Integrative and Comparative Biology

Integrative and Comparative Biology, volume 56, number 6, pp. 1170–1182
doi:10.1093/icb/icw003

Society for Integrative and Comparative Biology

SYMPOSIUM

Developmental Transcriptomics of the Hawaiian Anchialine Shrimp *Halocaridina rubra* Holthuis, 1963 (Crustacea: Atyidae)

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From the symposium "Tapping the Power of Crustacean Transcriptomes to Address Grand Challenges in Comparative Biology" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2016 at Portland, Oregon.

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Synopsis Many crustacean species progress through a series of metamorphoses during the developmental transition from embryo to adult. The molecular genetic basis of this transition, however, is not well characterized for a large number of crustaceans. Here, we employ multiple RNA-Seq methodologies to identify differentially expressed genes (DEGs) between "early" (i.e., $Z_1 - Z_2$) as well as "late" (i.e., $Z_3 - Z_4$) larval and adult developmental stages of *Halocaridina rubra* Holthuis (1963), an atyid shrimp endemic to the environmentally variable anchialine ecosystem of the Hawaiian Islands. Given the differences in salinity tolerance (narrow vs. wide range), energy acquisition (maternal yolk-bearing vs. microphagous grazing), and behavior (positively phototactic vs. not) between larvae and adults, respectively, of this species, we hypothesized the recovery of numerous DEGs belonging to functional categories relating to these characteristics. Consistent with this and regardless of methodology, hundreds of DEGs were identified, including upregulation of opsins and other light/stimulus detection genes and downregulation of genes related to ion transport, digestion, and reproduction in larvae relative to adults. Furthermore, isoform-switching, which has been largely unexplored in crustacean development, appears to be pervasive between *H. rubra* larvae and adults, especially among structural and oxygen-transport genes. Finally, by comparing RNA-Seq methodologies, we provide recommendations for future crustacean transcriptomic studies, including a demonstration of the pitfalls associated with identifying DEGs from single replicate samples as well as the utility of leveraging "prepackaged" bioinformatics pipelines.

Introduction

Many aquatic species undergo metamorphosis, usually reaching a final adult form after transitioning between several distinct larval stages. Larvae at each of these stages can be quite different from each other and the adult form in terms of anatomy, physiology, behavior, and ecology (reviewed by Anger 2006). Progression through each distinct stage is accomplished via molting, with new features often emerging during specific metamorphic molts (e.g., Charmantier et al. 1991). For crustaceans, methyl farnesoate, the equivalent of juvenile hormone in insects (Laufer et al. 1987; Laufer and Biggers 2001;), is thought to govern metamorphosis. However, the molecular genetic aspects of this progression of developmental stages are not well characterized, and even the specific role of methyl farnesoate during development remains underexplored (Abdu et al. 1998). Given this, the combination of advancements in generating DNA sequence data and comparative transcriptomics has the power to greatly enhance our understanding of larval metamorphosis (Medina 2009) within groups such as the Crustacea.

The application of RNA-Seq, or high-throughput DNA sequencing technologies to profile the

Advanced Access publication July 8, 2016

population of RNAs from a genome at a given point in time (Wang et al. 2009), is now being utilized more widely for investigating gene expression patterns within and between transcriptomes from various developmental stages of crustaceans. The amphipod *Parhyale hawaiensis* is an emerging model in this area, with its tractable reproduction and development in the laboratory leading to the availability of transcriptomic resources for multiple stages of development (Rehm et al. 2009; Zeng et al. 2011; Blythe et al. 2012). Another example is the giant river prawn *Macrobrachium rosenbergii*, where RNA-Seq of planktonic larval and benthic juvenile stages identified numerous differentially expressed genes (DEGs), several of which interact with methyl farnesoate or belong to well-known (e.g., Hedgehog and Wnt) developmental pathways (Ventura et al. 2013). Moreover, specific genes or pathways of interest have been targeted for study during crustacean development via transcriptomic analyses, like those of the peptidergic signaling systems in the copepod *Calanus finmarchicus* (Christie et al. 2013). Comparative transcriptomics can also illuminate the morphological and functional similarities and differences during development of species spanning a wide spectrum of relatedness and life-history strategies (reviewed by Roux et al. 2015). Thus, continued characterizations and comparisons of transcriptomes across diverse crustacean taxa from different environments with varying larval development will undoubtedly help in revealing the molecular mechanisms underpinning evolutionary developmental biology across crustaceans.

Shrimp from the family Atyidae provide an excellent opportunity to contextualize the molecular genetics of crustacean development from an interesting ecological perspective. Specifically, atyids as adults are for the most part restricted to freshwater, with no known or extant marine representatives (Fryer 1977). Although adult atyids are typically intolerant of seawater (Smith and Williams 1981; von Rintelen et al. 2012), many species exhibit an amphidromous life-history strategy, with larvae being exported into seawater for development and returning to freshwater as juveniles (Bauer 2013). There is an exception to this general rule, however, in the species of the "anchialine clade" (von Rintelen et al. 2012), a monophyletic group within the Atyidae whose members are endemic to the variable salinities of the anchialine ecosystem—which is made up of ponds, pools, and submerged caves sharing underground connections to both freshwater and seawater sources (Sket 1996). The Hawaiian anchialine atyid *Halocaridina rubra* (Holthuis 1963) is an exemplar of this clade, with adults tolerating salinities ranging from fresh (e.g., 0‰) to hypersaline (e.g., 50‰) waters via a novel mechanism of chronically upregulated osmoregulatory processes, thus leading *H. rubra* to be the strongest known osmoregulator among the Crustacea (Havird et al. 2013, 2014a). Notably, *H. rubra* larvae do not tolerate freshwater and lack gills capable of ion transport until the post-settlement juvenile stage or later (Havird et al. 2015). Therefore, the exclusion of larvae from the lower salinity epigeal (i.e., surface), and confinement to the higher salinity hypogeal (i.e., subterranean) component of Hawaii's anchialine ecosystem may reflect a difference in osmoregulatory ability as compared to adults (Havird et al. 2015).

