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HORIZONS

Transcriptomics and metatranscriptomics in zooplankton: wave of the future?

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Molecular tools have changed the understanding of zooplankton biodiversity, speciation, adaptation, population genetics and global patterns of connectivity. However, the molecular resources needed to capitalize on these advances continue to be limited in comparison with those available for other eukaryotic plankton. This deficiency could be addressed through an Ocean Zooplankton Open 'Omics Project (Ocean ZOOP) that would generate *de novo* assembled transcriptomes for hundreds of metazoan plankton species. A collection of comparable reference transcriptomes would generate a new framework for ecological and physiological studies. Defining species niches, identifying optimal habitats, assessing adaptive capacity and predicting changes in phenology are just a few examples of how such a resource could transform studies on zooplankton ecology.

KEYWORDS: gene expression; physiology; ecology; transcriptome

INTRODUCTION

In 2014, the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) transformed the study of phytoplankton physiological ecology by generating annotated transcriptomes for over 650 phytoplankton species and making these publicly available (Keeling *et al.*, 2014). The marine zooplankton community has not matched this achievement. The year was marked by the availability of two deep and annotated *de novo* transcriptomes, both for the copepod *Calanus finmarchicus* (Lenz *et al.*, 2014; Tarrant *et al.*, 2014). The number of high-quality and annotated transcriptomes for metazoan zooplankton available through the National Center for Biotechnology Information (NCBI) is growing but it continues to be small especially for holoplanktonic species (<50; September 2020).

Transcriptomics has transformed many areas of biological research. The analysis of expression profiles of messenger RNAs has elucidated developmental programs, has led to the discovery of novel cell types and has spurred the development of new diagnostic tools

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and therapies (Wang *et al.*, 2009). While the zooplankton research community has embraced genomics tools for biodiversity studies, transcriptomic approaches have been uncommon (Bucklin *et al.*, 2018). We argue that this has hampered zooplankton research, in particular in comparison with phytoplankton and microbial studies. By way of illustration we searched a database compiled by Lima and Rheuban (2018) of abstracts of grants funded by the US National Science Foundation's Biological Oceanography Program between 2008 and 2018. The term "transcriptome" or "transcriptomic" appeared in three abstracts (2.9%) among all 102 projects that included "zooplankton". A parallel search of "phytoplankton" projects yielded 22 out of 221 (10.4%). Transcriptomics and metatranscriptomics have changed our understanding of nutrient stress, niche partitioning and succession among phytoplankton species (Alexander *et al.*, 2015a; Lampe *et al.*, 2019; Zhang *et al.*, 2019). Resources developed by MMETSP were critical for this progress (Alexander *et al.*, 2015a, b; Helliwell *et al.*, 2016; Janouškovec *et al.*, 2017). In contrast, transcriptomics applications to zooplankton ecology have been limited in scope and focused mostly on single species (Scear *et al.*, 2012; Tarrant *et al.*, 2014; Roncalli *et al.*, 2016; Batta-Lona *et al.*, 2017; Mojib *et al.*, 2017; Roncalli *et al.*, 2019; Piccolin *et al.*, 2020). An Ocean Zooplankton Open 'Omics Project (Ocean ZOOP) is long overdue. Ocean ZOOP would be a community effort to identify hundreds of high-priority zooplankton species spanning diverse taxa from across the world's oceans to be targeted for RNA-Seq. Its goal would be to generate publicly available, curated and functionally annotated *de novo* transcriptomes for individuals from each species. Ocean ZOOP could be as transformative as MMETSP has been.

ZOOPLANKTON ECOLOGY

The unique ecologies of zooplankton need to be considered to optimally apply transcriptomic technologies. The community is diverse, dynamic, and characterized by complex trophic interactions. Evaluating how these communities are impacted by global warming and other concurrent changes to the environment is critical. Predictive ecological models have been parameterized using experimentally determined functional responses to food, temperature, pH, harmful algae, etc ... obtained by varying one or two factors under controlled conditions (Strader *et al.*, 2020). However, such experimental results fail to represent the broad spectrum of responses expected in a natural community having a multiplicity of interactions, behaviors such as diel vertical migration, and cascading ecological effects that can occur even with small changes to the physical/chemical environment. Here is where environmental transcriptomics can be combined with ecological studies to uncover physiological changes that affect fitness.

