864193	P85798.1	+	5E-23	94
Bursicon α	JQ864194	XP_003485714.1	+	3E-60	193
Bursicon β	JQ864195	ADI86243.1	+	5E-43	148
Calcitonin-like diuretic hormone-isoform A	JQ864196	EE299367.1	+	2E-14	71.6
Calcitonin-like diuretic hormone-isoform B	JQ864197	ACX47068.1	+	4E-21	90.5
Corticotrophin-like diuretic hormone	isotig15071 [#]	UniRef100_P82373	–	0.005	36.6
Eclosion hormone	JQ864198	EGI68318.1	+	3E-11	64.7
Insulin-related peptide	JQ864199	NP_001233285.1	+	1E-08	57.4
Ion transport peptide	GBQDZ6L01AXT7W [#]	UniRef100_E0VME9	–	5E-19	89.4
	GBQDZ6L01BA9KU [#]	UniRef100_E2BEL2	–	0.003	37.4
Neuropeptide F	JQ864200	UniRef100_C9EAB8	+	0.005	36.6
Orcokinin	JQ864201	EFX70781.1	+	3E-27	110
Pigment dispersing hormone	JQ864202	JC4756	+	4E-14	70.1
SIFamide	JQ864203	NP_001161182.1	+	7E-06	47.8
Sulfakinin	JQ864204	NP_492344.2	+	1E-06	51.2
Tachykinin-relate peptide	GBQDZ6L01CRRE4 [#]	BAD06363.1	–	0.0002	32
	JQ864205	BAC82426.1	+	3E-15	78.6

[&]Reference accession No. is the accession No. of the known neuropeptide that has the highest hit against barnacle transcript.

[#]NCBI accession numbers were not designated to sequences that were not confirmed by RACE.

doi:10.1371/journal.pone.0046513.t001

Bioinformatics-based mining of neuropeptides from Expressed Sequence Tag (EST) library, transcriptome and genome has been recently conducted and numerous novel peptides have been uncovered in several species [17,18]. The barnacle has been subjected to deep sequencing of EST recently [19]. In our study, we obtained the transcriptome of the barnacle *Balanus amphitrite* using 454 pyrosequencing technology, which contained more than 90,000 predicted open reading frames [20]. This rich source of transcriptomic information made large-scale *in silico* discovery of peptides feasible since we can overcome the difficulties of collecting and sectioning enough amounts of nervous tissues from *B. amphitrite* larvae for mass spectrometry analysis. In this study, we conducted *in silico* transcriptome mining of neuropeptides/peptide hormones in *B. amphitrite*, and quantified their expression levels at different developmental stages. We then examined the effect of proprotein convertase inhibitor on larval settlement to explore the possible function of the neuropeptides characterized. Our results provide a comprehensive catalog of neuropeptidome of *B. amphitrite*, and insights on the possible functional roles of some neuropeptides in barnacle larval settlement.

Materials and Methods

1. Sample Preparation

Adult barnacles were collected from the Pak Sha Wan public piers, Hong Kong (22°21'45" N, 114°15'35" E). No specific permits were required for the field collection. The field studies did not involve any endangered or protected species. Broods were isolated from adult barnacle in the laboratory and nauplii were hatched and cultured according to Thiyagarajan & Qian 2008 [21], and larvae were collected once they reached cypris stages after 4 days' culture. For expression analysis of peptide precursor

genes, nauplii II, nauplii VI, cyprids, newly metamorphosed juveniles, and adults were collected. Total RNA extraction and cDNA synthesis were conducted according to Chen et al. 2011 [20]. Briefly, total RNA of barnacles of different developmental stages was extracted with TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA). Trace DNA contaminants were removed by TURBO DNA-free[™] Kit (Ambion Inc, Austin, TX, USA). cDNA was synthesized using M-MLV reverse transcriptase (USB, Cleveland, OH, USA) with oligo dT primer for Rapid Amplification of cDNA Ends (RACE) reactions and real-time PCR assays.

2. Database Mining of Neuropeptide Precursors

Several methods from recent publications [17,18,22] were combined and modified to search for neuropeptide/peptide hormone encoding genes in barnacle transcriptome, which contains 23,451 contiguous sequences including 182 contigs, 23,269 isotigs and 77,785 singletons [20]. Protein sequences of the known neuropeptides and peptide hormones in arthropod were obtained from UniProt Knowledgebase (<http://www.uniprot.org/>) using "neuropeptide", "hormone" and "peptide" as search keywords without "receptor", "signal anchor", or "transmembrane". NCBI non-redundant protein sequences (<http://www.ncbi.nlm.nih.gov/>) were also used for known arthropod peptides extraction, since different databases tend to use different key-word searching criteria which may lead to different results. After removing the unrelated sequences, such as enzymes and transcription factors, the remaining sequences were transformed into FASTA format to generate a local arthropod neuropeptide database. The program "tBLASTn" was used to mine for putative cDNA sequences that encode for active peptides in the barnacle transcriptome via queries using

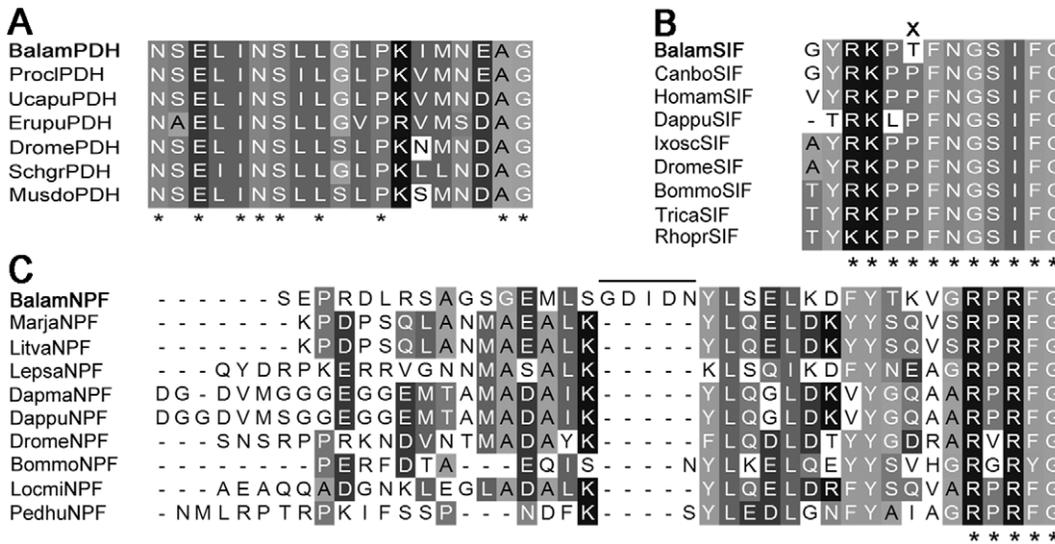


Figure 1. Sequence alignment of barnacle pigment dispersing hormone, SIFamide and neuropeptide F with arthropods. (A) Sequence alignment of mature pigment dispersing hormone. Decapods peptide sequences are from: *Uca pugilator* P08871, *Procambarus clarkii* Q9TWW7 and *Cancer productus* ABV68725; isopod peptide is from *Eurydice pulchra* ACX49752; insect peptides are from: *Drosophila melanogaster* AAL49303, *Schistocerca gregaria* ACY02888 and *Musca domestica* Q76JT9. (B) Alignment of barnacle SIFamide mature peptides. Sequences are from: decapods *Homarus americanus*, *Cancer borealis*; branchiopod *Daphnia pulex*; insects *Drosophila melanogaster*, *Tribolium castaneum*, *Bombyx mori*, *Rhodnius prolixus* and *Ixodes scapularis* [38]. (C) Alignment of barnacle NPF mature peptide. Insect NPF are from: *Drosophila melanogaster* AF117896, *Bombyx mori* NP_001124361, *Locusta migratoria* CO854418 and *Pediculus humanus* EEB15547. Decapods NPF are from shrimps *Marsupenaeus japonicus* C1998017 and *Litopenaeus vannamei* AEC12204; copepod NPF is from *Lepeophtheirus salmonis* FK933794; branchiopods NPF are from *Daphnia magna* EG565358 and *Daphnia pulex* EFX90018. Asterisks "*" represent typical motif residues of each peptide. "x" indicates unique amino acid residues of *B. amphitrite* compared to other arthropods. Line marks the unique five amino acids insertion of NPF in *B. amphitrite*. doi:10.1371/journal.pone.0046513.g001