Here, the molecular genetics of development were examined in *H. rubra* using a comparative transcriptomic approach to bring both novel insights into the biology of the species, such as the physiological context of juveniles transitioning from the hypogeal to epigeal environment, as well as crustacean developmental biology in general. In addition to differences in salinity tolerance, characteristics such as lecithotrophy (i.e., nutrition derived from maternally inherited yolk) and positive phototaxis distinguish larval and adult stages of *H. rubra* (Maciolek 1983; Bailey-Brock and Brock 1993). These distinctions in ecophysiology, energy acquisition, and behavior led us to hypothesize that hundreds of genes would be differentially expressed between *H. rubra* life stages, specifically those from functional categories related to osmoregulation, digestion, and light detection. To test this, we developed transcriptomic resources for adult and larval *H. rubra* and identified DEGs among different developmental stages using multiple RNA-Seq approaches.

Materials and Methods

Animals and holding conditions

Laboratory colonies of *H. rubra* have been previously described in detail by Vaught et al. (2014) and Havird et al. (2014a). Briefly, adult *H. rubra* collected from seven genetically diverged (i.e., ~0.05 uncorrected (*p*) genetic distance in mitochondrial cytochrome oxidase subunit I (COI) sequence) lineages, as well as one naturally mixed population of two lineages (Craft et al. 2008), have been maintained in separate 38-L aquaria at Auburn University, AL, USA since 2006. Following establishment, colonies have exhibited semi-predictable and continuous reproduction that significantly differs by lineage and time of year (Havird et al. 2015). To construct reference transcriptomes for *H. rubra*, a single adult and 88 larvae were sampled from the Windward Oahu (EP) lineage in April 2012. At this sampling,

larvae were not examined to determine their specific stage, but all were planktonic and likely a mix of Z_1 – Z_4 individuals (Iwai 2005). This pooling scheme was designed to both sample a broad diversity of larval development stages as well as provide sufficient RNA concentrations for library preparation and sequencing (see below). In May 2014, adults and larvae were also sampled from the East Hawaii (HILO) lineage. Larval stages were determined during this second sampling using morphological characters described in Iwai (2005), with larvae in the Z_1 and Z_2 stages grouped as "early" and Z_3 and Z_4 stages grouped as "late" for differential expression analyses (see below).

Table 1 Sequencing and assembly statistics for *Halocaridina rubra* transcriptomes.

	"Adult"	"Larvae"	"Adult+ larvae"
#s of 100 bp paired end (PE) reads	8,603,841	39,441,672	48,045,513
Mean quality score of reads	34.1	30.7	32.4
Total # contigs following assembly	68,295	109,572	138,453
Total # bp in contigs	75,503,607	87,793,800	148,192,453
%GC of contigs	40.2	39.4	39.7
Average contig length (bp)	1106	801	1070
Median contig length (bp)	511	432	516
N50 (bp)	2209	1399	2089

RNA extraction, cDNA library preparation, and transcriptome sequencing, assembly, and annotation

Construction of the reference transcriptomes for adult and larval *H. rubra* of the EP lineage generally followed the methods outlined in Havird et al. (2014b). For the adult transcriptome, a single individual was placed in ice-cold Trizol and homogenized via bead-beating. Larvae were treated similarly, except that the 88 individuals were pooled into a single sample. Total RNA was extracted and purified using an RNeasy kit (Qiagen) and DNAse I treatment according to the manufacturer's instructions, with RNA quality assessed on a Bioanalyzer 2100 (Agilent). Libraries were then made using the SMART cDNA construction kit (Clontech) with the provided 3′ oligo replaced by the Cap-Trsa-CV oligo. Resulting double-stranded cDNA was purified with a QIAquick PCR purification kit (Qiagen) and adapters trimmed via digestion with *Sfi*I (Clontech). The two cDNA libraries were then sequenced at the Genomic Services Lab at HudsonAlpha Institute for Biotechnology in Huntsville, AL, USA using Illumina Tru-Seq technology, with each sample utilizing approximately one-sixth of an Illumina HiSeq 2000 lane. Paired-end (PE) reads of 100 bp were received in FASTQ format and are available from the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) under BioProject accession PRJNA304256.

From the raw PE reads, three separate *H. rubra* transcriptome assemblies were generated *de novo*: (1) "adult"–consisting solely of reads from the adult individual; (2) "larvae"–consisting solely of reads from the 88 pooled larvae of mixed stages, and; (3) "adult + larvae"–consisting of reads combined from both the adult and larval samples, as recommended by Haas et al. (2013) for downstream differential expression analyses. Given the overall high quality of the PE reads (Table 1) and that aggressive quality score-based trimming can negatively impact assembly of RNA-Seq data (MacManes 2014), no preprocessing in the form of filtering or trimming was performed. The PE reads were digitally normalized using the normalize-by-median.py script (Brown et al. 2012) prior to assembly with Trinity version r20131110 (Grabherr et al. 2011) to improve computational efficiency (Haas et al. 2013) under default parameters and 6 CPU cores.

Nucleotide contigs resulting from each assembly were annotated by first extracting all putative open reading frames (ORFs) with TransDecoder version r20131117. These putative ORFs, along with the original nucleotide contigs, were then submitted to a local implementation of Trinotate version r20131110 (https://trinotate.github.io), which uses several methods to assign functional annotations including, among others, BLASTx, BLASTp (Altschul et al. 1997), and queries to UniProt (Apweiler et al. 2012). Trinotate annotation results were parsed into tab-delimited text files at a BLAST Expect (E) value of $1e^{-5}$.

