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Transcriptomic analysis of changes in gene expression of immune proteins of gill tissue in response to low environmental temperature in fathead minnows (*Pimephales promelas*)

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ABSTRACT

In the face of ongoing climate change, it is imperative to understand better the effects of temperature on immune function in freshwater teleosts. It is unclear whether previously observed changes were caused by temperature *per se*. We studied changes in the gill transcriptome of fathead minnows (*Pimephales promelas*) at low temperature to understand better the effects of temperature on immune function. *De novo* assembly of the transcriptome using Trinity software resulted in 73,378 assembled contigs. Annotation using the Trinotate package yielded 58,952 Blastx hits (accessions). Expression of 194 unique mRNA transcripts changed in gill tissue of fathead minnows acclimatized to 5° compared to controls at 22 °C. At 5 °C mRNAs coding for proteins involved in innate immune responses were up-regulated. Those included proteins that block early-stage viral replication and macrophage activation. Expression of mRNAs coding for pro-inflammatory molecules and mucus secretion were also enhanced. Messenger RNAs coding for proteins associated with adaptive immune responses were down-regulated at 5 °C. Those included antigen-presenting proteins and proteins involved in immunoglobin production. Messenger RNAs coding for proteins that stimulate the cell cycle were also down-regulated at 5 °C. Histological comparison revealed that gills of cold acclimated fish had fewer mucus cells but cells contained larger mucus droplets. We conclude that decreased temperature modifies the immune systems of freshwater teleosts, leading to genome-wide upregulation of innate immunity and down regulation of adaptive immunity. Such acclimation likely evolved as an adaptive strategy against seasonal changes in infectious insults.

1. Introduction

Seasonal environmental changes affect many homeostatic mechanisms in freshwater fish, including immune function (Bowden, 2008). As climate changes, temperature variations are likely to show wider excursions that may result in immune dysfunction. The aim of this study was to establish a baseline of temperature effects on immune function. To do so we acclimated fathead minnows to 22° and 5° C, temperatures seen seasonally in streams in North America.

Previous investigators have reported that changes in temperature cause changes in immune function. Plasma titers of IgM increased as acclimatization temperature increased in Nile tilapia (*Oreochromis niloticus*) (Dominguez et al., 2004), sea bass (*Dicentrarchus labrax*), and Atlantic cod (*Gadus morhua*) (Magnadóttir et al., 1999), and expression of several cytokines increased with temperature in rainbow trout (*Oncorhynchus nerka*) (Raida and Buchmann, 2007). Those responses are in line with the general view that at low temperatures innate immune responses predominate while at higher temperature the adaptive immune system becomes more important (Makrinos and Bowden, 2016). Exceptions to that pattern have been reported. Anti-viral genes are up-regulated in *Danio rerio* larvae maintained at 28 °C compared to 15 °C (Dios et al., 2010) but in a different study of sea bass exposed to natural seasonal changes, humoral immune factors showed little change with temperature (Valero et al., 2014). However, in the latter study temperatures varied only from 15 °C to 20 °C. Yang et al. (2016) demonstrated in *Oreochromus* niloticus that exposure to temperatures either

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higher or lower than acclimation temperature depressed expression levels of C-type lectin, proteins involved in immune responses and apoptosis. Transcriptomic analysis of a sub-tropical fish, *Melanotaenia duboulayi*, acclimated to 25° C for 30 days, then acclimated to 33° C for 14 days, showed that expression of hundreds of mRNAs was either up- or down-regulated by high temperature exposure (Smith et al., 2013).

The gills of fish are the primary organs of gas exchange and are important in ion balance. A single layer of gill epithelial cells covered by a thin mucus layer separates extracellular fluid from water, facilitating gas and ion exchange but making the tissue vulnerable to infection. Gill tissue is protected from infection by immunoglobins IgM and IgT.

In trout both B and T lymphocytes are collected in intrabranchial lymphoid tissue and respond to infection (Parra et al., 2015). Xu et al. have shown in rainbow trout that IgT is produced in gill IgT + B-cells in rsponse to *Ichthyophthirius multifilis* (Ich) parasite infection (Xu et al., 2016). They also demonstrated that the gill microbiota is coated with IgT molecules.

Fathead minnows (*Pimephales promelas*) are a widely used toxicology model (Ankley and Villeneuve, 2006). They are common across North America, living in estuaries as well as fresh water ecosystems that exhibit wide seasonal variations of temperature. Fatheads are commercially important in the aquarium trade and large numbers are raised and sold as bait fish for recreational fishing (Page and Burr, 2011). Despite the biological and commercial significance of fatheads, there are no studies of seasonal changes in their immune system at any level. Genome-wide transcriptomic analysis of the effects of acclimatization temperature on immunity should contribute to a more complete understanding of the cellular mechanisms underlying changes in immune function in response to temperature. Although there have been explorations of gene expression in fathead minnows through expressed sequence tag work and microarrays (Jovanović et al., 2011) and recent efforts to sequence the genome (*Pimephales promelas (ID 13167) - Genome - NCBI*, n.d.), no reference transcriptome has been sequenced and assembled for any tissues of this species including those that would be involved in immune function. We sequenced the transcriptome of gill tissue from *Pimephales promelas* acclimated to 5 °C and 22 °C for 4 weeks, and analyzed the expression of mRNAs associated with adaptive and innate immunity.