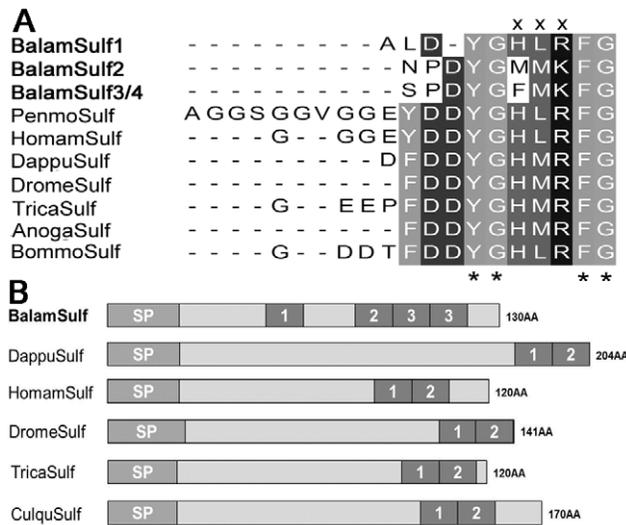


Figure 2. Comparison of barnacle sulfakinin peptide with other arthropods. (A) Sequence alignment of mature sulf. (B) Precursor structure of barnacle sulf compared with other crustaceans and insects. Insects Sulf are from: *Drosophila melanogaster* AAF52173, *Tribolium castaneum* EFA04708, *Anopheles gambiae* AAR03495, *Bombyx mori* NP_001124354 and *Culex quinquefasciatus* XP_001846221; branchiopod is from *Daphnia pulex* EFX80896; decapods Sulf are from *Homarus americanus* and *Penaeus monodon* [38]. Asterisks "*" represent typical motif residues of each peptide. "x" indicates unique amino acid residues of *B. amphitrite* compared to other arthropods. doi:10.1371/journal.pone.0046513.g002

arthropod neuropeptide sequences mentioned above. For each query, the top three blast hits with an E-value lower than 0.01 were screened out and chosen as candidates and manually checked for homology to known peptides.

3. Peptide Prediction

Neuropeptide candidate sequences generated by database mining were translated using ExPASy translate tool (<http://web.expasy.org/translate/>). Three typical neuropeptide precursor criteria, which are signal sequence, pro-hormone processing sites and length less than 300 amino acids, were applied to evaluate candidate sequences. Signal peptide identification was deduced by online program SignalP 3.0, using both the neural networks and Hidden Markov Model algorithms [23]. Pro-hormone cleavage sites were predicted based on work by Veenstra 2000 [24], and Neuropred online program (<http://neuroproteomics.scs.illinois.edu/neuropred.html>) and/or by homology to the known arthropod precursors. Sulfation state of Tyr residues was predicted using the online program Sulfinator [25] and/or by homology to known arthropod neuropeptides. In some cases, other post-translational modifications, e.g. cyclization of N-terminal Glu/Gln residues and C-terminal amidation were predicted mainly by homology to known peptide isoforms.

4. RACE Sequencing and Peptide Confirmation

Since neuropeptide precursor sequences generated from transcriptome are usually fragmented or incomplete, further confirmation on the predicted neuropeptide candidates by full length open reading frame (ORF) is required. Two sets of specific primers were designed from the partial cDNA sequences obtained from the transcriptome database. For RACE, a first run of amplification

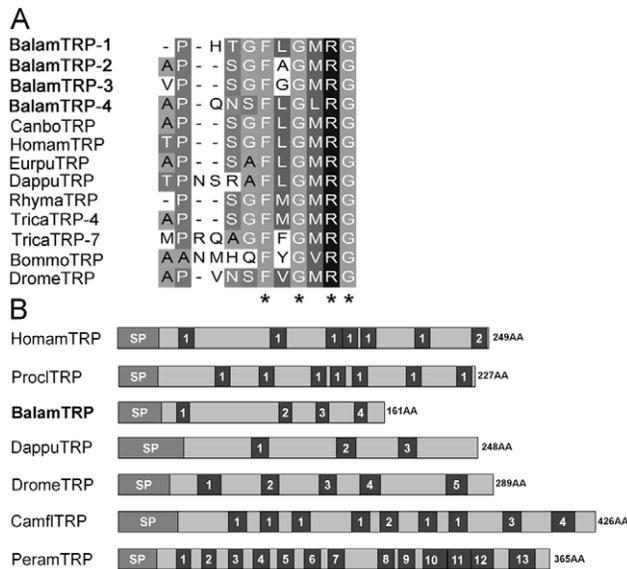


Figure 3. Comparison of barnacle tachykinin-related peptide with that of insects and crustaceans. (A) TRP sequence alignment. Decapods TRP are from: *Cancer borealis* [72] and *Homarus americanus* ACB41786; isopod TRP is from *Eurydice pulchra* CO869025; branchiopod TRP is from *Daphnia pulex* [38]. Insects TRP are from: *Tribolium castaneum* EFA09176, *Drosophila melanogaster* AAF89172, *Bombyx mori* NP_001124364 and *Rhyarobia maderae* AAX11211. (B) Barnacle TRP precursor structure compared with other crustaceans and insects. Decapods TRP are from: *Homarus americanus* ACB41786 and *Procambarus clarkii* BAC82426. Insects TRP are from: *Drosophila melanogaster* AAF89172, *Camponotus floridanus* EFN66667 and *Periplaneta americana* AAX11212. Branchiopod TRP is from *Daphnia pulex* [38]. Asterisks "*" represent typical motif residues of each peptide. doi:10.1371/journal.pone.0046513.g003

was performed using gene specific primer 1st and adaptor with oligo (dT)/oligo (dG) primer. Then gene specific primer 2nd (up or down to primer 1st) and adapter primers were used for second run PCR amplification. RACE products were cloned and sequenced. Complete amino acid sequences of candidate genes were submitted to NCBI BLAST again and checked manually for the precursor structure. If BLAST result of the new sequence didn't match the corresponding neuropeptide genes, it was excluded.

5. Sequence Analysis

Corresponding neuropeptide sequences from crustaceans and insects were searched and collected. All the crustacean neuropeptide genes and proteins discovered through *in silico* data mining, cloning or mass spectrometric approach were compiled. Predicted mature peptide sequences were used for alignment using ClustalW (version 2.0) with default parameters and manually checked.

6. Quantitative Real-time PCR

Gene specific primers were designed manually based on nucleotide sequences from the barnacle transcriptome database. Details of the primers are listed in Table S3 as additional information. The cytochrome b gene was used as the inner reference for normalizing the expression levels of target genes [26]. All real-time PCR assays for each peptide-encoding gene were performed on Stratagene Mx3005P QPCR System (Agilent, Santa Clara, CA, USA), using KAPA™ SYBR® FAST qPCR Kit Master Mix (2X) Universal (KAPA Biosystems, Woburn, MA, USA). For each neuropeptide gene, three replicates were conducted using each batch of larvae, and three batches of larvae

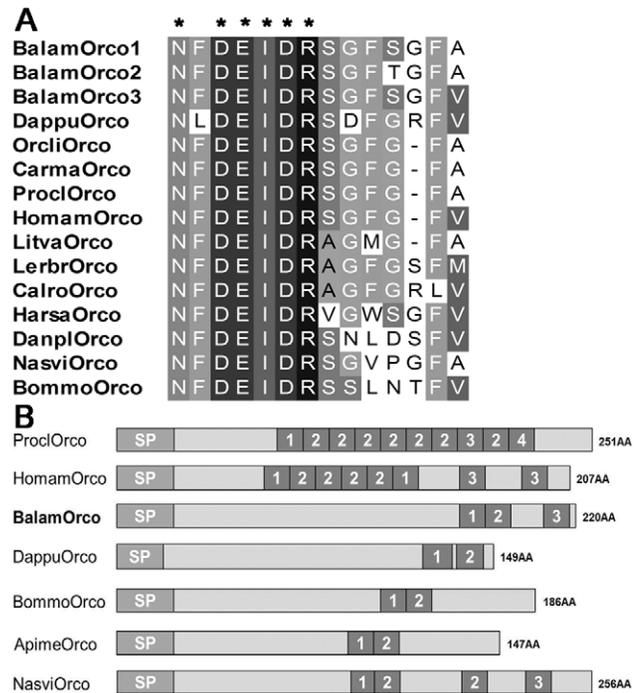


Figure 4. Comparison of barnacle orcokynin with other arthropods. (A) Sequence alignment mature orco. (B) Precursor structure of barnacle orco compared with other crustaceans and insects. Sequences of insects Orco are from: *Nasonia vitripennis* XP_003426062, *Bombyx mori* NP_001124366, *Danaus plexippus* EHJ77769, *Harpegnathos saltator* EFN80782 and *Apis mellifera* XP_001120650; copepods Orco are from: *Calius rogercresseyi* and *Lernaocera branchialis* [22]; decapods Orco are from: *Litopenaeus vannamei* [1], *Marsupenaeus japonicas* [73], *Homarus americanus* ACD13197, *Procambarus clarkii* [73], *Carcinus maenas* [74] and *Orconectes limosus* [75]. Branchiopod Orco is from *Daphnia pulex* EFX70781. Asterisks "*" represent typical motif residues of each peptide. doi:10.1371/journal.pone.0046513.g004

were collected for real-time PCR analysis. Generated qRT-PCR Ct values were analyzed by 2- $\Delta\Delta$ Ct method [27] and further tested by using one-way ANOVA, followed by Tukey test post-hoc analysis. Gene expression level in juvenile stage was standardized in order to better characterize genes that are up-regulated in cypris stage and down-regulated after metamorphosis, which might be involved in settlement regulation.