Quantification of DEGs between developmental stages

Two approaches were utilized in quantifying DEGs between adults and larvae of *H. rubra*. First, to generate a robust assessment of DEGs among developmental stages, adults as well as "early" and "late" larvae from the HILO (see Craft et al. 2008 for lineage abbreviations) lineage were utilized in a 3′-tag-based RNA-Seq experiment using multiple biological replicates. For "early" larvae, 10 individuals were pooled into a single biological replicate, with four

replicates investigated for this stage. For "late" larvae, three replicates were examined, each with five individuals being pooled. For adults, five individuals were treated as five separate biological replicates. Total RNA was extracted from each sample ($n = 12$ in total) using a similar method as described above for the reference transcriptomes except an E.Z.N.A. Total RNA Kit I (Omega Biotek) was utilized. RNA fragmentation, first-strand cDNA synthesis and amplification, adapter and barcode addition, and size selection were then performed according to Meyer et al. (2011) using the step-by-step protocol at http://people.oregonstate.edu/~meyere/tools.html. Resulting size-selected cDNA libraries with sample-specific barcodes were sequenced at the Genomic Services Lab of HudsonAlpha, with each of the 12 samples utilizing ~1/24th of an Illumina HiSeq 2000 lane. Single-end (SE) reads of 50 bp were received in FASTQ format and are available from the SRA at NCBI under BioProject accession number PRJNA304256.

Mapping of reads and estimates of transcript abundance followed Haas et al. (2013) using the utilities and scripts supplied with Trinity version r20131110. Briefly, read mapping utilized Bowtie version 1.1.1 (Langmead 2010), with the "adult + larvae" transcriptome assembly serving as the reference, and transcript abundances were estimated per sample replicate with RSEM version 1.2.12 (Li and Dewey 2011). Statistically significant DEGs were identified with DESeq (Anders and Huber 2010) and edgeR (Robinson et al. 2010) using default values (i.e., $P = 0.001$, with at least a four-fold difference), followed by Trimmed Mean of M-values (TMM) normalization (reviewed by Dillies et al. 2012) and generation of expression values measured as fragments per kilobase of transcript per million mapped reads (FPKM; Trapnell et al. 2010). Notably, DEGs were quantified at the "gene" level, with read counts on a per-Trinity-component, rather than per transcript (i.e., isoform), basis (Haas et al. 2013). Analyses were performed considering larvae as separate "early" and "late" stages as well as consolidating both into a single "larvae" stage relative to adults.

In contrast to the above experimental design, a number of recent RNA-Seq studies have utilized a single biological replicate to quantify DEGs between crustacean developmental stages (Havird and Santos, submitted for publication). However, the robustness of such analyses is unclear since the statistical methods employed by DESeq, edgeR, and CuffDiff (Trapnell et al. 2013) require multiple replicates to accurately model biological variability (but see Feng et al. 2012) and tend to generate high numbers of false positives otherwise (Sims et al. 2014; Tarazona et al. 2011). To explore this possibility, a second set of DEG analyses was conducted where PE reads from the adult and pooled larvae used in the transcriptome assemblies were treated as single replicate samples. In order to provide a direct comparison with the multiple replicate, SE read analyses, only one of the two paired reads were utilized in mapping and the read number for larvae was randomly subsampled with seqtk (https://github.com/lh3/seqtk) to equal that of the adult (i.e., ~8.6 M SE reads). Remaining analyses were done as previously described for the SE reads with multiple replicates and statistically significant DEGs identified via edgeR.

For both the multiple and single replicate approaches, UniProt accession numbers for statistically significant DEGs were extracted from the Trinotate text file reports and submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (Huang et al. 2007a, 2007b) to identify enriched biological themes by Gene Ontology (GO) terms along with functionally-related gene groups. Significant enrichment was determined using a Fisher's exact test (Hosack et al. 2003), and multiple test corrections were performed with a false discovery rate (FDR) of 0.05. The resulting "Functional Annotation Chart" outputs from DAVID for adult and larval *H. rubra* were utilized in gene-set enrichment analyses and network visualizations with the Enrichment Map version 2.1.0 (Merico et al. 2010) and WordCloud version 3.0 (http://www.baderlab.org/WordCloud) plugins to Cytoscape version 3.2 (Shannon et al. 2003), following the protocol from the Bader Lab (http://www.baderlab.org/Software/EnrichmentMap/DavidTutorial) and *P*- (i.e., statistical significance cut-off) and *Q*- (i.e., the FDR) values of 0.001 and 0.05, respectively.

Data availability

In addition to raw sequence reads being publically archived in the SRA at NCBI, data presented here as well as those from supplementary analyses can be downloaded from http://www.auburn.edu/~santosr/sequencedatasets.htm, including contig assemblies and their annotations for the three *H. rubra* transcriptomes, statistics from the DEG and DAVID analyses, as well as the identification of DEGs at an alternative (i.e., $P = 0.05$) statistical cut-off. Additionally, transcriptomes of *H. rubra* are web searchable via BLAST using the HALO-BLAST tool at http://www.auburn.edu/~santosr/halo_blast.htm.

Results

Transcriptome assemblies and annotations

Transcriptomes for *H. rubra* from adult and larval stages, as well as a composite of "adult + larvae," possessed contig numbers and N50 values ranging from 68,295–138,453 and 1399–2209 bp, respectively, with a %GC average of 39.7 (Table 1). Notably, the number of contigs in a transcriptome assembly positively correlated with the total amount of PE reads utilized in generating it (Fig. 1). For example, there were >2X as many contigs in the "adult + larvae" assembly, which included ~48.0 M PE reads, relative to the "adult" assembly encompassing ~8.6M PE reads (Fig. 1). Given that the composite "adult + larvae" assembly possessed many more contigs than the individual assemblies, it is likely that a number are unique to each transcriptome, which is supported by the differential expression analyses (see below).

