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Transcriptome analysis of the molting gland (Y-organ) from the blackback land crab, *Gecarcinus lateralis*

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ABSTRACT

In decapod crustaceans, arthropod steroid hormones or ecdysteroids regulate molting. These hormones are synthesized and released from a pair of molting glands called the Y-organs (YO). Cyclic nucleotide, mTOR, and TGFβ/Smad signaling pathways mediate molt cycle-dependent phase transitions in the YO. To further identify the genes involved in the regulation of molting, a YO transcriptome was generated from three biological replicates of intermolt blackback land crab, *Gecarcinus lateralis*. Illumina sequencing of cDNA libraries generated 227,811,829 100-base pair (bp) paired-end reads; following trimming, 90% of the reads were used for further analyses. The trimmed reads were assembled *de novo* using Trinity software to generate 288,673 contigs with a mean length of 872 bp and a median length of 1842 bp. Redundancy among contig sequences was reduced by CD-HIT-EST, and the output constituted the baseline transcriptome database. Using Bowtie2, 92% to 93% of the reads were mapped back to the transcriptome. Individual contigs were annotated using BLAST, HMMER, TMHMM, SignalP, and Trinotate, resulting in assignments of 20% of the contigs. Functional and pathway annotations were carried out via gene ontology (GO) and KEGG orthology (KO) analyses; 58% and 44% of the contigs with BLASTx hits were assigned to GO and KO terms, respectively. The gene expression profile was similar to a crayfish YO transcriptome database, and the relative abundance of each contig was highly correlated among the three *G. lateralis* replicates. Signal transduction pathway orthologs were well represented, including those in the mTOR, TGFβ, cyclic nucleotide, MAP kinase, calcium, VEGF, phosphatidylinositol, ErbB, Wnt, Hedgehog, Jak-STAT, and Notch pathways.

1. Introduction

Decapod crustaceans possess a rigid exoskeleton that must be shed periodically for organismal growth, a process called ecdysis or molting. Molting is regulated by two endocrine organs: the X-organ/sinus gland (XO/SG) complex, located in the eyestalk ganglia, and a pair of molting glands, or Y-organs (YOs), located in the cephalothorax (Skinner, 1985; Hopkins, 2012). The interaction between molt-inhibiting hormone (MIH), an inhibitory neuropeptide produced by the XO/SG complex, and steroid molting hormones (ecdysteroids), produced by the YO, drives the progression through the molt cycle (Webster et al., 2012; Webster, 2015). Hemolymph ecdysteroid titers are low during the intermolt stage, increase during the premolt stage, drop precipitously near the end of premolt, and remain at low levels during postmolt (Chang and Mykles, 2011; Mykles, 2011). The increase in ecdysteroid titers initiate and coordinate the physiological processes required for molting, such as degradation and reformation of the exoskeleton (Skinner, 1985), claw muscle atrophy (Mykles, 1997; Mykles and Medler, 2015), and limb regeneration (Skinner, 1985; Mykles, 2001; Hopkins and Das, 2015).

The YO undergoes transitions in physiological properties at critical stages of the molt cycle. During intermolt, the YO is kept in the basal state by pulsatile releases of MIH to maintain low hemolymph ecdysteroid titers (Nakatsuji et al., 2009; Chung et al., 2010). The repression of ecdysteroid synthesis via MIH signaling in the YO is mediated by cyclic nucleotide second messengers (Covi et al., 2009). MIH binding to a putative G protein-coupled membrane receptor is hypothesized to initiate a cAMP-dependent triggering phase, which is followed by an NO/cGMP-dependent summation phase for prolonged inhibition of ecdysteroidogenesis between MIH pulses (Chang and Mykles, 2011; Covi et al., 2012; Webster, 2015). A reduction in MIH during intermolt de-represses the YO. The activated YO hypertrophies to increase ecdysteroid synthesis; the hemolymph ecdysteroid titer increases and the animal transitions to the premolt stage (Chang and Mykles, 2011). The duration of early premolt depends on environmental and physiological conditions. Environmental stress, acting through the XO/SG complex, can prolong the premolt period, as the YO remains sensitive to MIH and a stress neuropeptide, crustacean hyperglycemic hormone (CHH) (Chang and Mykles, 2011; Shrivastava and Princy, 2014). Autotomy of a limb regenerate suspends premolt for a few weeks, which allows