7. Proprotein Convertase Inhibitor Assay

To test the hypothesis that neuropeptides/peptide hormones are involved in larval settlement, we incubated cyprids in solution of peptidyl chloromethylketone (Enzo life sciences, Farmingdale, NY, USA) and compared the percentage of metamorphose between the treatment and control. This compound is a highly specific and potent inhibitor against proprotein convertases responsible for maturation of bioactive peptides [28]. Specifically, stock solution (20 mM) was prepared by dissolving the compound in dimethyl sulfoxide (DMSO) and stored at -20°C . Experiments were conducted in triplicates and repeated three times with different batches of cyprids. Around 20 cyprids were added to each well of a 24-well plate (#3047, BD Falcon™, Franklin Lakes, NJ, USA) each containing 1 mL of test solution. The treatment group consisted of three concentrations (1, 10, and 100 μmolL^{-1}), with two negative controls, i.e. autoclaved filtered seawater (AFSW) only and 0.5% DMSO in AFSW. The plates were incubated at 28°C in darkness for 48 hours, and the number of metamorphosed

B-Chain

```

B-Balam  PVDKRG-----PA--MH--FCGPSLADALEMVC EFG--MG-----
B-Armvu  -YQVRGMR-----SDVLCGD--IRFTVQCICNELGYFPTERLDKPCWPVNRE--
B-Pordi  -YQVEGMK-----SDVICAD--IRFTVHCICNELGRFPTARLTKPCWPVNRE--
B-Marja  -YEIECLSL-----VDFDCGD--I TNLASVCLRHNHYINPGP---TYVSK---
B-Penmo  -YNVTGIP-----VDFDCGD--IGDTMSHICKT---FPTARPHSRMPSLSLTVS
B-Chequ  -YRVENLL-----IDFDCGH--LADTMSICRTYQEFNDT-----
B-Dappu  -----NARYCGSYLADALRMACSKSS--YLPLFG-----
B-Bommo  DVHDKELKIEEN----PRVYCGRHANARMVLC-----YDTVE-----
B-locmi  ---SCAPQ---PV---ARYCGEKL SNALKLVCGRN--YNTMF-----
B-Drome  MVTPTGSGHQLLPPG--NHKLCGPALSDAMDVVC PHG--FNTLPR-----
B-Anoga  TSTPNSDALI SQLT---RSRYCGRRLTETLAFLCQGR--Y PMLTHYRTDYVHDQANQ
B-Camfl  ---NGYPQFNQKPAQVPPQKYCGKLLSNALQIVCDGV--YNSMF-----

```

C-Chain

```

A-Balam  -----SPGIVEECC-----LNPCITLSTLAQYC-----
A-Armvu  -----EIAFYQECNIRTEHKCNRTTVSLYCRTY-----
A-Pordi  -----DIAFH EECNIRTEHKCNRTTVELYCRRYSP-----
A-Marja  -----SPRECCNNASFRRCNFEEVAEYCI ELRPGVNTCSSR
A-Penmo  -----SYNVQDECCNHVSQRTCVAEEILEYCEDP---YF-----
A-Chequ  NSD TTDNTSSTNVYDECCSEKTLKTCVFDEIAQYCEQL EDGI YVSS--
A-Dappu  -----GVHDECC---VKGCTFKELTSYCTRPN-----
A-Bommo  -----GPGLVDECC---LKPCYTYDLLNYC-----
A-Locmi  -----GVFDECC---RKSCSISELQTYCGRR-----
A-Drome  -----HLTGGVYDECC---VKTC SYLELAICYL PK-----
A-Anoga  -----SSG IYDECC---KKSCSYVELRAYCD-----
A-Camfl  -----GIHEECC---LNACTISELSSYCA-----

```

A-Chain

```

C-Balam  ---KR-----SRTAPRQNLRLP-EFSGGHLST S-SASTMPY YQTQLYQS-----ADSR TKR-----
C-Armvu  ---KR-----SAPEDLAFED-YEDQDYFHP--RALSIPSEI EHDNEK-----ESDAFSLSRGKR-----
C-Pordi  ---RR-----STDD EYL FEE-DEDEEFFHP--RALSIRPTAAKYDDEI-----LEDEVSFHSR TKR-----
C-Marja  ---RRSADIT YVPS-TKSPSLAHP--RATHLTMAD EETQKVSKVEEEEIQHMTLSREEANNMLHSKRFR RD SVRR
C-Penmo  RSAD TDDLWQDTRAGQ TTPIDL-LPRQYRLHPR-ALNPMRYFEMDLIRDHLVS-----PEAAHALVKT SRRRAKR-
C-Chequ  RAVRSARDASFSA SVSMYD PGSKI AVRQVYHPRGRKLG VKETVPDARLGKQEAMTVSR-----EA AHTFIKTONYNNRRR-
C-Dappu  ---KRSTLPGKSLGL-TTTAATPNA--ELGSWPFINDDKAHS-----ILSNHHLFHR YTR-----
C-Bommo  ---KRAQSYLDAN- IISAGDLS--WPGLSSQYAKT-----RAFALAEKSKR-----
C-Locmi  ---KKA SQDVSDSESEDNYWSG-QSAD EAAEAA-AAALPPYPI LARPSAG-----GLLTGAVFRRRTR-----
C-Drome  ---KRESL LGNSDDD EDTEQEV-QDDSSMQWTL-DGAGYSFSP LLTNLY-----GSEVLIKMRHR-----
C-Anoga  ---R V KVEV-ATLPDTLPP--GFYP-----GAG--VHRRSR-----
C-Camfl  ---KKS LDQEMEM-ADYFAYDS--PPFLPRARARGMLN-----GPFAGR YRQSR-----

```

Figure 6. Peptide alignment of insulin-related peptide of *Balanus amphitrite* with arthropods. Isopods IRP are from: *Armadillidium vulgare* AB029615 and *Porcellio dilatatus* AB089811; decapods IRP are from: *Marsupenaeus japonicus* AB029615, *Penaeus monodon* GU208677.1 and *Cherax quadricarinatus* DQ851163; branchiopod IRP is from *Daphnia pulex* [38]; insects IRP are from: *Bombyx mori* NP_001233285, *Locusta migratoria* P15131, *Drosophila melanogaster* CG14173, *Anopheles gambiae* AAQ89693 and *Camponotus floridanus* EFN61735. Asterisks "*" mark the conserved Cysteine residues, black frame indicates specific amino acids insertion and deletion in IRP of isopod and decapod species, compared to insects, branchiopod and barnacle.

doi:10.1371/journal.pone.0046513.g006

based on precursor structure of both the TRP and Orco genes of *B. amphitrite*. Decapods' TRP precursor generally gave rise to several copies of a single TRP isoform or only one additional TRP isoform [37], while the TRP precursor of insects tended to encode multiple diverse isoforms [38]. The TRP precursor of *B. amphitrite* contained four copies of different TRP mature peptides (Figure 3, Table S2), which is structurally more similar to insects. In the case of Orco, mature Orco of both insects and *B. amphitrite* encoded for 14 amino acids, while the Orco of decapods was strictly 13 amino acids long (Figure 4A). Besides, comparison of their precursor structures also indicated that barnacle Orco resembled that of insects, with less mature peptides than crustaceans (Figure 4B).

The barnacle ASTB revealed in this study (Figure 5) lends further support to our postulation above. Among the 10 peptide isoforms encoded by ASTB precursor of *B. amphitrite*, 9 of them showed high similarity with the ASTB of insects. In addition, sequence alignment of IRP also indicated a similar result (Figure 6). In general, the IRP precursor contains contiguous B-C-A peptides. After maturation, A- and B-chain peptides are linked together by two interchain disulfide bonds and one intrachain disulfide bond. C-chain peptide assists the formation of linkage and will be clipped

off at the cleavage sites afterwards [39]. IRP of *B. amphitrite* structurally resembled that of insects, with one more amino acid between the two Cys on B-chain and four amino acids gap between the second and third Cys of the A-chain (Figure 6). One contradiction came from the ASTA of *B. amphitrite*, which resemble that from decapods. Among predicted ASTA peptides of *B. amphitrite*, ASTA-1/4/5/6/8/9 were similar to ASTA peptides from the giant fresh water prawn *Macrobrachium rosenbergii*, while the rests shared similarities with the lobster *Panulirus interruptus* (Figure 7). Sequence comparison of other neuropeptides including BurA/B, Calci and EH were shown in Figure 8 and 9, revealing their conserved structures among arthropod species.

