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Neuropeptides encoded within a neural transcriptome of the giant triton snail *Charonia tritonis*, a Crown-of-Thorns Starfish predator

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ABSTRACT

Neuropeptides represent a diverse class of signaling molecules originating from neural tissues. These chemical modulators orchestrate complex physiological events including those associated with growth and reproduction. *De novo* transcriptome sequencing of a cerebral ganglion library of the endangered giant triton snail (*Charonia tritonis*) was undertaken in an effort to identify key neuropeptides that control or influence its physiology. The giant triton snail is considered a primary predator of the corallivore *Acanthaster planci* (Crown-of-Thorns Starfish) that is responsible for a significant loss in coral cover on reefs in the Indo-Pacific. The transcriptome library was assembled into contigs, and then bioinformatic analysis was used to identify a repertoire of 38 giant triton snail neuropeptide precursor genes, and various isoforms, that encode conserved molluscan neuropeptides. *C. tritonis* neuropeptides show overall precursor organisation consistent with those of other molluscs. These include those neuropeptides associated with mollusc reproduction such as the APGWamide, buccalin, conopressin, gonadotropin-releasing hormone (GnRH), NKY and egg-laying hormone. These data provide a foundation for further studies targeted towards the functional characterisation of neuropeptides to further understand aspects of the biology of the giant triton snail, such as elucidating its reproductive neuroendocrine pathway to allow the development of knowledge based captive breeding programs.

1. Introduction

The giant triton snail (*Charonia tritonis*) is one of the largest of all gastropods with a shell size reaching a length of 50 cm. In addition to its sheer size, this marine snail is highly sought after and collected for its attractive shell and appears to be significantly depleted throughout its range and protected in many countries of the Indo-Pacific [22]. It has been hypothesized that the giant triton snail has a role in moderating Crown-of-Thorns Starfish (COTS, *Acanthaster planci*) populations as it is a primary predator of this starfish [14]. It has been argued that excessive collection of giant tritons may contribute to the repeated occurrence of COTS outbreaks that can devastate Indo-Pacific coral reefs including the Great Barrier Reef.

A deeper understanding of the biology of the giant triton snail would assist in not only understanding their basic physiology but also the potential development of methods to culture them for restocking selective reefs, as has been done with other gastropod species [41]. For example, restocking of juvenile trochus *Trochus niloticus* on coral reefs in Western Australia, sub-adults at stocked sites were 54% more abundant compared to control sites. Closed-life cycle production of the giant triton has so far been unsuccessful partially due to a lack of understanding in the basic rearing requirements of the larvae [39]. There is no information of the genes, proteins and metabolites that regulate larval development, growth and reproduction in this species. Critical to these functions are neuropeptides, which comprise a diverse class of cell signaling molecules with varied roles: from neurotransmitters and/or neuromodulators (when neuropeptides functionally modulate neuronal circuit activity) to neurohormones (when neuropeptides are released from neurons into the circulatory system in order to reach and influence cell targets in distance) [38].

In the past, the identification of neuropeptides has been from purified neuronal tissue extracts using mass spectrometry and peptide sequencing [45,51]. In recent times, the number of genes identified encoding known/putative neuropeptides in both model and non-model species has increased significantly since the introduction of next-generation sequencing technology com-

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bined with bioinformatics [16,24,36]. To date, few investigations have established the repertoire of neuropeptide genes from the genomes/transcriptomes of molluscs, i.e., *Lottia gigantea* (limpet) [59], *Theba pisana* (land snail) [1], *Pinctata fucata* (pearl oyster) and *Crassostrea gigas* (oyster) [48], because of the limited number of available molluscan genomes and neural-specific tissue transcriptomes. No neuropeptides have been identified previously in the *C. tritonis*. In this study, a cerebral ganglia transcriptome of *C. tritonis* was interrogated to identify and annotate the neuropeptide genes. Among the neuropeptides identified are those known to be involved in molluscan reproduction and growth that may provide the basis for reproductive manipulation and assist in the recovery of *C. tritonis* populations.

2. Materials and methods

2.1. Animal and tissue collection

Specimens of *Charonia tritonis* were collected from sites surrounding Kavieng, Papua New Guinea (April 2015) and kept within culture facilities at the Nago Island marine hatchery facility. Snails were provided food (various echinoderms) *ad libitum* and maintained at ambient temperature. Snails (gender unknown) were anaesthetized with isotonic $MgCl_2$. The cerebral ganglia were removed by dissection and immediately stored in RNAlater (Ambion).

