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SYMPOSIUM

Tapping the Power of Crustacean Transcriptomics to Address Grand Challenges in Comparative Biology: An Introduction to the Symposium

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Synopsis Crustaceans, and decapods in particular (i.e., crabs, shrimp, and lobsters), are a diverse and ecologically and commercially important group of organisms. Understanding responses to abiotic and biotic factors is critical for developing best practices in aquaculture and assessing the effects of changing environments on the biology of these important animals. A relatively small number of decapod crustacean species have been intensively studied at the molecular level; the availability, experimental tractability, and economic relevance factor into the selection of a particular species as a model. Transcriptomics, using high-throughput next generation sequencing (NGS, coupled with RNA sequencing or RNA-seq) is revolutionizing crustacean biology. The 11 symposium papers in this volume illustrate how RNA-seq is being used to study stress response, molting and limb regeneration, immunity and disease, reproduction and development, neurobiology, and ecology and evolution. This symposium occurred on the 10th anniversary of the symposium, "Genomic and Proteomic Approaches to Crustacean Biology", held at the Society for Integrative and Comparative Biology 2006 meeting. Two participants in the 2006 symposium, the late Paul Gross and David Towle, were recognized as leaders who pioneered the use of molecular techniques that would ultimately foster the transcriptomics research reviewed in this volume. RNA-seq is a powerful tool for hypothesis-driven research, as well as an engine for discovery. It has eclipsed the technologies available in 2006, such as microarrays, expressed sequence tags, and subtractive hybridization screening, as the millions of "reads" from NGS enable researchers to *de novo* assemble a comprehensive transcriptome without a complete genome sequence. The symposium series concludes with a policy paper that gives an overview of the resources available and makes recommendations for developing better tools for functional annotation and pathway and network analysis in organisms in which the genome is not available or is incomplete.

Introduction

Over the last 7 years, a number of position papers published in *Integrative and Comparative Biology* have conceptualized "Grand Challenges" in organismal biology, with the aim of providing a direction for future research and developing the infrastructure needed for carrying out that research. Schwenk et al. (2009) articulated a framework of five major challenges in organismal biology: (1) understanding the organism's role in organism-environment linkages; (2) utilizing the functional diversity of organisms; (3) integrating living and physical systems analysis; (4) understanding how genomes produce organisms; and (5) understanding the tightrope between stability and change. The Grand Challenges articles that followed expanded on these topics from different disciplines, including an article by Mykles et al. (2010) on comparative physiology. A key question

emerging from this and other papers is: How does a genome determine the phenotypic plasticity of an organism? A true mechanistic understanding of the genome to phenome continuum requires integration across all levels of biological organization (Mykles et al. 2010). The first step by which information encoded in the genome is accessed is represented by the transcriptome, a spatially and temporally defined collection of mRNA and microRNAs (McGettigan 2013). Transcriptome diversity and dynamics are important determinants of phenotype, forming a vital link between environmental drivers and cellular function.

Transcriptomics using high-throughput next generation sequencing (NGS) technologies to characterize the suite of mRNAs in a sample (RNA-seq) has the potential to achieve a greater understanding of the interplay between gene expression and organismal adaptation and evolution (Kircher and Kelso 2010; Jung et al. 2013; McGettigan 2013; Rathburn et al. 2013; Stillman and Armstrong 2015). The cost of RNA-seq has dropped to the point where it is now feasible for individual research groups to use this technology; service facilities are increasingly available at many research institutions and there are numerous commercial suppliers of NGS services. With further cost reductions brought about by "Third Generation" technologies (McGettigan 2013; Sanchez-Flores and Abreu-Goodger 2014), in the near future it will be routine to conduct transcriptome analyses to assess individual phenotypic plasticity in physiological response across increasingly varied and smaller experimental contexts. Indeed, the number of reports using RNA-seq technology has increased dramatically in recent years. This growth is not only attributable to lower costs, but also to the application of the technology to non-genomic model organisms. "Deep sequencing" and *de novo* assembly has enabled researchers to catalog, annotate, and quantify all the transcripts present in an organism for which a genome sequence is incomplete or not available.

The purpose of the collection of papers here is to illustrate how transcriptomics can be used to address fundamental questions in integrative organismal biology. The inductive approach of analyzing the transcripts of all genes, both known and unknown, can reveal gene networks and gene interactions. Such analyses on a genome scale are simply not feasible using a gene-by-gene approach. RNA-seq based natural history is reminiscent of Sir Francis Bacon's idea of the "inductive machine", in which information (data) gathered through unbiased, yet methodical, observations are the basis for discovery and invention (Agassi 2013). The outcomes of the symposium were to identify common problems in transcriptome analysis, exchange best-practices and engage the community in discussing the support that will be needed to link gene expression to phenotype, such as better tools for functional annotation and pathway and network analysis.