Based on molecular studies in the past decades, it is now widely accepted that hexapods are associated with crustaceans, forming a group called Pancrustacea within arthropods. However, the inner relationships among Pancrustacean constituent lineages are far from being resolved [40]. A recent phylogenetic analysis of protein-coding nuclear genes demonstrates that Hexapoda is most closely related to the crustacean Branchiopoda, Cephalocarida and Remipedia, while Malacostraca including decapods are grouped with Cirripedia (barnacles) making the traditional

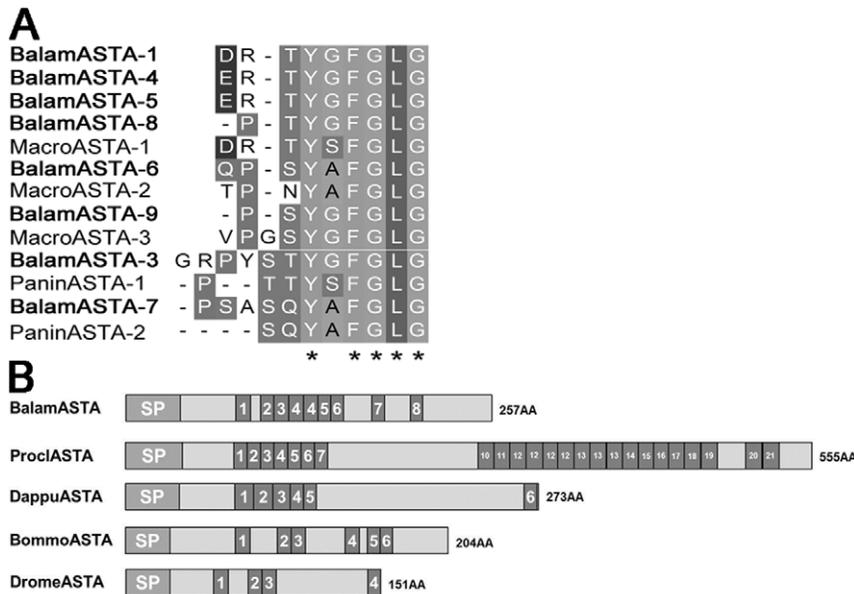


Figure 7. Sequence comparison of barnacle A-type allatostatin with homologs in arthropods. Decapod ASTAs are from *Macrobrachium rosenbergii* Q1AHE3, *Panulirus interruptus* A6BL33 and *Procambarus clarkii* Q3LI53; insect ASTAs are from *Bombyx mori* NP_001037036, *Drosophila melanogaster* AAF97792. Asterisk "*" represents typical motif residues of ASTA peptide.
doi:10.1371/journal.pone.0046513.g007

crustacean class Maxillopoda with copepod [41]. However in *B. amphitrite*, neuropeptides such as TRP, Orco, IRP and ASTB, were much similar to their homologs from insects rather than decapods, as supported by structure comparison and sequence alignment. High sequence similarity among different species may represent independent evolution under shared evolutionary constraints, which maintains structural and functional conservation of protein products [38]. The closer relationship of these neuropeptides between *B. amphitrite* and insects inferred much about their similar function in these two groups. Since molecular neuroendocrine information of arthropods other than decapods and insects is limited, data on neuropeptides from other taxa would be helpful.

4. Neuropeptides that are Specifically Up/down Regulated in Cyrid Stage

To further explore the potential involvement of neuropeptide genes in larval settlement of *B. amphitrite*, late nauplius VI, cyprid, early juvenile and adult were chosen to assess developmental variation of neuropeptide genes. Fourteen predicted neuropeptide genes were subjected to quantitative real-time PCR (Figure 10). Since the expression level of bursicon α subunit has already been measured by Chen et al. [20], this gene was omitted in the current developmental profiling. Using gene expression level in the juvenile stage as a standard, *calcia* (A isoform of calcitonin-like diuresis hormone), *sif* (SIFamide), *pdh* (pigment dispersing hormone) and *astb* (B-type allatostatin) were found to be specifically up-regulated in either late naupliar stage or cyprid stage but down-regulated in early juvenile and adult stages (Tukey test, $p < 0.05$). *trp* (Tachykinin-related peptide) was down-regulated in cyprid and early juvenile stage compared to naupliar and adult stages (Tukey test, $p < 0.05$); *irp* (Insulin-related peptide) was down regulated in early juvenile compared to other stages ($p < 0.05$, One-way ANOVA); expression level of *npf* (neuropeptide F), *burb* (bursicon subunit β), *calcib* (B isoform of calcitonin-like diuresis hormone), *orco* (orcokinin), *sulf* (sulfakinin), *eh* (eclosion hormone),

asta and *astc* (A- and C- type allatostatin) remained unchanged among naupliar VI, cyprid, juvenile stages, but were down-regulated in adults. The down regulation of neuropeptide genes in adults could be due to the degenerated neural system since the central nervous system of adult barnacle is highly reduced in the sessile mode [29,42]. There was, however, an exception, which is the high expression of tachykinin-related peptide (*trp*) in both naupliar VI and adult stages. Since the nervous system of cyprid larva is specifically cater for sensing settlement cues [43], we thus expect that peptide B-type allatostatin, A isoform of calcitonin-like diuresis hormone (Calci-A), pigment dispersing hormone and SIFamide are involved in cyprid attachment and metamorphosis, or at least performs a specific function during these two stages, since they were higher expressed in larval stage but down-regulated after metamorphosis.

4.1 B-type allatostatin. All three families of ASTs were found in the transcriptome of *B. amphitrite*, but only ASTB was up-regulated in both naupliar VI and cyprid stages, being almost 3-fold higher than the juvenile stage (Figure 10B). Different developmental expression patterns of these three types of ASTs in *B. amphitrite* indicates that each type may be respectively involved in different physiological processes, such as juvenile hormone synthesis, stomatogastric or cardiac neuromuscular functions reported in insects [44,45]. One major function of ASTs in the insects studied is inhibiting juvenile hormone (JH) synthesis [46]. JH is responsible for the maintenance of juvenile characteristics during development, and prevents metamorphosis during larval stage. Methyl farnesoate (MF), the unepoxidised form of JH III discovered in crustacean, was recently considered as functionally equivalent to insect JH [47]. In barnacle, high concentration of exogenous MF induced precocious metamorphosis without attachment [14], while physiologically-relevant concentration of natural isomer of MF inhibited larval settlement [16]. Furthermore, Yamamoto et al. [13] suggested that the balance of JH and 20-Hydroxyecdysone could regulate cyprid metamorphosis, especially molting. Since ASTB was reported to have JH-inhibiting

A

```

BalamBurA - DEQK LTPVVHVLQYPGCVPKPIPSYACVGHCTS YVQVSGSKLWQTERS CMCCQESGQREASVSI FCPKA
EupsuBurA VDECSLTPV IHLSPGCKSKPIPSFACQGRCTS YVQVSGSKLWQTERS CMCCQESGEREA AVTLNCPKA
LitvaBurA -DECSLTPV IHLSPGCKSKPIPSFACQGRCTS YVQVSGSKLWQTERS CMCCQESGEREA SVTLNCPKA
HomgaBurA -DECSLTPV IHLSPGCVSKPIPSFACQGRCTS YVQVSGSKLWQTERS CMCCQESGEREA SVTLNCPKV
CarmaBurA -DECSLRPV IHLSPGCTSKPIPSFACQGRCTS YVQVSGSKLWQTERS CMCCQESGEREA I TLNCPKP
DappuBurA -DECQLTPV IHV LQYPGCI PKPIPSFAC TIGKCTS YVQVSGSKLWQTERS CMCCQESGEREA TVSL LCPKA
TricaBurA -DECQVTPV IHV LQYPGCVPKPIPSFAC IGRGCASY IQVSGSK IWQMERS CMCCQESGEREA SVSLFCPKA
DromeBurA -DDCQVTPV IHV LQYPGCVPKPIPSFACVGRGCASY IQVSGSK IWQMERS CMCCQESGEREA AVSLFCPKV
ApimeBurA -DECQA TPV IHFLQYPGCVPKPIPSYACRGRGCSSYLQVSGSK IWQMERS CMCCQESGEREA SVSLFCPRA
MusdoBurA -DDCQVTPV IHV LQYPGCVPKPIPSFACVGRGCASY IQVSGSK IWQMERS CMCCQESGEREA AVSLFCPKV
BommoBurA -QECQMTAV IHV LKHRGCKPKA IPSFAC IGRGCASY YVQVSGSK IWQMERTCNCCQESGEREA TVV LFCPDA
IxoscBurA -ESCQLRPV IHV LKQPGCKPKPIPSFACQGS CSSYLQVSGSRYWQV ERS CMCCQEMGEREA TKAVFC PKG