SINGLE-SPECIES ECOLOGICAL TRANSCRIPTOMICS: PROGRESS, BUT NOT ENOUGH

Gene expression profiles determined by RNA-Seq in field-collected individuals vary across space and time (Batta-Lona *et al.*, 2017; Roncalli *et al.*, 2019; Semmouri *et al.*, 2020). The magnitude of expression differences often greatly exceeds those reported in response to known stressors under experimental conditions (Roncalli *et al.*, 2016). Furthermore, transcriptional phenotype differences in oceanic zooplankton cannot be easily explained by regional genetic differences, which occur in species with fragmented populations (e.g. the intertidal copepod *Tigriopus californicus*) (Schoville *et al.*, 2012; Barreto *et al.*, 2018).

Diel vertical migration transports biomass and nutrients over hundreds of meters contributing to vertical carbon flux, but it has been difficult to investigate physiologically. Transcriptomic and targeted gene expression studies have started to elucidate the mechanisms that control this behavior (endogenous rhythms vs. environmental cues) in krill (Piccolin *et al.*, 2020), caridean shrimp (Deleo & Bracken-Grissom, 2020) and copepods (Häfker *et al.*, 2017). In addition, transcriptomics is characterizing other cyclical patterns in physiology such as metabolic rates (Piccolin *et al.*, 2020), data that are foundational to any quantitative studies on vertical nutrient fluxes in the ocean.

Seasonal changes in gene expression are another source of transcriptional variability, as documented in several taxa. In a seasonal study of the copepod *Temora longicornis*, differences in transcriptional signatures between sampling dates were indicative of changes in physiology and reproductive activity, which in turn were correlated with the population cycle in the North Sea (Semmouri *et al.*, 2020). In the Antarctic salp, *Salpa thompsoni*, gene expression not only changed with the seasonal cycle, but heterogeneity in expression profiles increased between the spring and summer (Batta-Lona *et al.*, 2017). This gave rise to the hypothesis that during the initial growth phase, the population is highly synchronized developmentally. Then, as the season progresses, variation in gene expression may result from local environmental conditions that affect the salp's physiology and fitness. A microarray study of seasonal differences in gene expression in Antarctic krill, *Euphausia superba*, included those involved in

digestion (Seear *et al.*, 2012). Expression of genes encoding digestive enzymes may be key indicators in zooplankton, not just for food abundance but also food type, as shown experimentally in a copepod's response to a toxic algal diet (Roncalli *et al.*, 2016). While limited in scope, these time series demonstrate how gene expression profiling could be used to discover physiological transitions that can lead to the early detection of impending changes in the population and potentially link them to environmental triggers.

At shorter temporal scales, transcriptional responses in zooplankton have been related to changes in the phytoplankton community. Such changes have been reported in the copepod *Temora stylifera* to changes in the diatom community and increases in ambient oxylipin concentrations (Russo *et al.*, 2020). Blooms of filamentous cyanobacteria like *Trichodesmium* can disrupt zooplankton communities, but the effect may not be direct. A bulk RNA-Seq study of neustonic samples collected prior, during and after a *Trichodesmium* bloom reported large-scale changes in gene expression at the metatranscriptomics level (Mojib *et al.*, 2017). Copepods and appendicularians (*Acartia fossae* and *Oikopleura dioica*) showed disproportionately steep declines in abundances. In a species-specific analysis, gene expression analysis of *A. fossae* implicated viral activity in the high mortality, rather than a direct response to the cyanobacterium. The presence of virus-like particles was confirmed by transmission electron microscopy.

Spatial differences in transcriptomic signatures can also be substantial, even in populations with highly synchronized life histories. Surprisingly large differences in transcriptional phenotypes have been reported in a single developmental stage (pre-adult, copepodid CV) of the copepod *Neocalanus flemingeri* from the northern Gulf of Alaska (Roncalli *et al.*, 2019). These were found to be correlated with the steep environmental gradients in the quantity of large phytoplankton cells. A functional analysis of gene expression patterns concluded that these copepods experienced nutritional stress in low chlorophyll *a* regions, while also underscoring the copepod's extreme resilience to environmental variability (Roncalli *et al.*, 2019). Zooplankton population models based on patterns of production in the Gulf of Alaska have shown a 65% agreement between model predictions and observed spring copepod abundances (Coyle *et al.*, 2019). Transcriptomic profiling suggests that the residual 35% discrepancy may in good part reflect the copepods' ability to persist in regions with low primary production.