Symposium topics

Crustaceans are a diverse and polyphyletic group of animals that live primarily in aquatic habitats. They play important ecological roles at various trophic levels and provide excellent model systems for the study of developmental and physiological processes. The 11 topics and speakers selected for this symposium (Fig. 1) represent a broad spectrum of research conducted on crustaceans using transcriptomic approaches. Much of the research has economic applications, in terms of aquaculture and sustainable fisheries. The symposium papers in this issue focus on how transcriptomics is being applied in decapod crustaceans and phylogenetically-related species in the areas of stress response; molting and growth; immunity and disease; reproduction and development; neurobiology; and ecology and evolution.

The use of transcriptomics in studies of crustaceans is rapidly growing despite lack of a coordinated set of protocols or guidelines for RNA-seq analyses. The symposium collection begins with contributions from Havird and Santos (2016a) and Das et al. (2016b), which report their analyses of RNA-seq studies conducted on crustaceans in the past few years. Havird and Santos (2016a) identify suites of software for transcriptomic analyses. They emphasize the critical importance of replication in RNA-seq studies. Trading off sequencing depth with increased biological replicates is likely to yield greater inferences about crustacean molecular physiology, as they demonstrate by analysis of venom genes (Havird and Santos 2016a). Das et al. (2016b) examined software and resources used for the identification and annotation of differentially expressed genes in *de novo* assembled crustacean transcriptomes published in 2014–2015. Most of the 23 papers did not include critical information on the software versions, parameters, and databases used for data analysis. This is of utmost importance, as software and databases are continually changing and parameters used for filtering, assembly, and assigning statistical significance can affect the outputs. Certainly, the community should no longer accept studies of differential gene expression when there is no biological replication for statistical analysis and/or insufficient details on the analytics. As all

Fig. 1 Speakers in the 2016 symposium. Front row, left to right: Scott Santos, Andrew Christie, David Durica, Fraser Clark, Sunetra Das, Daniel Powell, and Jennifer Chandler. Back row, left to right: Jill Johnson, Ann Tarrant, and Eric Armstrong. Justin Havird not pictured.

reports should be considered provisional, authors should make the original datasets available for re-analysis with updated software and databases.

One powerful use of RNA-seq is to identify all of the expressed variants of a particular gene, as illustrated by Johnson et al. (2016) in their analysis of the crustacean hemocyanin gene family. They show that the isoforms of hemocyanin expressed in shrimp are more diverse than previously thought, and that there is greater interspecific diversity in which isoforms are expressed than expected. Relative expression of specific hemocyanin isoforms under conditions of environmental change may be related to interspecific differences in tolerance to abiotic stressors such as hypoxia and hypercapnia (Johnson et al. 2016).

Interspecific difference in transcriptomic response to multivariate environmental conditions is a powerful tool for understanding the genomic bases of physiological adaptation. Armstrong and Stillman (2016) take that approach in studies of porcelain crab congeners adapted to different vertical intertidal zones. They present RNA-seq *de novo* transcriptomes for two porcelain crab congeners and compare those to a transcriptome made from an EST library with Sanger sequencing from one of the same species, as well as to other crustacean *de novo* transcriptomes made using RNA-seq. In a cautionary note, they show that RNA-seq can produce unexpected outcomes, as *de novo* transcriptomes from two closely related species can differ substantially even though they are made from the same amount of RNA from the same tissues from the same numbers of individuals exposed to the same conditions.

Molting is under the control of ecdysteroid molting hormones produced by a pair of Y-organs (YO) in the cephalothorax (Chang and Mykles 2011). Cyclic nucleotide, mTOR, and TGFβ signaling pathways drive phase transitions in the YO over the molt cycle; these are designated the basal (intermolt), activated (early premolt), committed (mid premolt), and repressed (late premolt) states (Chang and Mykles 2011). RNA-seq is being used to annotate and quantify differentially expressed genes (DEGs) in the YO over the molt cycle. YO transcriptomes are now available from intermolt *Pontastacus leptodactylus* (Tom et al. 2013), *Eriocheir sinensis* (Hao et al. 2014), and *Gecarcinus lateralis* (Das et al. 2016a). Das and Mykles (2016) report the annotation of the *G. lateralis* YO transcriptome from five molt stages (intermolt, early premolt, mid premolt, late premolt,

and postmolt) and recommend a combination of software packages and databases for gene identification and functional annotation.