```

```

BalamBurA KQSDQK FRKIVTRAPVECMCRPCTQA EESKAI PQEVAGFVSGMSLESMPFV - - - - -
EupsuBurA RSGEPK L - - - - -
LitvaBurA RPPGPRMRKILTRAPIDCMCRPCTDVEEGTVLAQEIANFI EDSP-MENV PFL - - - - -
HomgaBurA RKGEPTRRKILTRAPIDCMCRPCTDVEEGTVLAQEIANFI HDSP-MGNV PFLK - - - - -
CarmaBurA RPPGPK EKKV LTRAPIDCMCRPCTDVEEGTVLAQKIANFI QDSP-MDSV PFLK - - - - -
DappuBurA APGEPK LRRV VTRAPVDCMCRPCTALEESAVMPQEIARFLDD - - - - GSFPFKL - - - - -
TricaBurA KPGERK FTKVT KAPLECMCRPCTGV EESA V I PQE IAGY ADEGPLSNHFLKSHSQ - - - - -
DromeBurA KPGERK FKKV LTKAPLECMCRPCTS I EESG I I PQE IAGYSDEGPLNNHFRR IALQ - - - - -
ApimeBurA KPGEK FRKV ITKAPLECMCRPCTSV E EY A I I PQE IAGFADEGPF TTS AHFRSSDLQ - - - - -
MusdoBurA KHGERK FKKV LTKAPLECMCRPCTS I EESG I V PQE IAGYSDEGPLNNHFRR IALQ - - - - -
BommoBurA QNEEK RFRK VSTKA PLQCMCRPCTGS I EESS I I PQEVA GYS EEGPLYNHFRKSL - - - - -
IxoscBurA - - PGPK FRK LITRAPVECMCRPCTA PDEAS I LPQEFVGL - - - - -

```

B

```

BalamBurB - - - - - ARPQCETIPSTIHIITKDEFSSSGLERTCEGDIPVNKCEGTCS SQQVQPSV I SPSGFNKECS CC
HomamBurB - - - - - RRYDL ECETLPST IHVAKKEEFDEAGR VERTCEEDLAVNKCEGACVSKVQPSVNTPSGFLKDCRCC
LitvaBurB GPSRAHTYGS ECETLPSTMHVAKKEEFDDAGR LVRTCEEDLAVNKCEGACVSKVQPSVNTPSGFLKDCRCC
HomgaBurB - - - - - RRYDL ECETLPST IHVAKKEEFDEAGR VERTCEEDLAVNKCEGACVSKVQPSVNTPSGFLKDCRCC
CarmaBurB - - - - - RSYGVE CETLPST IHSKEEYDDTGR LRVCEEDVAVNKCEGACVSKVQPSVNTPSGFLKDCRCC
DappuBurB - - SKTNLSMGT CETLPST IHIITKEEYTDGG T LSR TCEGDIGVAKCEGSCSSQVQPSVHPSGFLKECMCC
TricaBurB - - - VSEIS EETCETLMSD INLIKEEFDELGR LQR I CNGEVAVNKCEGSKS QVQPSVITPTGFLKECYCC
DromeBurB - RYSQGTG DENCETL KSEIHLIKEEFD ELGRMQRTCNADV I VNKCEGLCNSQVQPSVITPTGFLKECYCC
ApimeBurB - - - - - QVTDD ENCE TLQSEVHITKDEYDE IGR LKRTCSGD ISVTKCEGFCNSQVQPSVASTTGFSKECYCC
MusdoBurB L RYSQGTG DENCETI KSEIHLIKEE LDELGRMQRTCNADV I VNKCEGLCNSQVQPSVITPTGFLKECYCC
BommoBurB - - - - - - EENCE TLVASEVHVTKEEYDEMGR L LRS CSEGEVSVNKCEGMCNSQVHPSISSPTGFQKECFCC
IxoscBurB R PRA TL DGGSS CR LQ PTT I R I TRDQNDELGSL TRTCEGTVLVSRC EGT I SQVQPSITLPHGFLKECNCC

```

```

BalamBurB KETGLRVREITLTRCFNPDGQQVAG - DQGR LTVKL - - REPSDCRCS RCEN - - -
HomamBurB RETHLRAREVTLTHCYDADGNRLTG - DRGTLV IKL - - REPADCQC FKCGDSTR
LitvaBurB RETHLRARDV V LTHC CDGDNRI TG - DNGK LTVK LTV REPADCQC FKCGNS I -
HomgaBurB RETHLRAREVTLTHCYDADGNRLTG - DRGTLV IKL - - REPADCQC FKCGDSTR
CarmaBurB REVHLRARD I TLTHCYDGGARL SG - AKATQHVKL - - REPADCQC FKCGDSTR
DappuBurB RESFLRER V VTLTHCYDANGNRLTG - KSSSLDVKM - - REPADCKC FRCGDSAE
TricaBurB RESFLRERT I TLTHCYDPDGVRLT AETVNSMDVKL - - REPAECKCYKCGDFSR
DromeBurB RESFLKEKV I TLTHCYDPDGTRLTSP EMGSM D IRL - - REPTECKC FKCGDFTR
ApimeBurB RESYLKERH I TLHHCYDADGI KLMNEENGVM EIKI - - REPVECKC IKCGD ISQ
MusdoBurB RESFLKEK I I TLSHCYDPDGTRLNSPE MATMD IRL - - REPTDCKC FKCGDFTR
BommoBurB REKFLRERLV TLTHCYDPDGI RFED EENALMEVRL - - REPDECECKYKCGDFSR
IxoscBurB RETYMNKRE IQLQDC FDPNGQKLYG - AEGSM TIFL - - EEPQDCSCHKCG - - -

```

Figure 8. Sequence alignment of barnacle bursicon peptides. (A) Alignment of bursicon α subunit. Sequences are from: euphausiacean *Euphausia superba* [22]; decapods *Litopenaeus vannamei* [22], *Homarus gammarus* ADI86242.1 and *Carcinus maenas* EU139428; branchiopod *Daphnia pulex* [38]; insects *Tribolium castaneum* DQ138190, *Drosophila melanogaster* NM_142726, *Apis mellifera* NM_001098234, *Musca domestica* EF424614 and *Bombyx mori* NM_001098375; chelicerate *Ixodes scapularis* XM_002407468. (B) Alignment of bursicon β subunit. Sequences are from: decapods *Homarus americanus* [22], *Homarus gammarus* ADI86243.1, *Litopenaeus vannamei* [22] and *Carcinus maenas* EU139429; branchiopod *Daphnia pulex* [38]; insects *Tribolium castaneum* DQ156997, *Drosophila melanogaster* NM_135868, *Apis mellifera* NM_001040262, *Musca domestica* EF424613 and *Bombyx mori* NM_001043824; chelicerate *Ixodes scapularis* XM_002407469. Asterisk "*" mark the conserved cysteine that will form dimers. doi:10.1371/journal.pone.0046513.g008

effects in other species, we presume that high expression level of ASTB in late cyprid stage would suppress JH synthesis and subsequently regulates metamorphosis.

4.2 Calcitonin-like diuretic hormone. In our study, two isoforms of calcitonin-like diuretic hormone (CalciA and CalciB) located at different transcripts were identified and distinct expression patterns were detected. CalciA was highly expressed in both Nauplii VI and cyprid compared with juvenile stage, while

CalciB's expression didn't significantly change during settlement (Figure 10E, F). Calci belongs to the diuretic hormone (DH) family and could promote fluid secretion of Malpighian tubule in insects [48], acting as mosquito natriuretic peptide that can stimulate Na-rich urine [49], or modulated diuresis-related hindgut activity [50]. In crustaceans, Calci was characterized in the American lobster *Homarus americanus* and functioned as an intrinsic modulator of cardiac output [51]. Thus barnacle CalciA may be involved in