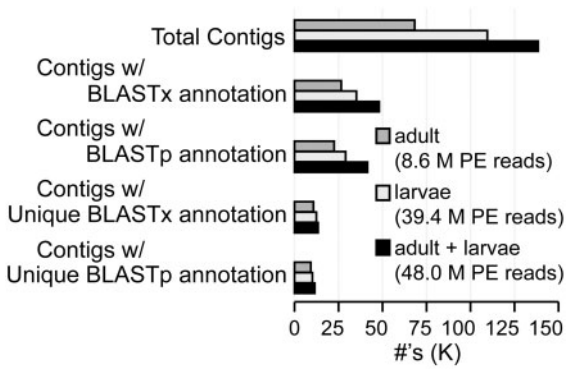

Fig. 1 Summary statistics for *Halocaridina rubra* transcriptomes and their annotation from adult, larvae, and composite "adult + larvae" assemblies. Annotation via BLASTx and BLASTp to the UniProt database was conducted with Trinotate (see text for additional details).

However, most putative ORFs extracted from contigs of the *H. rubra* transcriptomes had no homology to entries in publically available databases, with the percentage of contigs annotated via BLASTx and BLASTp queries to UniProt (as implemented in Trinotate) averaging 32.5% (Fig. 1). When only unique accession numbers are considered from the BLASTx and BLASTp annotation (i.e., collapsing potential isoforms to a single entry), the average percentage drops to 11.3%, or 9261–13,342 contigs having putative ORFs with identifiable, unique homologs in UniProt (Fig. 1).

Numbers of DEGs between developmental stages

For the 3′-tag-based RNA-Seq experiment with multiple replicates (see Supplementary Table S1 for read statistics), DESeq and edgeR identified 258 and 1117 DEGs, between adults and larvae respectively (Table 2), with more being upregulated in adults relative to larvae. When considered separately, "early" larvae possessed more DEGs than "late" larvae compared to adults while few DEGs were identified between "early" and "late" larval stages (Table 2). Overall, edgeR identified higher numbers of DEGs than DESeq. In sharp contrast, analogous analyses utilizing the single replicate samples from the transcriptome assemblies identified 1674 statistically significant DEGs with edgeR between adult and larval stages of *H. rubra* (Table 2), with more being upregulated in larvae relative to adults.

Functional categories and gene-set enrichment of DEGs between developmental stages

When considering the functional categories of DEGs between adults and larvae of *H. rubra*, we choose to conservatively focus on the analyses utilizing multiple replicates and generally highlight the lower number identified by DESeq (Fig. 2). In this context, those

Table 2 Numbers of differentially expressed genes (DEGs) and their annotation percentages among *Halocaridina rubra* developmental stages and RNA-Seq experimental designs

Comparison	Replicate method	DE program	#DEGs	% of DEGs annotated
All larvae vs. adults	Multiple replicates	DESeq	258	38
	Multiple replicates	edgeR	1117	45
	Single replicates	edgeR	1674	32
"Early" larvae vs. adults	Multiple replicates	DESeq	220	45
	Multiple replicates	edgeR	832	34
"Late" larvae vs. adults	Multiple replicates	DESeq	75	61
	Multiple replicates	edgeR	353	41
"Early" vs. "late" larvae	Multiple replicates	DESeq	1	100
	Multiple replicates	edgeR	3	33

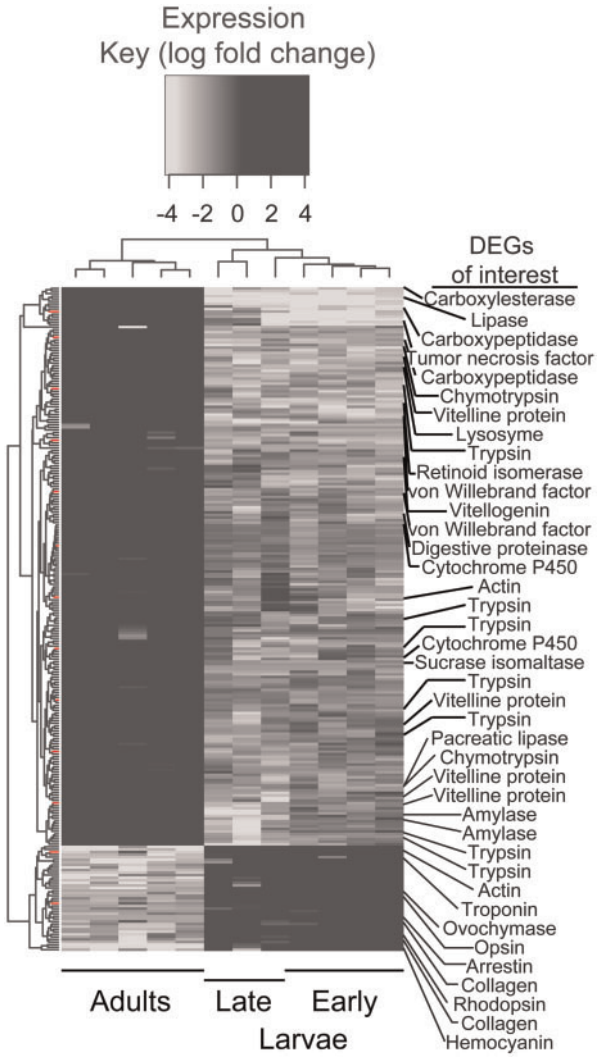

Fig. 2 Heatmap of differentially expressed genes (DEGs) between developmental stages (i.e., adult, "early" and "late" larvae) of *Halocaridina rubra* based on multiple replicate samples. Statistically significant DEGs were identified using DESeq at $P = 0.001$, with a minimum four-fold difference in expression. Log fold change in expression is given in shades of gray (under-expressed) to black (over-expressed). Unsupervised hierarchical clustering of genes with similar expression patterns is presented to the left of the heatmap. Similarly, unsupervised hierarchical clustering of the 12 biological samples according to their gene expression patterns is shown above the heatmap. Notable genes based on their annotation and discussed in the text are identified to the right of the heatmap. (This figure is available in black and white in print and in color at *Integrative And Comparative Biology* online).