Crustaceans are critical components of natural food webs and vital to food production around the world. As a consequence, much research has been directed at identifying processes of disease and disease resistance that shape the health of natural populations and developing best practices for breeding, growth, and survival in farmed populations. Clark and Greenwood (2016) and Powell et al. (2016) take the position that efforts to connect assembled RNA-seq data to immune function phenotypes are hampered by the lack of gene ontology assignments for immune defense in crustaceans. This constraint is attributed to a general lack of information regarding the constituent pathways and molecular bases for immunity in non-model organisms. Despite these constraints, Clark and Greenwood (2016) demonstrate a combinatorial approach for the annotation of transcriptomic data from whole larvae of the American lobster, *Homarus americanus*, that maximizes the number of contigs that can be identified and assigned to specific functional pathways of immune defense. Bypassing the lack of annotation for crustacean immune defense pathways, Powell et al. (2016) instead used transcriptomic profiles coupled with differential expression analysis to identify contig biomarkers that might distinguish selected lines of the banana shrimp, *Fenneropenaeus merguiensis*, expressing high versus low levels of natural infection with the hepatopancreatic parvo-like virus. In addition to demonstrating the use of RNA-seq data in selective breeding for aquaculture, this report also illustrates how differential expression analysis of selected sib families with distinctive immune phenotypes might be used to explore the molecular bases that underlie disease resistance in crustaceans.

Pancrustacea represent one of nature's most diverse group of organisms, which is clearly exhibited in the varied sexual strategies, modes of reproduction and the enormous range of sexually dimorphic characteristics observed across the clade. Sex determination mechanisms and the process of sexual maturation have been actively investigated in insects, but examination of genetic pathways involved in programming sexual development and maturation in the Crustacea has been limited. Comparative studies are now being addressed through the adoption of RNA-seq analyses, and Chandler et al. (2016) describe recent studies on the important commercial species, *Sagmariasus verreauxi*, the Eastern spiny lobster.

Male sexual differentiation and behavior is governed by the androgenic gland (AG) and its production of insulin-like androgenic gland hormone (IAG). The authors' transcriptomic studies catalog a dramatic range of IAG expression, correlating with very low levels at the first sign of sexual differentiation and dramatic increases accompanying maturation (Chandler et al. 2016). RNA-seq analyses have revealed additional insulin like peptides (ILP) and the presence of ancillary molecules - insulin-like growth factor binding proteins, putative chaperones, and membrane-anchored protein (MAG)—that may be mediating IAG/ILP cellular activity. A tyrosine kinase insulin receptor (TKIR) identified in the *S. verreauxi* transcriptome functions in sexual development. Over 50 genes, however, showed a greater than two-fold male-biased expression in mature individuals, including an ortholog of a gene essential for male sex-pheromone synthesis in *Drosophila*. As Chandler et al. (2016) clearly demonstrate the advantages of transcriptomic analyses as a tool in candidate gene identification, the authors conclude by advocating for the increasing integration of proteomics and genomics into the unlocking of physiological potential moving from genome to phenome.

Understanding physiological shifts associated with life history stage transitions, for example, developmental metamorphosis or diapause, are enabled by using RNA-seq. That approach was employed to understand metabolic transitions during development and diapause phases of calanoid copepods (Tarrant et al. 2016). Up regulation of specific metabolic genes matched expectations from tissue physiological studies. Complete inferences of physiological regulation from RNA-seq transcriptomics are limited, however, by the low rate of functional annotation common to studies of non-model organisms, and in particular, crustaceans.

Crustaceans living in estuarine and freshwater habitats have acquired the ability to osmoregulate. Anchialine pools are isolated bodies of water with subterranean connections to the sea. The pools are usually stratified, with less dense fresh or brackish water at the surface and denser salt water below. Each pool is a separate ecosystem—a natural experiment for studying the effects of salinity on the ecology and evolution of endemic species, such as the anchialine shrimp, *Halocaridina rubra*. The contribution from Havird and Santos (2016b) reviews the use of RNA-seq to identify DEGs during larval development and metamorphosis. Larvae have a narrow salinity tolerance, obtain their nutrition from yolk, and are positively phototactic. By contrast, adults have a wider salinity tolerance, are microphagous grazers,

and are not positively phototactic. The DEGs reflect the differences in osmoregulatory ability; feeding, digestion, and metabolism; and behavior between larvae and adults. In addition, isoform switching is associated with the metamorphosis.