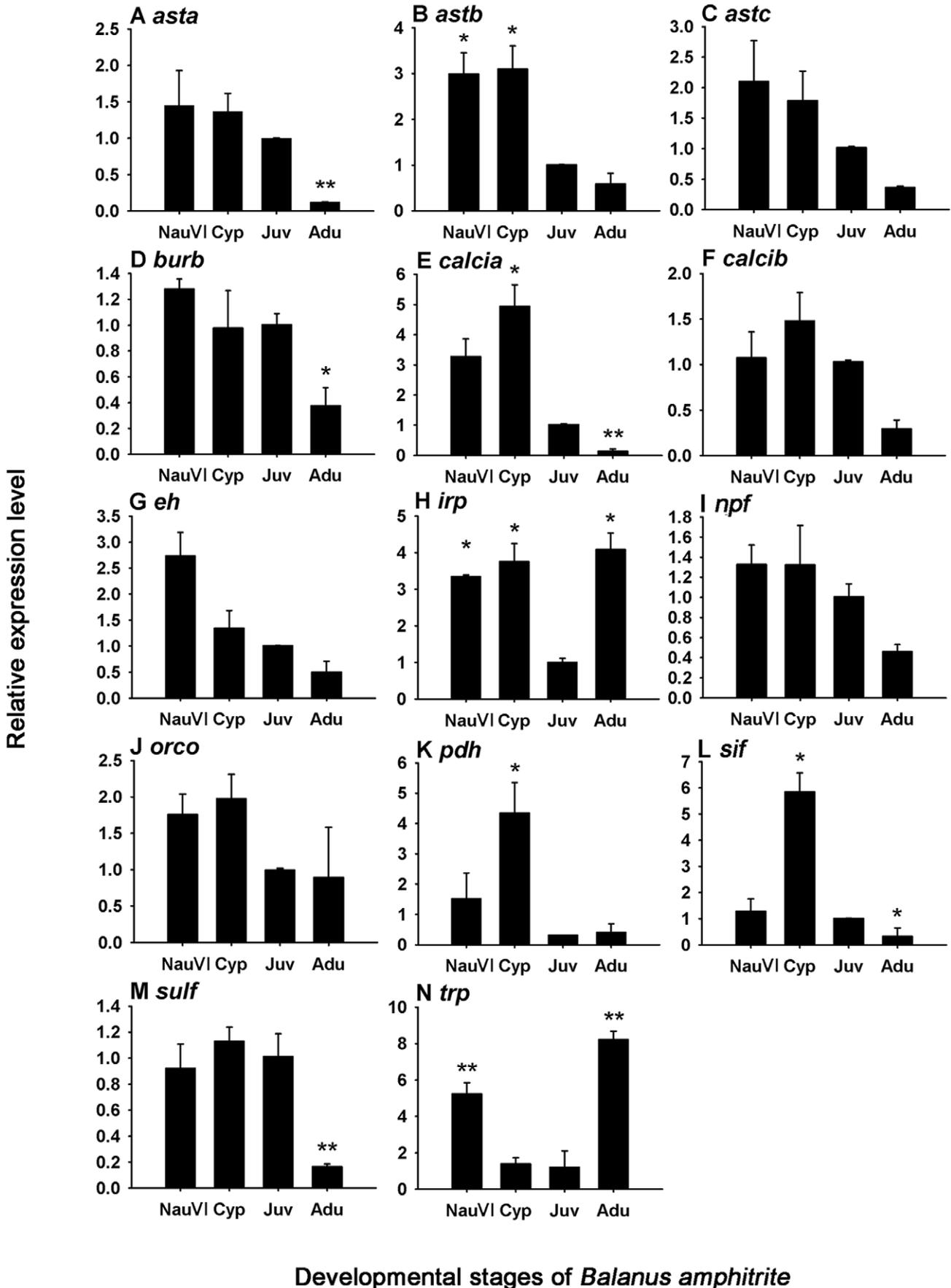


Figure 10. Real-time PCR results of predicted neuropeptide genes in four developmental stages. Stages include the late nauplius VI (NauVI), cyprid (Cyp), young juvenile (Juv) and the adult (Adu). Gene expression was measured for (A) A-type allatostatin (*asta*), (B) B-type allatostatin (*astb*), (C) C-type allatostatin (*astc*), (D) bursicon subunit β (*burb*), (E) calcitonin-like diuretic hormone isoform A (*calcia*), (F) calcitonin-like diuretic hormone isoform B (*calcib*), (G) eclosion hormone (*eh*), (H) insulin-related peptide (*irp*), (I) neuropeptide F (*npf*), (J) orcokinin (*orco*), (K) pigment dispersing hormone (*pdh*), (L) SIFamide (*sif*), (M) sulfakinin (*sulf*), (N) tachykinin-related peptide (*trp*). Values are shown as mean \pm SD from three biological replicates. Asterisks indicate significant difference detected by one-way ANOVA comparing to gene expression level in juvenile stage (Tukey test, * $p < 0.05$, ** $p < 0.01$). doi:10.1371/journal.pone.0046513.g010

feeding status in locust evident by a decrease in immunoreactivity after 48 hours of starvation [66]. In crustaceans, TRP was first discovered in the crab *Cancer borealis* and exactly the same sequence was then found in other seven crab species [37]. In general, TRP is related to food intake and digestion related functions. Since the cyprid larvae do not feed, the subsequent habitat selection and settlement behavior are dependent on energy reservation [67], i.e. lipids and vitellin-like protein [68]. During settlement, barnacle larvae undergo tremendous morphological changes and begin to feed from 2 to 5 days afterward [69]. TRP expression attained a relatively low level in the two non-feeding stages, cyprid and early juvenile, indicating its paracrine/hormonal control of feeding-related behavior of barnacle.

5. Prohormone Convertase Inhibitor Effectively Delayed Larval Settlement of *B. amphitrite*

Neuropeptides are derived from larger proprotein precursors which carry one or more mature peptides. Highly regulated posttranslational transformation is required for generating mature peptides with biological functions. After cleavage of N-terminal signal peptide, proprotein convertase (PC) cleaves at the mono- or di-basic cleavage sites of the remaining part of precursor, giving rise to peptide products that will undergo subsequent peptidase modification [70]. Two members of proprotein convertase family, PC2 and PC1/PC3, appear to play a preeminent role in neuroendocrine precursor maturation process in both mammalian and invertebrates [71]. In *Caenorhabditis elegans*, HPLC-MALDI-TOF analysis indicated a drastic reduction of types and

abundance of neuropeptides in KPC-2/KPC-3 (PC homologs) mutant strains compared to wild type strains [70]. The *kpc-2/egl-3* mutant was still viable, but its responsiveness to mechanical stimuli and egg-laying behavior were impaired [70].

To further explore peptidergic control of larval settlement of *B. amphitrite*, we performed settlement bioassay. This inhibitor is the most potent commercial compound that specifically inhibits peptide production and maturation [28]. Bioassay result showed that this inhibitor effectively delayed larval attachment and metamorphosis of *B. amphitrite*, on a dose dependent manner. After 24 hours, larval metamorphosis was significantly inhibited when the inhibitor concentration $\geq 10 \mu\text{molL}^{-1}$ (Tukey test, ** $p < 0.01$, *** $p < 0.001$), compared with cyprids incubated in AFSW or 0.5% DMSO as the control, while no significant effect was observed at $1 \mu\text{molL}^{-1}$ (Tukey test, $p = 0.081$). The inhibition was unlikely to be caused by toxicity of PC inhibitor since the unsettled cyprids in treatment group were swimming normally. After incubation for 48 hours, most of the swimming cyprids in the treatment group settled and metamorphosed normally into early juveniles, and larval metamorphosis percentage among the controls and treatments was not different (Tukey test, $p > 0.05$) (Figure 11). No mortality was observed for all the tested concentrations within the experimental duration. We may deduce that PC inhibitor restrained peptide maturation and thus cyprids delayed metamorphosis into juvenile. The real concentration of the compound in cyprids might be lower than the nominal concentration [11], and the peptide may be degraded by enzymes in hemolymph 48 hours after treatment. The complimentary peptide maturation pathways in addition of

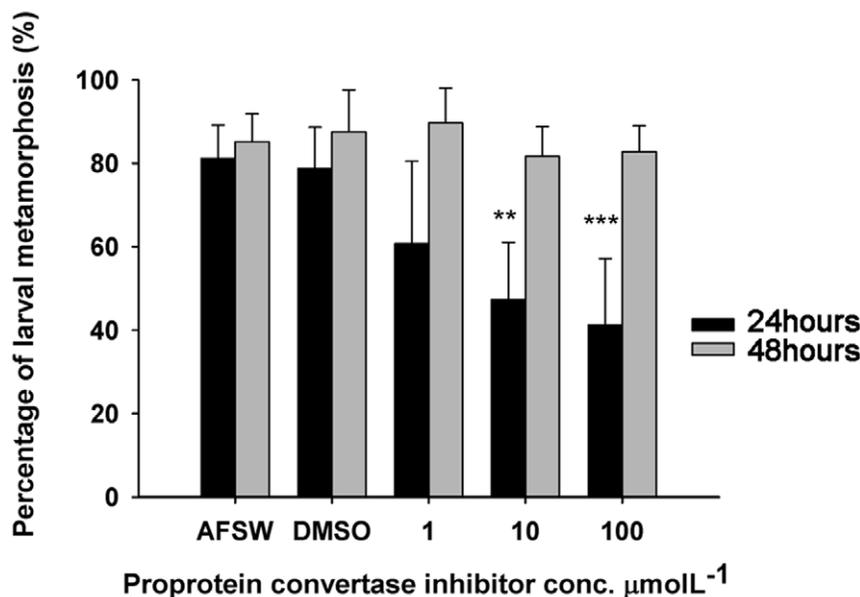


Figure 11. Proprotein convertase inhibitor bioassay result. Autoclaved filtered seawater (AFSW) and 0.1% DMSO served as positive control. Error bar represents mean \pm SD for three biological replicates. Asterisks indicate significant difference detected by one-way ANOVA comparing the treatments to the positive control (Tukey test, ** $p < 0.01$ and *** $p < 0.001$). doi:10.1371/journal.pone.0046513.g011

PC could be another reason why settlement was not completely blocked by inhibitor.