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time for a new regenerate to form and grow, and the animal molts with a complete set of walking legs (Mykles, 2001; Yu et al., 2002; Chang and Mykles, 2011). A critical transition occurs at mid-premolt, when the animal becomes committed to molt. The YO increases ecdysteroid production further and becomes insensitive to MIH and CHH (Chang and Mykles, 2011; Covi et al., 2012). The animal progresses through to ecdysis without delay. If a limb regenerate is autotomized at this stage, there is no regeneration and the animal molts without a full complement of legs (Mykles, 2001; Yu et al., 2002).

Two highly conserved signaling pathways mediate the molt cycle-dependent transitions of the YO. The activation of the YO in early premolt requires mechanistic Target of Rapamycin (mTOR)-dependent protein synthesis. mTOR is a protein kinase that controls global translation of mRNA into protein in eukaryotic cells (Baretic and Williams, 2014). Its activity is controlled by a variety of signals that regulate energy allocation to protein synthesis, such as nutrients, cellular energy status, growth factors, and stress (Shimobayashi and Hall, 2014; Albert and Hall, 2015; Cetrullo et al., 2015). Cycloheximide, an inhibitor of mRNA translation, and rapamycin, an mTOR inhibitor, repress YO ecdysteroid secretion *in vitro* (Mattson and Spaziani, 1986, 1987; Abuhagr et al., 2014b). Moreover, increases in mRNA levels of mTOR signaling components *Gl-mTOR* and *Gl-Akt* as well as *Gl-elongation factor 2* (*Gl-EF2*) in mid-premolt and late premolt stages coincide with the increase in ecdysteroid production in the committed YO (Abuhagr et al., 2014b). The transition of the YO from the activated to the committed state involves TGFβ/Smad signaling via an Activin-like membrane receptor (Chang and Mykles, 2011). An Activin receptor inhibitor (SB431542) blocks YO commitment but has no effect on YO activation in eyestalk-ablated *Gecarcinus lateralis in vivo* (Abuhagr et al., 2012). Both signaling pathways are also important in insect molt regulation. mTOR activity controls the size and ecdysteroid synthetic capacity of the prothoracic gland (PG) by prothoracicotropic hormone (PTTH), bombyxin, and insulin-like peptides (ILPs) (Teleman, 2010; Covi et al., 2012; Yamanaka et al., 2013; Smith et al., 2014; Gu et al., 2015; Hatem et al., 2015). A recent report of an ortholog of *Drosophila* Ilp7 and eight insulin-like growth factor binding proteins in the rock lobster, *Sagmariasus verreauxi* (Chandler et al., 2015), lends support for the importance of ILP/mTOR signaling in crustaceans and raises the possibility that the YO, like the insect PG, is regulated by insulin-like growth factors (Yamanaka et al., 2013). Moreover, TGFβ signaling, which is mediated by Smad transcription factors (Macias et al., 2015), is necessary for the PG to respond to PTTH and insulin. Blocking Activin/Smad signaling in the *Drosophila* PG prevents the PTTH-triggered ecdysteroid peak that initiates metamorphosis (Pentek et al., 2009; Gibbens et al., 2011). Taken together, the data suggest that mTOR and TGFβ/Smad pathways play essential roles in the regulation of crustacean and insect molting glands by neuropeptides.