At the interface between population genetics and physiological acclimatization, we can highlight a recent study on *Oithona similis* using *Tara* Oceans Expedition metagenomic and metatranscriptomic data (Laso-Jadart *et al.*, 2020). While genetic differentiation was low among populations, the study reports a mismatch between gene expression and allelic frequencies for some loci. This study offers insights into local adaptation and links it to natural selection on genes involved in a variety of functions including neuronal regulatory pathways.

These pioneering studies have brought new insights into physiological adaptation and resilience of planktonic organisms to a marine environment that is locally and temporally unpredictable. The studies also had to surmount several obstacles. Development of well-annotated reference transcriptomes using shotgun assembly approaches is nontrivial; predicted transcripts need to be translated into predicted proteins and then annotated based on similarity to known proteins from often distantly related taxa. In addition to these uncertainties, reference transcriptomes have varied in quality and depth (completeness). While this might not affect the validity of mapping short-sequence reads from different samples against a common reference, it does impact the interpretation of the expression signal. Nevertheless, robust protocols have been developed for generating and assessing the quality of reference transcriptomes for non-model species including zooplankton (Roncalli *et al.*, 2017; Tarrant *et al.*, 2019). Manual annotations of genes of interest have provided additional validation of *de novo* transcriptomes (Christie *et al.*, 2013; Roncalli *et al.*, 2015; Porter *et al.*, 2017).

Interpretation of gene expression data has been evolving with new analysis and bioinformatics tools. Clustering of samples by similarity of gene expression can reveal physiological commonalities (Batta-Lona *et al.*, 2017). Dimensionality-reduction tools (e.g. t-SNE) that incorporate machine learning excel at clustering developmental, experimental and field samples and thus are exceptionally promising for zooplankton studies (Cieslak *et al.*, 2020). One application of such a tool resulted in the development of a protocol that incorporated functional filters to differentiate between direct-developing and diapause-bound *C. finmarchicus* (Lenz *et al.*, 2020). However, functional interpretation of gene expression continues to lag. Statistical approaches such as enrichment and differential network analyses can focus data interpretation on groups of genes with similar expression or function. However, relating these differences in expression to organism performance or fitness will require additional validation from field and laboratory experiments to pave the way for transcriptomic approaches to reach their full potential.

THE PROMISE AND CHALLENGES OF COMPARATIVE AND METATRANSCRIPTOMICS

Ocean ZOOP would generate reference transcriptomes for hundreds of species of interest. The resource would put in motion comparative transcriptomic and metatranscriptomic eco-physiological investigations of multiple and co-occurring zooplankton species. It would enable a broad ecosystem-wide view not available from current studies on just a few species. While there are challenges to the broad application of multispecies transcriptomics, progress on single species provides a roadmap for developing robust protocols. Ocean ZOOP would open the door to exploiting existing and publicly available metatranscriptomic data generated for bulk zooplankton during the *Tara* Oceans Expedition (Pesant *et al.*, 2015; Alberti *et al.*, 2017; Laso-Jadart *et al.*, 2020).

Cross-mapping among species

Access to high-quality transcriptomes will open the way to metatranscriptomics of zooplankton communities. With this approach, the transcriptional phenotypes of different taxonomic groups of zooplankton can be determined simultaneously from bulk RNA-Seq samples. However, metatranscriptomics using short-sequence reads depends on taxon-specific mapping of each read. Taxonomic assignments for mapped reads derived from highly conserved genes within a mixed-species assemblage can be challenging, especially between closely related taxa. Cross-species mapping rates can be used to estimate taxonomic ambiguity in read identity that could confound species-specific measures of gene expression. As a demonstration we employed an established metazoan workflow for *de novo* transcriptome assembly of short-sequence reads and their mapping (Roncalli *et al.*, 2019; Cieslak *et al.*, 2020) to examine cross-mapping rates between a selection of species separated by a range of genetic distances from copepods (protostomes) to salps (deuterostomes; Fig. 1A, Supplementary material). In our example, cross-mapping rates decreased with taxonomic distance, with median rates below 1% between species belonging to different families. Thus, cross-mapping errors are minor at that taxonomic distance and higher. However, significant cross-mapping within genus and within family (>20%) could affect interpretation of metatranscriptomic data.