DEGs upregulated in adults tended to fall into functional categories associated with digestion and reproduction, including several annotated as trypsins, amylases, peptidases, and ones related to vitellogenesis (Fig. 2). Notably, some of these DEGs were either upregulated hundreds to thousands of times in adults versus larvae of *H. rubra* or were undetected in larval stages. Oxygen-transport proteins, including a heme-binding protein and hemocyanin (Fig. 2), were also upregulated in adults. Finally, a number of structural proteins including actins and chitinase were upregulated in adults, along with ones participating in general cellular functions such as protein degradation and stress response (Fig. 2). These functional categories were consistent in comparisons of either "early" or "late" larvae to adults, although more DEGs were recovered from comparisons to the "early" stage.

Upregulated DEGs from larvae relative to adults also fell into several categories, including crustacyanin and other lipocalins as well as structural proteins like actins, collagens, and chintinase (Fig. 2). Notably, light/stimulus detection was another functional category recovered, with DEGs being annotated to opsins, rhodopsins, or arrestins (Fig. 2). In fact, annotation for the only statistically significant DEG identified between "early" and "late" larvae using the DESeq method (Table 2) was to a compound eye opsin, which was upregulated 219-fold in "early" relative to "late" larvae. Visualization of significant gene-set enrichments from the DESeq-identified DEGs (Fig. 3A) generally recovered these functional categories, with those upregulated in adults and larvae corresponding to nodes related to digestion and reproduction (i.e., von Willebrand factors, see below) or light/stimulus detection, respectively.

Comparison between multiple and single replicate approaches

Differing numbers of DEGs and functional categories were recovered from adults and larvae of *H. rubra* when multiple versus single replicate samples were examined with edgeR, with each approach producing distinct gene-set enrichments (Fig. 3B and C). While the gene-set enrichment network from multiple replicates (Fig. 3B) generally reflected the one based on DEGs identified via DESeq (Fig. 3A), the one from single replicate samples (Fig. 3C) had unique properties, including a large number of nodes suggesting upregulation of ribsosomal protein genes in larvae, which was not recovered from either analysis using multiple replicates. Moreover, peptidases were upregulated in larvae for the single replicate samples; in contrast, this same functional category was upregulated in adults when using multiple replicates. Another striking difference between the approaches was more genes tending to be upregulated in adults when utilizing multiple replicates, while the opposite was observed for the single replicate samples (Fig. 3C compared to 3A and B). In spite of this, a number of functional categories were shared between DEGs in