Conclusion

In conclusion, we discovered fourteen neuropeptide and peptide hormone families/subfamilies through *in silico* transcriptome mining of *Balanus amphitrite*. The analysis of mature structure and sequence of the predicted neuropeptides provided a new evidence on evolution of barnacle neuropeptides. B-type allatostatin, calcitonin, pigment dispersing hormone and SIFamide were up-regulated in cypris stage and down-regulated after metamorphosis. Together with our bioassay result of proprotein convertase inhibitor, we demonstrated the involvement of neuropeptides in larval metamorphosis. Our neuropeptidome data also provide a platform for further elucidating the physiological functions of individual peptide. Specifically, synthetic peptide could be raised based on the predicted peptide structure, for exploring their spatial expression pattern through specific antibodies, or for *in vivo* test of their functions in barnacle through peptide treatment. Given that *B. amphitrite* is an important biofouling species worldwide, neuropeptide genes and their postulated functional role in larval settlement revealed in this study may shed light on the future development of novel antifouling compounds.

References

- Ma M, Gard AL, Xiang F, Wang J, Davoodian N, et al. (2010) Combining *in silico* transcriptome mining and biological mass spectrometry for neuropeptide discovery in the pacific white shrimp *Litopenaeus vannamei*. *Peptides* 31: 27–43.
- Boonen K, Landuyt B, Baggerman G, Husson SJ, Huybrechts J, et al. (2008) Peptidomics: the integrated approach of MS, hyphenated techniques and bioinformatics for neuropeptide analysis. *J Sep Sci* 31: 427–445.
- Li B, Predel R, Neupert S, Hauser F, Tanaka Y, et al. (2008) Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red flour beetle *Tribolium castaneum*. *Genome Res* 18: 113–122.
- Grimmelikhuijzen CJP, Williamson M, Hansen GN (2002) Neuropeptides in cnidarians. *Can J Zool* 80: 1690–1702.
- Clark CA, del Campo ML, Ewer J (2004) Neuroendocrine control of larval ecdysis behavior in *Drosophila*: complex regulation by partially redundant neuropeptides. *J Neurosci* 24: 4283–4292.
- Riehle MA, Garczynski SF, Crim JW, Hill CA, Brown MR (2002) Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science* 298: 172–175.
- Montagné N, Desdevises Y, Soyev D, Toullec JY (2010) Molecular evolution of the crustacean hyperglycemic hormone family in cdysozoans. *BMC Evol Biol* 10: 62.
- Fusetani N (2004) Biofouling and antifouling. *Nat Prod Rep* 21: 94–104.
- Matsumura K, Nagano M, Fusetani N (1998) Purification of a larval settlement-inducing protein complex (SIPC) of the barnacle, *Balanus amphitrite*. *J Exp Zool* 281: 12–20.
- Matsumura K, Nagano M, Kato-Yoshinaga Y, Yamazaki M, Clare AS, et al. (1998) Immunological studies on the settlement inducing protein complex (SIPC) of the barnacle *Balanus amphitrite* and its possible involvement in larva-larva interactions. *Proc R Soc Lond B* 265: 1825–1830.
- Yamamoto H, Tachibana A, Kawai S, Matsumura K, Fusetani N (1996) Serotonin involvement in larval settlement of the barnacle, *Balanus amphitrite*. *J Exp Zool* 275: 339–345.
- Yamamoto H, Shimizu K, Tachibana A, Fusetani N (1999) Roles of dopamine and serotonin in larval attachment of the barnacle, *Balanus amphitrite*. *J Exp Zool* 284: 746–758.
- Yamamoto H, Kawai S, Yoshimura E, Tachibana A, Fusetani N (1997) 20-Hydroxyecdysone regulates larval metamorphosis of the barnacle, *Balanus amphitrite*. *Zool Sci* 14: 887–892.
- Yamamoto H, Okino T, Yoshimura E, Tachibana A, Shimizu K, et al. (1997) Methyl farnesoate induces larval metamorphosis of the barnacle, *Balanus amphitrite* via protein kinase C activation. *J Exp Zool* 278: 349–355.
- Freeman JA, Costlow JD (1983) The cyprid molt cycle and its hormonal control in the barnacle *Balanus amphitrite*. *J Crustacean Biol* 3: 173–182.
- Smith PA, Clare AS, Rees HH, Prescott MC, Wainwright G, et al. (2000) Identification of methyl farnesoate in the cyprid larva of the barnacle, *Balanus amphitrite*, and its role as a juvenile hormone. *Insect Biochem Mol Biol* 30: 885–890.
- Christie AE (2008) Neuropeptide discovery in Ixodoidea: an *in silico* investigation using publicly accessible expressed sequence tags. *Gen Comp Endocrinol* 157: 174–185.
- Liu F, Baggerman G, D'Hertog W, Verleyen P, Schoofs L, et al. (2006) *In silico* identification of new secretory peptide genes in *Drosophila melanogaster*. *Mol Cell Proteomics* 5: 510–522.
- De Gregoris TB, Rupp O, Klages S, Knaust F, Bekel T, et al. (2011) Deep sequencing of naupliar-, cyprid- and adult-specific normalised Expressed Sequence Tag (EST) libraries of the acorn barnacle *Balanus amphitrite*. *Biofouling* 27: 367–374.
- Chen Z-F, Matsumura K, Wang H, Arellano SM, Yan X, et al. (2011) Toward an understanding of the molecular mechanisms of barnacle larval settlement: a comparative transcriptomic approach. *PLoS One* 6: e22913.
- Thiyagarajan V, Qian P-Y (2008) Proteomic analysis of larvae during development, attachment, and metamorphosis in the fouling barnacle, *Balanus amphitrite*. *Proteomics* 8: 3164–3172.
- Christie AE, Durkin CS, Hartline N, Ohno P, Lenz PH (2010) Bioinformatic analyses of the publicly accessible crustacean expressed sequence tags (ESTs) reveal numerous novel neuropeptide-encoding precursor proteins, including ones from members of several little studied taxa. *Gen Comp Endocrinol* 167: 164–178.
- Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340: 783–795.
- Veenstra JA (2000) Mono- and dibasic proteolytic cleavage sites in insect neuroendocrine peptide precursors. *Arch Insect Biochem Physiol* 43: 49–63.
- Monigatti F, Gasteiger E, Bairoch A, Jung E (2002) The Sulfinator: predicting tyrosine sulfation sites in protein sequences. *Bioinformatics* 18: 769–770.
- Bacchetti De Gregoris T, Borra M, Biffali E, Bekel T, Burgess JG, et al. (2009) Construction of an adult barnacle (*Balanus amphitrite*) cDNA library and selection of reference genes for quantitative RT-PCR studies. *BMC Mol Biol* 10: 62.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods* 25: 402–408.
- Basak A (2005) Inhibitors of proprotein convertases. *J Mol Med* 83: 844–855.
- Gallus L, Ferrando S, Bottaro M, Diaspro A, Girosi L, et al. (2009) Presence and distribution of FMRFamide-like immunoreactivity in the cyprid of the barnacle *Balanus amphitrite* (Cirripedia, Crustacea). *Microsc Res Tech* 72: 101–109.
- Webster SG (1998) Peptidergic neurons in barnacles: An immunohistochemical study using antisera raised against crustacean neuropeptides. *Biol Bull* 195: 282–289.
- Semmler H, Wanninger A, Hoeg JT, Scholtz G (2008) Immunocytochemical studies on the naupliar nervous system of *Balanus improvisus* (Crustacea, Cirripedia, Thecostraca). *Arthropod Struct Dev* 37: 383–395.
- Verleyen P, Huybrechts J, Schoofs L (2009) SIFamide illustrates the rapid evolution in arthropod neuropeptide research. *Gen Comp Endocrinol* 162: 27–35.
- Weaver RJ, Audsley N (2009) Neuropeptide regulators of juvenile hormone synthesis: structures, functions, distribution, and unanswered questions. *Ann N Y Acad Sci* 1163: 316–329.
- Holmgren S, Jensena J (2001) Evolution of vertebrate neuropeptides. *Brain Res Bull* 55: 723–735.

Supporting Information

Table S1 Mature neuropeptides/peptide hormones predicted from *Balanus amphitrite*.

(PDF)

Table S2 Precursor sequences of neuropeptides and peptide hormones from *Balanus amphitrite*. Signal peptides are marked as green letter, precursor related peptides are marked as blue letter, and mature neuropeptides are marked as red letter. The putative mono-, di- or tribasic cleavage sites are underlined, and amino acid residues that predicted to be sulfated are shaded as pink.

(PDF)

Table S3 Primers used for real-time PCR amplification.

(PDF)

Author Contributions

Conceived and designed the experiments: X-CY P-YQ. Performed the experiments: X-CY Z-FC. Analyzed the data: X-CY JS ZF-C KM. Wrote the paper: X-CY Z-FC JS KM RW P-YQ.