In addition, cross-mapping rates in within-group comparisons can be variable (Fig. 1A). A modified workflow that removed the inevitable highly conserved ribosomal RNA (rRNA) from each short-sequence read set reduced this variability (Supplementary material). Removal of

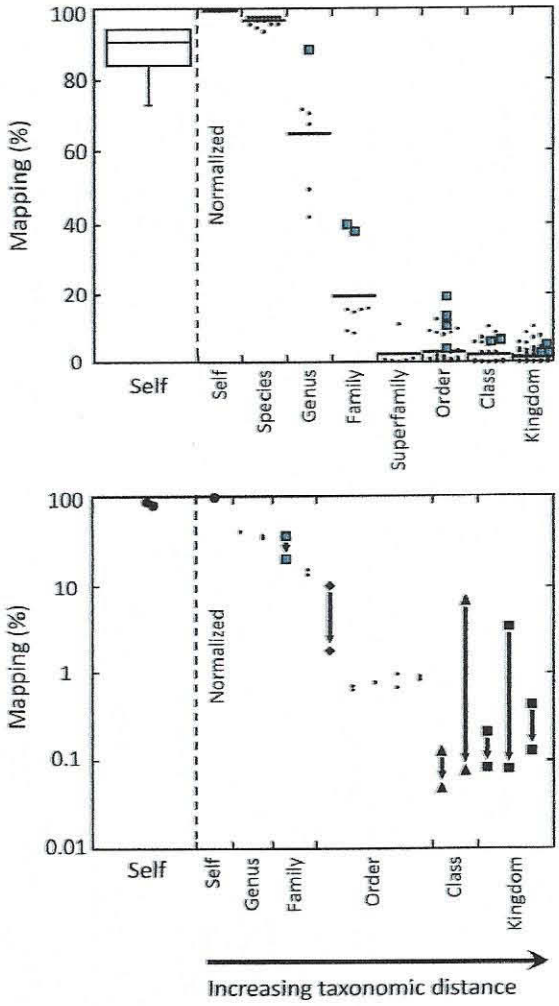

Fig. 1. Mapping of short sequence reads against self and cross-mapping to other species with increasing taxonomic distance between the reference transcriptome and the source of short sequence reads. A. *Left column:* self-mapping percentages of short-sequence reads mapped against the *de novo* assemblies generated from each dataset (linear scale). Box and whiskers plot median (line), lower and upper quartile (box) and range (bars) indicated. Species (n = 13)—Crustacea: Maxillopoda, Calanoida: *Calanus finmarchicus, Calanus sinicus, Neocalanus flemingeri, Neocalanus plumchrus, Labidocera madurae, Eurytemora affinis, Acartia tonsa, Pleuromamma xiphias;* Malacostraca: *Euphausia superba, Euphausia crystallorophias;* Mollusca, Gastropoda: *Clione limacina;* Tunicata: Appendicularia: *Oikopleura dioica;* Thaliacea: *Salpa thompsoni*). *Right of dashed line:* cross-mapping percentages normalized to self-mapping rates. Dot plot shows individual mapping rate points (blue squares: *N. flemingeri*; circles: others; horizontal lines: median for each group). Number of observations: within species (n = 16; *N. flemingeri*), within genus (n = 6; 3 genera: *Calanus, Neocalanus, Euphausia*); within family (n = 8; Calanidae); within superfamily (n = 6; Centropagoidea); within order (Calanoida) across superfamily (n = 38; 3 superfamilies: Augaptiloidea, Centropagoidea, Megacalanoidea); within class (Crustacea) across orders (n = 32; 2 orders: Maxillopoda, Malacostraca); within kingdom (Animalia), across phyla (n = 63; 3 phyla: Crustacea, Mollusca, Tunicata). B. *Left column:* log scale of mapping percentages of short-sequence reads to the *C. finmarchicus* reference transcriptome before and after the removal of

rRNA sequences is routine in microbiome and microbial studies (Kopylova *et al.*, 2012). In our example, this correction reduced cross-mapping substantially (Fig. 1B). Gene library preparation for RNA-Seq includes the removal of rRNA sequences, however, the effectiveness of this step varies. Thus, the largest reductions occurred in samples with unusually high cross-mapping rates for their taxonomic distance (Fig. 1). The removal of rRNA sequences reduced cross-mapping rates to ca. 1% within the order Calanoida and to 0.1% with greater taxonomic distances (Fig. 1B). However, habitats with multiple congeners, such as the Norwegian Sea (*Calanus helgolandicus*, *C. finmarchicus*, *C. glacialis*, *C. hyperboreus*) (Strand *et al.*, 2020), may require additional modifications to the reference transcriptomes to reduce cross-mapping below 1%.