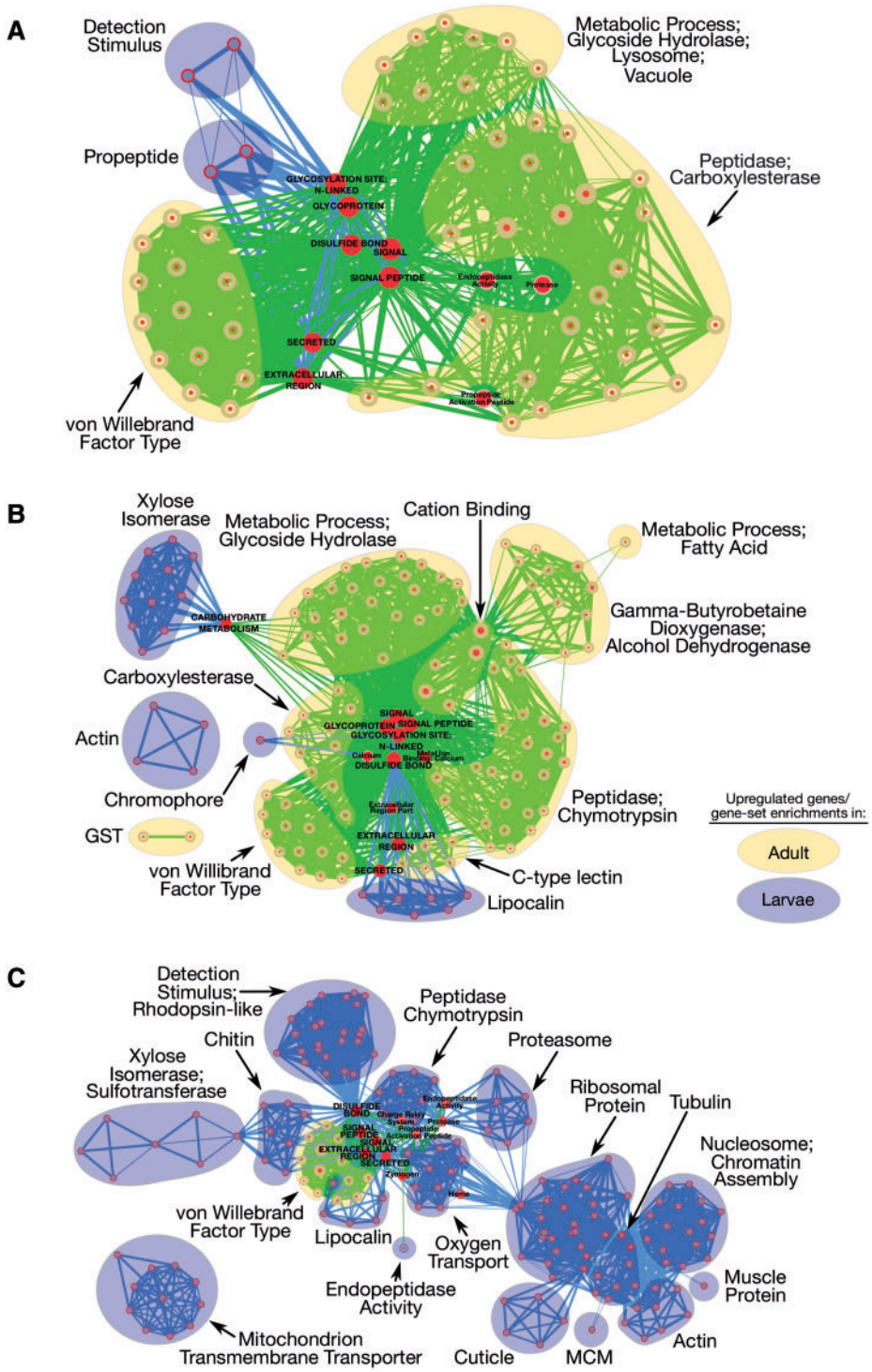

Fig. 3 Gene-set enrichment networks between developmental stages (i.e., adult versus all larvae) of *Halocaridina rubra* (as in Figure 1) Enriched biological themes by Gene Ontology (GO) terms along with functional-related gene groups were inferred with the Database for Annotation, Visualization and Integrated Discovery (DAVID) from differentially expressed genes (DEGs) identified under three different experimental designs: (A) DESeq with multiple replicate samples (see Fig. 2 caption); (B) edgeR with multiple replicate samples; and (C) edgeR with single replicate samples. Each node in a network depicts a gene-set, with sizes corresponding to the number of DEGs from *H. rubra* adults (inner circle) or larvae (outer circle) in a gene-set. Labeled nodes indicate ones with high interconnectivity between biological themes. Color intensity of red for inner (adults) and outer (larvae) circles of a node is proportional to enrichment significance in that developmental stage. Edge size corresponds to the number of genes that overlap between the two connected gene-sets, with green and blue representing adults and larvae, respectively. Clusters of functionally related gene-sets were manually assigned labels, with upregulated DEGs and gene-set enrichments of adults and larvae highlighted in yellow and purple, respectively. See text for additional details.

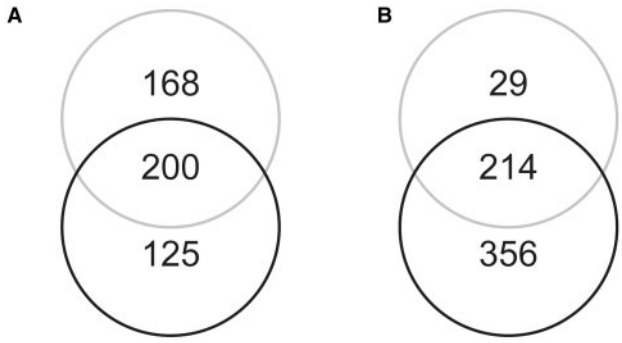

Fig. 4 Venn diagrams depicting overlapping annotation terms for upregulated DEGs in (A) adult and (B) all larval developmental stages of *Halocaridina rubra* (as in Figure 1) inferred from multiple (light gray) and single (black) replicate samples.

the multiple versus single replicate analyses (Fig. 4), including upregulation of light/stimulus detection genes such as opsins in larvae and von Willebrand factors in adults (Fig. 3B and C).

Discussion

Behavioral, physiological, and ecological contexts of DEGs between developmental stages of *H. rubra*

Consistent with our hypothesis, hundreds of DEGs were identified during development in *H. rubra*. Furthermore, many belong to functional categories correlating with known behavioral, physiological, and ecological differences among developmental stages of this anchialine shrimp. For instance, significantly higher expression of opsins and other genes related to light detection in *H. rubra* larvae supports observations for their positively phototactic nature, as well as adults being generally indifferent to the same stimulus (Couret and Wong 1978; Bailey-Brock and Brock 1993; Iwai 2005; Craft et al. 2008). However, it remains to be determined what benefit(s) larvae of *H. rubra* derive from being positively phototactic when they are apparently confined to the dark, hypogeal waters of Hawaii's anchialine ecosystem due to its higher salinity (Havird et al. 2015). In relation to this, querying the multiple replicate data under an alternative P (i.e., $P = 0.05$ vs. 0.001) value identifies both anion and cation transporters as being upregulated in adults relative to larvae (data available at http://www.auburn.edu/~santosr/sequencedatasets.htm), suggesting a significant change in osmoregulatory ability between larvae, which are intolerant of freshwater, and adults, which tolerate a wide range of salinities (Havird et al. 2014a; Havird et al. 2015). Another indicator of maturation in *H. rubra* appears to be the larval upregulation of crustacyanin and other lipocalins (Figs. 2 and 3B), known to be major contributors in crustacean coloration (Cianci et al. 2002; Wade et al. 2009) and directors of insect cell growth (Flower 1996), which are highly expressed immediately after moltings in species like *M. rosenbergii* (Wang et al. 2007).

In the case of *H. rubra* adults, upregulated DEGs again correlate with characteristics of the life-stage. Here, genes for proteins like trypsins, amylases, and peptidases were highly upregulated. While these enzymes can serve non-digestive functions (Page and Di Cera 2008), this difference likely reflects a combination of low digestive activity in larvae due to being lecithotrophic (i.e., yolk-bearing) and non-feeding, while adults function primarily as microphagous grazers (Holthuis 1973; Bailey-Brock and Brock 1993; Dalton et al. 2013). Support for this includes increased digestive enzyme gene expression and/or activity in later, feeding stages of gastropods, fishes, the white shrimp *Litopenaeus schmitti*, and the marine crab *Charybdis japonica* compared to earlier, non-feeding stages (Izquierdo et al. 2000; Lemos et al. 2002; Wei et al. 2014; Collin and Starr 2013; Xu et al. 2013) as well as a lack of such differences between feeding larval stages in the spiny lobster *Panulirus argus* (Perera et al. 2008). Other DEGs potentially linked to feeding in adults are those belonging to the cytochrome P450 family (Fig. 2), with members being differentially induced or upregulated to degrade ingested xenobiotics (David et al. 2003; Matzkin 2012). It is also unsurprising that expression of reproductive genes was only detected in adults of *H. rubra*. The von Willebrand factors fall into this category since they are homologous to vitellogenins, or egg yolk precursor proteins, in invertebrates (Baker 1988).