The series concludes with a policy paper authored by members of the Animal Genome to Phenome (AG2P) Research Coordination Network (RCN; Mykles et al. 2016). The AG2P RCN gathered comments at the 2016 SICB meeting through a feedback booth in the exhibit hall and at a workshop held after the symposium. The number of RNA-seq studies is growing rapidly, but the infrastructure to support the analysis and interpretation of data resulting from these studies is not keeping pace with metadata collection. Acquiring raw sequence "reads" from Illumina and similar platforms is relatively easy. The time-consuming and computationally challenging component is the assembly and analysis of millions of sequences, which requires processing power that most universities do not have. Moreover, practices vary and there is no consensus as to which protocols to use in order to create standardization and to facilitate comparisons across datasets and studies for non-model organisms. The paper (1) describes the software and resources currently available and (2) makes recommendations for analytical tools and resources needed for annotating genes and capturing changes in gene pathways and networks in organisms for which well-defined genomes are not available.

Reflection: genomic and proteomic approaches to crustacean biology symposium (2006)

The 2016 symposium was the 10th anniversary of the 2006 SICB symposium "Genomic and Proteomic Approaches to Crustacean Biology" that was organized by Donald Mykles and David Towle; nine of the 10 presentations were published in *Integrative and Comparative Biology* (volume 46, issue 6) (Fig. 2). A review of the speakers and topics provides an opportunity to look back and review the progress of "omics" research over the last 10 years and to project how NGS technology will shape the future of research in comparative organismal biology.

The 2006 symposium program

In 2006, genomics research on crustaceans largely relied on expressed sequence tag (EST) libraries, subtractive hybridization screening, quantitative PCR, and microarrays to characterize genes and quantify their expression. Nuala O'Leary reported on their efforts to identify genes involved in immune function

Fig. 2 Speakers in the 2006 symposium. Left to right: Donald Mykles, Tim McClintock, David Durica, John Colbourne, Nora Terwilliger, David Towle, Robert Chapman, Nuala O'Leary, Jonathon Stillman, and Tom Shafer.

in the Pacific whiteleg shrimp, *Litopenaeus vannamei*, which is the primary shrimp species used in aquaculture worldwide (O'Leary et al. 2006). Timothy McClintock reported on using subtractive screening of cDNA libraries to identify genes that function in specific cell types in the olfactory organ of the American lobster, *H. americanus* (McClintock et al. 2006). David Durica sequenced EST libraries from early blastema stage (4 days post-autotomy) to study ecdysteroid regulation of limb regeneration in the fiddler crab, *Uca pugilator* (Durica et al. 2006). Donald Mykles presented the only proteomics paper, using two-dimensional polyacrylamide gel electrophoresis and mass spectrometry to characterize molt-dependent changes in proteins and NO synthase phosphorylation in the YO of *G. lateralis* (Lee and Mykles 2006). Tom Shafer reported on the discovery of genes involved in cuticle synthesis and calcification in the blue crab, *Callinectes sapidus* (Shafer et al. 2006). Nora Terwilliger characterized cDNAs encoding hemocyanin, cryptocyanin, and phenoloxidase in Dungeness crab, *Cancer* (*Metacarcinus*) *magister*; these proteins are involved in oxygen transport, the innate immune response, and molting (Terwilliger et al. 2006). Robert Chapman reported his group's efforts to use artificial neural networks and fractal geometry to study the dynamic, and often non-linear, interactions between thousands of genes in response to environmental stress (Chapman et al. 2006). John Colbourne reported on the *Daphnia* genome project and their progress in developing EST libraries and microarrays to assess the effects of ecological stressors (e.g., toxic metals, UV radiation, hypoxia, starvation, and predation; Colbourne et al. 2005). David Towle spoke on using ESTs for the discovery of genes expressed in the tissues of green shore crab, *Carcinus maenas*, and *H. americanus* (Towle and Smith 2006). Jonathon Stillman reported on the use of cDNA microarrays to study spatial and temporal responses to thermal stress of the porcelain crab, *Petrolisthes cinctipes* (Stillman et al. 2006).

There have been significant technological advances in genomics and transcriptomics over the last 10 years. In many cases, RNA-seq technology has replaced EST sequencing and microarrays for the analysis of gene expression. Moreover, the depth and coverage far exceeds that of ESTs, making it possible to produce a comprehensive transcriptome. The efficiencies in time and cost now make it feasible to obtain the mRNA sequence of any expressed gene from a *de novo* assembled transcriptome. RNA-seq is supplanting the gene-by-gene approach that uses conventional RT-PCR cloning.