Developmental signals

Crustacean zooplankton, which often dominate communities in species diversity and abundance (Humes, 1994), typically pass through multiple developmental stages with distinct transcriptional phenotypes (Cieslak *et al.*, 2020). Thus, when a mixture of stages is included in a sample, species-specific expression profiles would represent an average across developmental stages that cannot be easily interpreted. Two solutions can be proposed: (1) physical separation of developmental stages through size-fractionation (Jungbluth *et al.*, 2013; Pesant *et al.*, 2015; Sommer *et al.*, 2017); and (2) gene-selection filters constructed to minimize developmental signals while focusing on gene expression signals of interest (Cieslak *et al.*, 2020).

Reference transcriptomes for cross-species comparisons

Cross-species comparisons require validation given the potential for errors due to differences in the completeness of reference transcriptomes, quality of annotation and mapping rates. Currently available *de novo* assemblies for zooplankton vary in depth and quality (Supplementary material) (Tarrant *et al.*, 2019), a problem that would be corrected through a coordinated sequencing project like Ocean ZOOP. A consistent RNA-Seq protocol followed by bioinformatic processing of *de novo* assemblies would ensure comparable reference transcriptome quality and annotation for quantitative analyses of relative gene expression across species. In the diatom study using metatranscriptomics, the workflow for cross-species comparisons included a validation step using a second normalization protocol based on "housekeeping" genes, followed by laboratory experiments (Alexander *et al.*, 2015a). The authors' strategy was to focus on regulated pathways that were common to two competing species. Such an approach has already been demonstrated experimentally in the freshwater cladoceran, *Daphnia magna*, for pathways associated with lipid metabolism (Windisch & Fink, 2018). However, environmental transcriptomics data suggest that many more pathways are regulated. Thus, a better understanding is required for robust comparisons across species and interpretations within an ecological context.

LOOKING FORWARD

The potential for transcriptomics and metatranscriptomics to advance basic understanding of metazoan zooplankton ecology is undeniable. Obstacles are surmountable. Investing now in zooplankton transcriptomics, cross-species comparisons and metatranscriptomics is timely: the 'Omics field has advanced to the point where application of molecular and bioinformatic tools can answer basic questions in niche differentiation, habitat quality and boundaries, population cycles and organism–environment interactions.

Transcriptomics is highly relevant to answering basic questions of zooplankton ecology provided that we do the necessary experiments to ground-truth gene expression data, and link them to traditional and nontraditional measures of growth, fitness and secondary production. Reference transcriptomes generated by Ocean ZOOP would open the door to within and across species comparisons of gene expression signatures for hundreds of species. Used creatively Ocean ZOOP promises to revolutionize our understanding of zooplankton evolution, population cycles and community ecology.

SUPPLEMENTARY DATA

Supplementary data mentioned in the text are available to subscribers in *PLANKT* online.

AUTHOR CONTRIBUTIONS

P.H.L. and D.K.H. conceived the study and wrote the manuscript; P.H.L., D.K.H., V.R., B.L. and M.C.C. contributed to data acquisition,

reads mapped to rRNA transcript. *Right of dashed line:* cross-mapping percentages normalized to the percentage of reads mapping to the *C. finmarchicus* reference before and after removal of rRNA reads (87.1 and 86.7%, respectively). Vertically aligned symbols (small circles and larger symbols) show the effect of the removal of rRNA reads on cross-mapping. Observations by taxonomic distance from *C. finmarchicus*: within *Calanus* (n = 2; *C. sinicus*, *Calanus glacialis*); within Calanidae (n = 2; *N. flemingeri* [blue squares], within Calanoida (n = 6; *L. madurae* [diamonds], *E. affinis*, *A. tonsa*, *Acartia fossae*, *P. xiphias*); within Crustacea (n = 2; black triangles; *E. superba*, *E. crystallorophias*); across Phyla (n = 3; black squares; *C. limacina*, *O. dioica*, *S. thompsoni*). Arrows and large symbols highlight comparisons where the proportional decline in cross-mapping was large.

analysis and interpretation, and manuscript preparation. All authors approved the final manuscript.

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