RNA-Seq technology has quickly become a powerful tool in decapod crustacean physiology, as it provides a deeper and broader range of transcripts than other methods (Wang et al., 2009). As the field lacks a decapod species with a fully mapped and annotated genome, the *de novo* assembly of RNA-Seq data allows the cataloging of all the genes expressed in a tissue, essentially leapfrogging the genome to the direct analysis of genes that define a specific function. This methodology can identify gene ontologies and networks associated with a physiological process, as well as quantify levels of mRNA abundance for all genes transcriptionally activated within that physiological state. Transcriptomic approaches have revealed genes associated with reproduction (Gao et al., 2014), development (Wei et al., 2014a; Chandler et al., 2015; Christiaens et al., 2015; Li et al., 2015), chitin metabolism in integumentary tissues (Tom et al., 2014; Abehsera et al., 2015), digestion (Wei et al., 2014b), neuroendocrine regulation (Christie, 2014; Ventura et al., 2014), and molting and growth (Tom et al., 2013; Lv et al., 2014). In the present study, Illumina high-throughput sequencing and *de novo* assembly was used to create a YO transcriptome database of the blackback land crab, *G. lateralis*. The species is an important model for the study of the endocrine regulation molting by neuropeptides and other factors (Chang and Mykles, 2011). A pipeline for the validation, analysis, and functional assignment of contigs is described. Genes encoding signal transduction pathways were characterized. As expected, the genes in the cyclic nucleotide, insulin/mTOR, and TGFβ/Smad pathways were well represented in the annotated database. Genes in the MAP kinase, calcium, VEGF, phosphatidylinositol, ErbB, Wnt, Hedgehog, Jak-STAT, and Notch pathways were also expressed. The diversity of signaling pathways raises the possibility that the YO can integrate endocrine, paracrine, and autocrine signals in order to respond appropriately to environmental and physiological conditions that affect molting.

2. Methods

2.1. Animals

Adult male *G. lateralis* were collected in the Dominican Republic, shipped to Colorado State University, and maintained as described (Covi et al., 2010). YOs were dissected from the branchial chamber side of the anterior branchiostegite region of the cephalothorax and stored in 300 μL RNAlater (Life Technologies, Grand Island, NY, USA) at −20 °C until processing. Hemolymph ecdysteroid titers were quantified using a competitive enzyme-linked immunosorbent assay (ELISA) (Kingan, 1989; Abuhagr et al., 2014a).

2.2. mRNA isolation, library preparation, and sequencing

Total RNA was isolated using RNeasy™ Mini Kits (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. RNA was quantified using Quant-iT™ RiboGreen® RNA Assay (Life Technologies, Carlsbad, CA, USA). The mRNA purification and cDNA synthesis was carried out with a TruSeq™ Stranded mRNA Library Prep Kit (Illumina). Three cDNA libraries, designated Im1, Im2, and Im3, were generated; each library was derived from mRNA from six YOs pooled from three intermolt animals. Paired-end sequencing of the cDNA libraries using an Illumina HiSeq™ 2000 instrument was performed at the Oklahoma Medical Research Foundation. All samples were run in a single sequencing lane with three adaptor tags.

2.3. Transcriptome assembly and annotation

The quality of paired-end raw reads in fastq format was assessed using the FASTQC program (Babraham Institute, Cambridge, UK). Quality reads with a minimum phred (nucleotide base call) score of 28 and length ranging from 36 bp to 100 bp were extracted by trimming of low quality reads and adapter sequences via Trimmomatic software (version number: 0.32) (Bolger et al., 2014). The trimmed reads obtained from three different biological YO replicates were concatenated into two files containing forward and reverse sequences, respectively. Further, both paired and unpaired reads were used for downstream analyses. The trimmed forward and reverse reads were then assembled via Trinity software with default settings (version number: r20130814) (Haas et al., 2013). The minimum contig length was set at 201 bp. Following assembly, the contigs were clustered based on a 90% sequence similarity threshold using the CD-HIT-EST program (version number: 4.6.1) (Li and Godzik, 2006). The output of CD-HIT-EST was used as the reference transcriptome to map the reads from individual libraries. We designated the assembled data as the YO baseline transcriptome.

For annotation, both nucleotide sequences and predicted protein sequences were used to run BLAST queries against NCBI non-redundant (NR), Swiss-Prot (SP), TrEMBL (Uniprot), and Uniprot Uniref90 protein databases (http://www.uniprot.org/downloads; Fig. 1) (Altschul et al., 1990; Bairoch and Apweiler, 2000). The NR and SP databases were downloaded on April 1, 2015, TrEMBL on October 19, 2015, and Uniref90 on June 26, 2015. Stand-alone software was used for running BLAST (version: 2.2.28) against the above-mentioned databases