Unexpectedly, we failed to recover DEGs characteristic of metamorphosis that have been identified in other RNA-Seq based experiments of crustacean development (De Gregoris et al. 2011; Ventura et al. 2013). For example, among seven Wnt-related genes expressed in adults or larvae of *H. rubra* at measurable levels, none were identified as DEGs, even in our most liberal analyses. While a general trend of increased Wnt expression in larvae could be reasonably argued (Fig. 5), our analyses apparently lacked the statistical power to detect this trend. This may be because Wnt-related genes are only weakly overexpressed (e.g., two- to eight-fold) during development, as noted in metamorphosis of *M. rosenbergii* (Ventura et al. 2013). Furthermore, we did not examine larvae as they explicitly transitioned between zoeal stages, when hormonally-regulated genes such as Wnt tend to be most upregulated (Cheong et al. 2015). This was not specifically investigated here

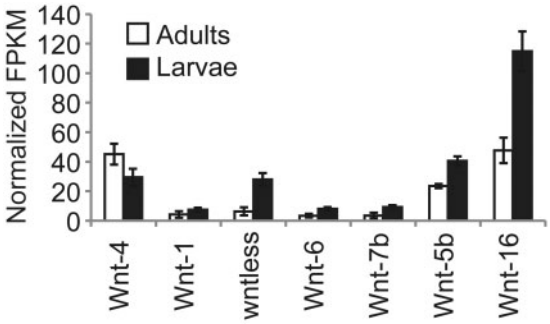

Fig. 5 Expression of genes annotated as Wnt-related in adult versus all larval developmental stages of *Halocaridina rubra* (as in Figure 1). None of these genes were identified as being statistically differentially expressed in DEG analyses.

since determining molting status in *H. rubra* larvae is difficult, but would represent an interesting future avenue. Similar to our analyses, Wnt-related genes were not identified among DEGs during development in the spiny lobster *Sagmariasus verreauxi* (Ventura et al. 2015). Last, some DEGs characteristic of crustacean metamorphosis may have gone undetected due to high divergence to homologs in the databases utilized for annotation (see section ''Materials and Methods'').

We also recovered relatively few DEGs between ''early'' and ''late'' larvae of *H. rubra* compared with other studies examining larval developmental stages in crustaceans (Blythe et al. 2012; Ventura et al. 2013). These particular DEGs mirrored trends generally seen in larval and adult comparisons, suggesting development in *H. rubra* represents a continuum rather than discrete physiological and ecological states, consistent with the fact that *H. rubra* larvae are planktonic and lecithotrophic in both ''early'' and ''late'' stages. It would not be surprising, however, if RNA-Seq experiments comparing Z_1 through Z_4 larvae specifically, rather than pooled ''early'' and ''late'' samples as done here, identify additional DEGs among developmental stages of *H. rubra*.

Isoform-switching among developmental stages of *H. rubra*

Interestingly, different genes belonging to the same functional categories were upregulated in both *H. rubra* adults and larvae, especially under the alternative $P = 0.05$ analyses. Notable among these were structural (e.g., actins, myosins, collagens, and chitinases) and oxygen-transport (e.g., heme-binding and hemocyanin; Fig. 6) proteins. For example, particular actins were upregulated in adults, while other actins were upregulated in larvae. This suggests that while both developmental stages may be performing the same physiological functions, they are using different variants, or isoforms, of homologous genes to do so. In other words, *H. rubra* apparently uses one specific isoform in the larvae life stage but then switches to another isoform as adults across an apparently wide range of genes.

The application of high-throughput sequencing and RNA-Seq technologies has demonstrated that isoform-switching during development is likely a general phenomenon. For example, 330 genes showed a complete isoform switch during differentiation, while 1304 genes showed a subtler shift, in a mouse myoblast cell line (Trapnell et al. 2010). By using Trinity for transcriptome assembly and its associated utilities to identify DEGs, it is possible to explicitly examine differential expression among isoforms and alternative splice variants (Haas et al. 2013). Here, DEGs were conservatively quantified at the ''gene'' level, with read counts on a per-Trinity-component, rather than per transcript (i.e., isoform), basis (Haas et al. 2013). However, when these latter ''subcomponents'' are examined, isoforms of structural and oxygen-transport genes are recovered as being differentially expressed between the adult and larval stages of *H. rubra* (results available at http://www.auburn.edu/~santosr/sequencedatasets.htm). Because these functional categories exhibited signs of isoform switching in both types of analyses, they represent prime candidates for further gene-targeted studies.

While the phenomenon of isoform-switching has been largely unexplored in crustacean development, the data presented here for *H. rubra* suggests it is potentially pervasive, as has been documented in other systems. For example, troponin, another candidate for isoform-switching in *H. rubra* based on our analyses (Fig. 6), undergoes isoform-switching in adult cardiac tissue of mammals (Cooper and Ordahl 1984; 1985; Gorza et al. 1993; Cooper 1998). Furthermore, it is notable that: (1) myosin was previously considered a candidate for isoform-switching in lobster development and molting (Magnay et al. 2003; Mykles 1997); (2) actin undergoes isoform switching in sea urchin development (Shott et al. 1984); and (3) structural genes, including myosin and actin, were among those most differentially expressed between white shrimp developmental stages (Wei et al. 2014). Moreover, isoform-switching of oxygen-transport genes is known for vertebrates and invertebrates (Hardison 1998; Storz et al. 2011; Strobel et al. 2012), including different hemocyanin subunits being expressed during ontogeny in the Dungeness crab *Metacarcinus magister* (Terwilliger and Ryan 2001), which is consistent with our results.