The 10 speakers in the 2006 symposium availed themselves of the latest technologies to advance the field and served as the groundwork for results reported in the 2016 symposium. Three of the speakers in the 2006 symposium (Durica, Mykles, and Stillman) participated in the 2016 symposium. Paul Gross succumbed to cancer in 2008. David Towle died suddenly in 2011. Both individuals had tremendous impact on the emerging field of crustacean transcriptomics, as both were among the first comparative physiologists to exploit molecular techniques in their research. Both Paul and David shaped the careers of many comparative physiologists, including many of the participants in the 2016 symposium.

Paul Gross led the effort at the Hollings Marine Laboratory in Charleston, SC, to study the molecular basis of crustacean immunity. His drive extended both nationally and internationally, garnering awards from USDA, NSF and South Carolina Sea Grant that supported an extensive effort in Marine Genomics and leading to the sequencing and annotation of more than 250,000 ESTs from tissues of the Pacific whiteleg shrimp, *L. vannamei*. Microarrays based on those EST libraries supported numerous research programs, including Karen Burnett's research on the transcriptomic response of the shrimp hepatopancreas to low O_2/high CO_2 conditions (Rathburn et al. 2013). Jill Johnson did her first graduate rotation with Paul Gross at the Medical University of South Carolina, where she was first introduced to microarrays. Paul is remembered for his intellectual drive, as well as for his concern for all around him. He cultured a vibrant group of postdoctoral fellows and graduate students who shared his drive and passion for research. Had he not been taken from us so early in his career, Paul's international credibility, evident as coordinator for shrimp genomics in the USDA NSRP8 program and his groundbreaking studies on the evolutionary origins of invertebrate immunity, would have led to an international effort to sequence the shrimp genome. Our explorations of how the crustacean genome shapes phenotypic outcomes would be well underway.

As Senior Investigator and Director of the Marine DNA Sequencing and Analysis Center at Mt. Desert Island Biological Laboratories (MDIBL), David Towle trained a generation of scientists from ~1990 to 2009. The Center served the larger research community by providing EST clones at no cost. David assisted Fraser Clark and Spencer Greenwood's research program in transcriptomics at the Atlantic Veterinary College Lobster Science Centre, University of Prince Edward Island. Together they developed the Lobster Microarray 2.0, which they used extensively before

transitioning to RNA-seq. Justin Havird knew David through MDIBL, where he spent three summers as an undergraduate student in David Evans' laboratory. David was a knowledgeable resource on molecular biology protocols and helped Justin run his first qPCR. Andy Christie met David in 2006 when he, David, and Patsy Dickinson co-taught an IDeA Network of Biomedical Research Excellence course for Bowdoin College at MDIBL. Subsequently, Andy joined MDIBL as a visiting summer scientist and then as a year-round scientist. It was David who convinced Andy that transcriptomics could be an important tool for comparative physiology; their collaboration produced nine co-authored publications. Jonathon Stillman remembers David as encouraging of his early efforts in EST library construction and microarray generation. David was well connected to the crustacean research community and was instrumental in identifying speakers and topics for the 2006 symposium. All the 2016 symposium participants remember David as a true collaborator and gentleman scientist who always had time to discuss and engage on diverse topics from science to life in general.

The passion for marine research and education and training exemplified by Paul and David had an immense influence on students and investigators and remains an inspiration to us all. The symposium is a tribute to them and what they started. We dedicate this symposium series in recognition of the scientific legacies of Paul Gross and David Towle.

Conclusions

High-throughput sequencing is now the standard methodology for genomic and transcriptomic research. NGS technologies are democratizing biological research by enabling individual researchers to do what was recently limited to multimillion dollar sequencing consortia. Moreover, it has allowed for the study of physiological, ecological, and evolutionary research questions in ''non-model'' organisms. The papers in this symposium illustrate the power, as well as the limitations, of transcriptomics as a tool for discovery.

While software and computing power to support transcriptome assembly are now widely and freely available, this workflow still only produces a set of genes that are differentially expressed under experimental conditions. For researchers to derive a better understanding of their datasets, they must be able to model the biological context of gene expression data (i.e., understand functional enrichment, pathways, and molecular networks that are affected). A challenge of transcriptomics is to integrate correlational information cataloging gene expression at a particular time and place to physiological function. New methods are needed to directly link gene expression to a phenotype. There should be a concerted effort to develop a decapod crustacean model, in which transgenic tools are applied for experimentally testing gene functions.

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