35. Tobe SS, Zhang JR, Bowser PRF, Donly BC, Bendena WG (2000) Biological activities of the allatostatin family of peptides in the cockroach, *Diploptera punctata*, and potential interaction with receptors. *J Insect Physiol* 46: 231–242.
36. Weaver RJ, Edwards JP, Bendena WG, Tobe SS (1998) Structures, functions and occurrence of insect allatostatin peptides. In: Coast GM, Webster SG, editors. Recent advances in arthropod endocrinology. Cambridge: Cambridge University Press. 3–32.
37. Stemmler EA, Peguero B, Bruns EA, Dickinson PS, Christie AE (2007) Identification, physiological actions, and distribution of TPSGFLGMRamide: a novel tachykinin-related peptide from the midgut and stomatogastric nervous system of *Cancer* crabs. *J Neurochem* 101: 1351–1366.
38. Dirksen H, Neupert S, Predel R, Verleyen P, Huybrechts J, et al. (2011) Genomics, transcriptomics, and peptidomics of *Daphnia pulex* neuropeptides and protein hormones. *J Proteome Res* 10: 4478–4504.
39. Wu Q, Brown MR (2006) Signaling and function of insulin-like peptides in insects. *Annu Rev Entomol* 51: 1–24.
40. Regier JC, Shultz JW, Zwick A, Hussey A, Ball B, et al. (2010) Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. *Nature* 463: 1079–1083.
41. Cook CE, Yue Q, Akam M (2005) Mitochondrial genomes suggest that hexapods and crustaceans are mutually paraphyletic. *Proc Biol Sci* 272: 1295–1304.
42. Gwilliam GF, Cole ES (1979) The morphology of the central nervous system of the barnacle *Semibalanus curvatus* (Pallas). *J Morphol* 159: 297–310.
43. Harrison PJH, Sandeman DC (1999) Morphology of the nervous system of the barnacle cypris larva (*Balanus amphitrite* Darwin) revealed by light and electron microscopy. *Biol Bull* 197: 144–158.
44. Skiebe P, Schneider H (1994) Allatostatin peptides in the crab stomatogastric nervous system: inhibition of the pyloric motor pattern and distribution of allatostatin-like immunoreactivity. *J Exp Biol* 194: 195–208.
45. Jorge-Rivera JC, Marder E (1997) Allatostatin decreases stomatogastric neuromuscular transmission in the crab *Cancer borealis*. *J Exp Biol* 200: 2937–2946.
46. Lorenz MW, Kellner R, Hoffmann KH (1995) Identification of two allatostatins from the cricket, *Gryllus bimaculatus* de Geer (Ensifera: Gryllidae): additional members of a family of neuropeptides inhibiting juvenile hormone biosynthesis. *Regul Peptides* 57: 227–236.
47. Nagaraju GPC (2007) Is methyl farnesoate a crustacean hormone? *Aquaculture* 272: 39–54.
48. Furuya K, Milchak RJ, Schegg KM, Zhang J, Tobe SS, et al. (2000) Cockroach diuretic hormones: characterization of a calcitonin-like peptide in insects. *Proc Natl Acad Sci U S A* 97: 6469–6474.
49. Coast GM, Garside CS, Webster SG, Schegg KM, Schooley DA (2005) Mosquito natriuretic peptide identified as a calcitonin-like diuretic hormone in *Anopheles gambiae* (Giles). *J Exp Biol* 208: 3281–3291.
50. Brugge VA, Schooley DA, Orchard I (2008) Amino acid sequence and biological activity of a calcitonin-like diuretic hormone (DH31) from *Rhodnius prolixus*. *J Exp Biol* 211: 382–390.
51. Christie AE, Stevens JS, Bowers MR, Chapline MC, Jensen DA, et al. (2011) Identification of a calcitonin-like diuretic hormone that functions as an intrinsic modulator of the american lobster, *Homarus americanus*, cardiac neuromuscular system. *J Exp Biol* 213: 118–127.
52. Nässel DR (2002) Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog Neurobiol* 68: 1–84.
53. Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A PDF neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* 99: 791–802.
54. Petri B, Stengl M (1997) Pigment-dispersing hormone phase-shifts the circadian pacemaker of the cockroach *Leucophaea maderae*. *J Neurosci* 17: 4087–4093.
55. Meinertzhagen IA, Pyza E (1996) Daily rhythms in cells of the fly's optic lobe: taking time out from the circadian clock. *Trends Neurosci* 19: 285–291.
56. Rao KR, Riehm JP, Zahnov CA, Kleinholz LH, Tarr GE, et al. (1985) Characterization of a pigment dispersing hormone in eyestalks of the fiddler crab *Uca pugnator*. *Proc Natl Acad Sci U S A* 82: 5319–5322.
57. Meeleop E, Temmerman L, Schoofs L, Janssen T (2010) Signalling through pigment dispersing hormone-like peptides in invertebrates. *Prog Neurobiol* 93: 125–147.
58. Terhzaz S, Rosay P, Goodwin SF, Veenstra JA (2007) The neuropeptide SIFamide modulates sexual behavior in *Drosophila*. *Biochem Biophys Res Commun* 352: 305–310.
59. Vázquez-Acvedo N, Rivera NM, Torres-González AM, Rullan-Matheu Y, Suiz-Rodríguez EA, et al. (2009) GYRKPPFNGSIFamide (Gly-SIFamide) modulates aggression in the freshwater prawn *Macrobrachium rosenbergii*. *Biol Bull* 217: 313–326.
60. Stemmler EA, Cashman CR, Messinger DI, Gardner NP, Dickinson PS, et al. (2007) High-mass-resolution direct-tissue MALDI-FTMS reveals broad conservation of three neuropeptides (APSGFLGMRamide, GYRKPPFNGSIFamide and pQQLDHSVFLRFamide) across members of seven decapod crustacean infraorders. *Peptides* 28: 2104–2115.
61. Polanska MA, Yasuda A, Harzsch S (2007) Immunolocalisation of crustacean SIFamide in the median brain and eyestalk neuropils of the marbled crayfish. *Cell Tissue Res* 330: 331–344.
62. Clare AS, Matsumura K (2000) Nature and perception of barnacle settlement pheromones. *Biofouling* 15: 57–71.
63. Severini C, Improta G, Falconieri-Erspamer G, Salvadori S, Erspamer V (2002) The tachykinin peptide family. *Pharmacol Rev* 54: 285–322.
64. Winther AM, Nässel DR (2001) Intestinal peptides as circulating hormones: release of tachykinin-related peptide from the locust and cockroach midgut. *J Exp Biol* 204: 1269–1280.
65. Vanden Broeck J, Torfs H, Poels J, Van Poyer W, Swinnen E, et al. (1999) Tachykinin-like peptides and their receptors. A review. *Ann N Y Acad Sci* 897: 374–387.
66. Lange AB (2001) Feeding state influences the content of FMRFamide- and tachykinin-related peptides in endocrine-like cells of the midgut of *Locusta migratoria*. *Peptides* 22: 229–234.
67. Lucas MI, Walker G, Holland DL, Crisp DJ (1979) An energy budget for the free-swimming and metamorphosing larvae of *Balanus balanoides* (Crustacea: Cirripedia). *Mar Biol* 55: 221–229.
68. Shimizu K, Satuito CG, Saikawa W, Fusetani N (1996) Larval storage protein of the barnacle, *Balanus amphitrite*: biochemical and immunological similarities to vitellin. *J Exp Zool* 276: 87–94.
69. Rainbow PS, Walker G (1977) The functional morphology and development of the alimentary tract of larval and juvenile barnacles (Cirripedia: Thoracica). *Mar Biol* 42: 337–349.
70. Husson SJ, Clynen E, Baggerman G, Janssen T, Schoofs L (2006) Defective processing of neuropeptide precursors in *Caenorhabditis elegans* lacking proprotein convertase 2 (KPC-2/EGL-3): mutant analysis by mass spectrometry. *J Neurochem* 98: 1999–2012.
71. Rouillé Y, Duguay SJ, Lund K, Furuta M, Gong Q, et al. (1995) Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convertases. *Front Neuroendocrinol* 16: 322–361.
72. Christie AE, Lundquist CT, Nässel DR, Nusbaum MP (1997) Two novel tachykinin-related peptides from the nervous system of the crab *Cancer borealis*. *J Exp Biol* 200: 2279–2294.
73. Yasuda-Kamatani Y, Yasuda A (2000) Identification of orckinin gene-related peptides in the brain of the crayfish *Procambarus clarkii* by the combination of MALDI-TOF and on-line capillary HPLC/Q-ToF mass spectrometry and molecular cloning. *Gen Comp Endocrinol* 118: 161–172.
74. Ma M, Bors EK, Dickinson ES, Kwiatkowski MA, Sousa GL, et al. (2009) Characterization of the *Carcinus maenas* neuropeptidome by mass spectrometry and functional genomics. *Gen Comp Endocrinol* 161: 320–334.
75. Dirksen H, Burdzik S, Sauter A, Keller R (2000) Two orckinins and the novel octapeptide orcomyotropin in the hindgut of the crayfish *Orconectes limosus*: identified myostimulatory neuropeptides originating together in neurones of the terminal abdominal ganglion. *J Exp Biol* 203: 2807–2818.