2.2. RNA preparation, transcriptome sequencing, assembly and quantitation

RNA was extracted from from 4 *C. tritonis* cerebral ganglia using TRIzol Reagent (Invitrogen, Mt. Waverley, Australia), as per the manufacturer's protocol. Extracted RNA was assessed for quality by visualisation on a 1.2% denaturing formaldehyde agarose gel, quantified using a Nanodrop spectrophotometer 2000c (Thermo Scientific, Waltham, MA). RNA samples were pooled equally and provided to the Australian Genome Research Institute (Australia, Brisbane) for library construction and paired-end sequencing using an Illumina HiSeq 2500 platform. Raw sequences were trimmed of adaptors and assembled into contigs using the Genomic Workbench 8 software (default settings). Protein coding regions were determined using the open reading frame (ORF) predictor [35]. Relative expression of genes in the transcriptome was determined based on reads per kilobase of exon per million mapped reads (RPKM) values, utilising the *de novo* RNA-seq CLC Genomic Workbench 8 software [46].

2.3. Neuropeptide prediction and sequence analysis

Neuropeptides derived from the molluscs *L. gigantea* [59], *C. gigas* [48] and *T. pisana* [1] were used in a BLASTp search to identify neuropeptide-like proteins from the *C. tritonis* cerebral ganglion transcriptome. Matches were manually assessed to determine conservation of putative bioactive regions. Four bioinformatics tools were initially applied to all putative neuropeptides of *C. tritonis* to predict the presence of a signal peptide (SignalP 3.0 [4] and PrediSi [21]) and any transmembrane domain(s) (TMHMM 2.0 [27] and HMMTOP 2.1 [55]). These putative neuropeptides were then analysed using the NeuroPred tool to predict cleavage products [47]. Schematic diagrams of protein domain structures were prepared using Domain Graph (DOG, version 2.0) software [60]. Protein sequences from *C. tritonis* were aligned against a database prepared from known sequences from NCBI (January 2014) using the MEGA 5.1 [52] platform with the ClustalW protocol utilising the Gonnet protein weight matrix.

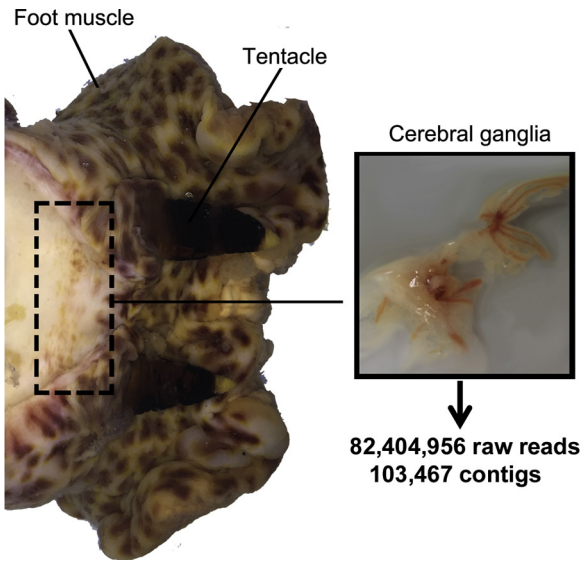

Fig. 1. Anatomy and transcriptome summary from the *Charonia tritonis* cerebral ganglion. Figure shows the cephalic region, cerebral ganglion and summary of sequences obtained from RNA-seq.

3. Results

3.1. De novo assembly of Charonia tritonis cerebral ganglia transcriptome and neuropeptide identification

A single transcript library was generated from 4 *C. tritonis* cerebral ganglia, then sequenced using Illumina HiSeq technologies and sequence reads were assembled into contigs. A summary of the location of the *C. tritonis* cerebral ganglion used for RNA isolation, the number of high quality raw sequence reads and contigs assembled is shown in Fig. 1. An N50 of 641 bp was achieved and the maximum contig length was 18,780 bp. From this cerebral ganglion transcriptome, genes encoding putative full-length or partial-length neuropeptide precursors were identified (Table 1). Numerous peptides are predicted to be cleaved from these precursors. Fig. 2 shows the organisation of full-length *C. tritonis* neuropeptide precursors, and compared with the land snail *T. pisana*. Overall, there is general high conservation in the spatial organisation of bioactive peptide sequences observed, as well as in precursor size, cleavage sites and position of cysteine residues.

A comparison with other molluscan *in silico* neuropeptide studies shows that the majority of neuropeptide precursors can be accounted for in this *C. tritonis* transcriptome (Table 2). The exceptions include AST-B, FWISamide, LRFNVamide, luqin, NPF/NPY and tachykinin. Some neuropeptide precursors that were not identified in other molluscan *in silico* studies include the adipokinetic hormone (AKH) and fulicin. Importantly, reproduction- and growth-related neuropeptides have been identified and will be discussed.

3.2. APGWamide

A transcript for an APGWamide (*Ctr-APGWamide*; Ctr = *Charonia tritonis*) was identified that encodes a 230 amino acid precursor protein, including putative cleavage sites that may release 12 APGWamide peptides (Fig. 3). The APGWamide are very well conserved with other molluscs, although variation exists in the bivalve *C. gigas* (i.e. RPGWamide). *Ctr-APGWamide* represents one of the most highly abundant neuropeptide transcripts in the cerebral ganglion (RPKM = 222.9, Table 2).