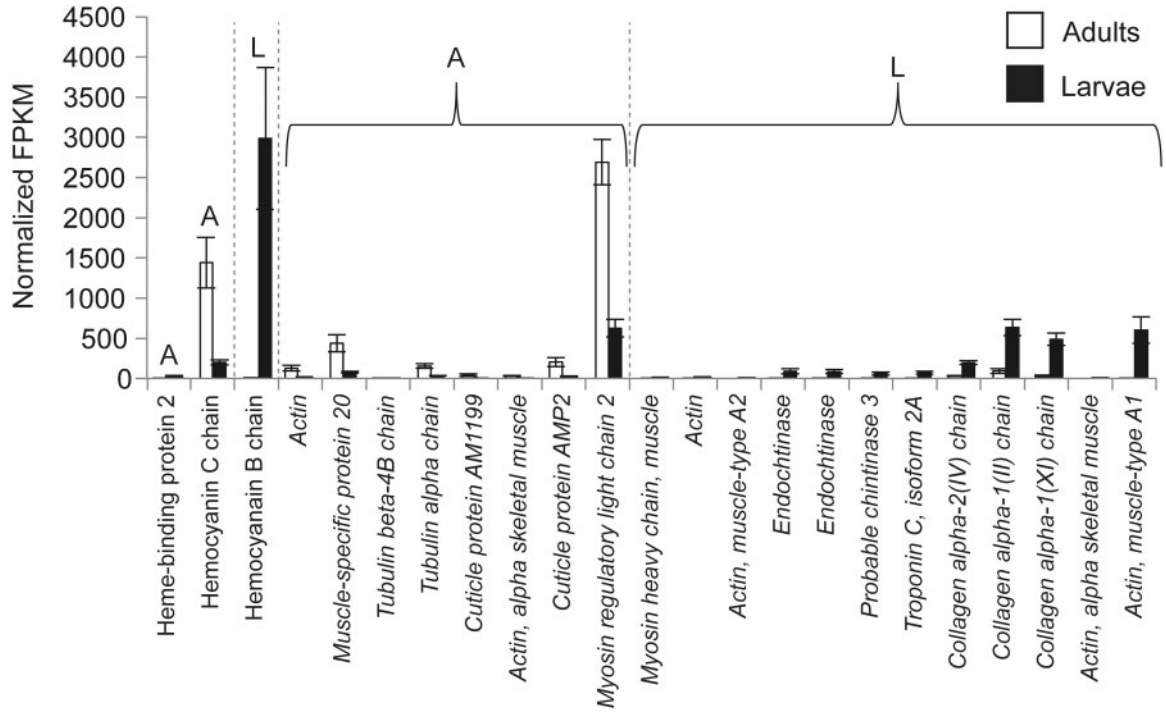

Fig. 6 Possible instances of isoform-switching between developmental stages (i.e., adult versus all larvae) of *Halocaridina rubra* (as in Figure 1) for genes corresponding to structural (names in italics) and oxygen-transporter proteins. Letters above columns indicate whether genes were upregulated in adults (A) or larvae (L), with these life-stages separated by dashed lines. All genes were identified as being differentially expressed between larvae and adults when using DESeq and $P = 0.05$.

Evaluation of differing methodological approaches in crustacean transcriptomics

To provide recommendations for future RNA-Seq studies of crustacean development, we compared results from different experimental designs as well as bioinformatics pipelines and software. First, specific focus is given to the utilization of multiple versus single replicate samples since a number of crustacean transcriptomic studies have reported DEGs solely from single samples (Havird and Santos, submitted for publication). While some of the same functional categories were inferred from both approaches (e.g., larval upregulation of light/stimulus detection genes), many other categories identified in analyses of the single replicate samples (e.g., larval upregulation of ribosomal protein genes) lacked support in the multiple replicate scheme. Even more problematic is the potential for erroneous biological interpretations given the identification of opposing trends in upregulated DEGs between *H. rubra* adult and larval stages from single versus multiple replicate samples (Fig. 3C compared to 3A and B). Therefore, while preliminary data could be gathered by using single replicate samples, we strongly recommend such results be vetted through a more rigorous, multiple replicate experimental design to account for biological variability before being considered valid for interpretation.

Second, we obtained largely consistent results from different bioinformatic pipelines and software. Specifically, the numbers and identities of DEGs between adults and larvae of *H. rubra* were nearly identical whether the ''prepackaged'' pipeline as provided with Trinity and outlined in Haas et al. (2013) was utilized (results presented here) or if data were subjected to a ''custom'' pipeline that involved processing via separately acquired open source programs for quality filtering, read mapping, FPKM estimates, and DE analyses (results available at http://www.auburn.edu/~santosr/sequencedatasets.htm). It is again also notable that edgeR identified consistently higher numbers of DEGs in all comparisons (Table 2), which likely stems from the more stringent default thresholds of DESeq (Love et al. 2014). However, most DEGs unique to the edgeR analyses either lacked annotation or belonged to the same functional categories as ones identified by DESeq, resulting in identical conclusions regardless of which software was employed to test for differential expression. Overall, these results lend further support for our conclusions on the developmental transcriptomics of *H. rubra* as well as the robustness of RNA-Seq

as a tool for investigating gene expression patterns within and between developmental stages of crustaceans in general.

Acknowledgments

We thank two anonymous reviewers for comments that improved the manuscript. We are also grateful to Donald Mykles, Karen Burnett, David Durica, and Jonathon Stillman for organizing the "Tapping the Power of Crustacean Transcriptomes to Address Grand Challenges in Comparative Biology" symposium at the 2016 meeting of the Society for Integrative and Comparative Biology and inviting us to participate. This represents contributions #139 and #47 to the Auburn University (AU) Marine Biology Program and Molette Biology Laboratory for Environmental and Climate Change Studies, respectively.

Funding

This work was supported in part by the National Science Foundation (NSF) [DEB #0949855 to SRS] and its Doctoral Dissertation Improvement Grant program [DEB #1311500 to JCH]. Funding support to JCH also came from the Auburn University Cellular and Molecular Biosciences Peaks of Excellence Graduate Fellowship program, a Professional Association of Diving Instructors (PADI) Foundation Research Award (#5089), a Fellowship in Graduate Studies for Genetics/Physiology from The Crustacean Society, and a Doctoral Graduate Research Fellowship from the Alabama Council on Higher Education (ACHE). Travel support for JCH to the Society for Integrative and Comparative (SICB) Annual Meeting 2016 came from NSF [IOS #1551003], The Crustacean Society, and the Division of Comparative Physiology & Biochemistry.

Supplementary data

Supplementary Data available at *ICB* online.

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