2. Results and discussion

2.1. Transcriptome sequencing and assembly

The initial assembly and annotation of the transcriptome was done using gills from control fish acclimated to 22 °C. *De novo* assembly was performed on quality controlled reads with *in silico* normalization using default Trinity parameters and kmers 25 bps long. The Trinity assembly included a total length of 111 megabase pairs (Mb) and 73,378 transcripts. From these transcripts an N50 value of 2685 bps was obtained with mean and median transcript lengths of 1525 bp and 943 bp respectively. The distribution of transcript lengths is shown in Fig. 1. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession **GCVQ00000000**. The version described in this paper is the first version, **GCVQ01000000**.

We cleaned the transcriptome prior to annotation and analysis. First, we removed reads of non-targeted sequences (*e.g.* mtDNA, bacterial DNA, viral DNA, and fungal DNA) to reduce the likelihood of assembling transcripts containing non-targeted reads. Second, we removed any transcripts with very low abundance to cull those that may have been poorly assembled or weakly supported. Last, we translated only the sequences that contained open reading frames of > 100 amino acids. The read coverage of our transcriptome is high. We estimate ~398× coverage, with an average transcript per million reads (TPM) value of 13.49. Our N50 value of 2658 indicates a high quality assembly.

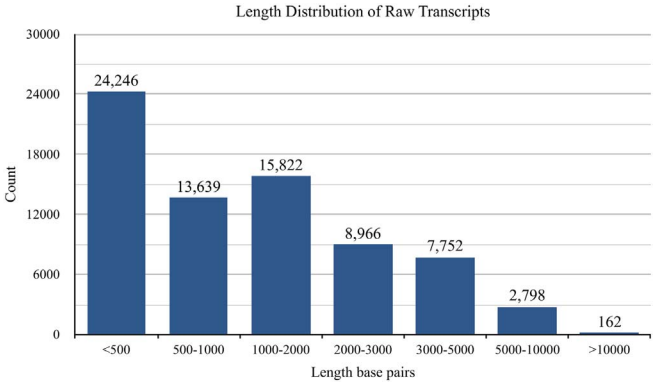

Fig. 1. Length Distribution of Raw Transcripts: A distribution of transcript lengths for the filtered transcriptome.

In terms of completeness, 7913 transcripts were described as full length, meaning they cover the entire length of the Blastx hit, with an additional 2511 being > 80% complete. These 10,424 transcripts make up approximately 17% of all annotated transcripts, and 31,738 out of 58,952 annotations (32.9%) made the cutoff of having > 80% identity with their best hit.

2.2. Functional gene annotation and classification

The complete Uniprot database (UniProt-Consortium, 2014) was used as a reference gene dataset because there is no extensively annotated genome for any close relative of the fathead minnow. Transcript contigs were blasted against the Uniprot database resulting in 58,952 total hits having an e-value of less than 1E-03. Many transcripts had hits to the same gene, resulting in 31,918 unique gene hits. Out of the 73,378 transcripts, 80.1% of transcripts were annotated. E-value distribution for the hits is shown in Fig. S1. A small portion of hits with non-eukaryotic origins were culled and a similarity distribution of the eukaryotic hits showed that 57.2% of hits had > 50% sequence similarity (Fig. S2). Additionally, a count was performed to test the number of transcripts covering a full-length matched protein with at least 80% overall alignment. Approximately 10,000 transcripts contained mRNA coding for > 90% of the length of their respective proteins (Fig. 2). However, it is important to note that while BLAST alignments may

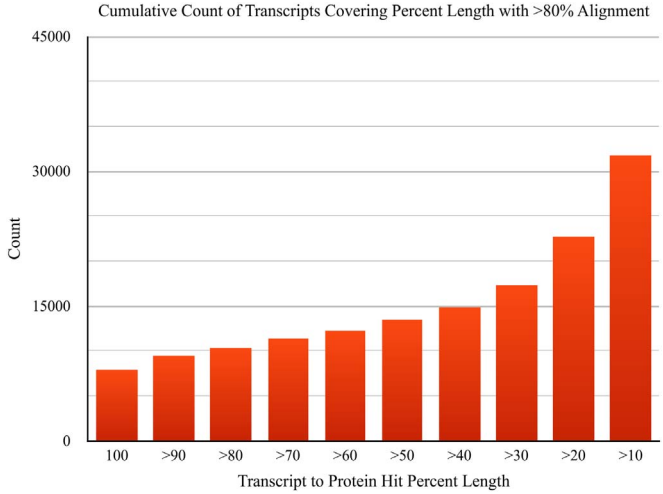

Fig. 2. Cumulative Count of Transcripts Covering Percent Length with > 80% Alignment: Presented above is a graph of the cumulative count of the filtered transcripts which comprise greater than the listed percent lengths of their protein hits, however, it must be noted that transcripts are only included on this graph provided they have > 80% identity with their protein hits, so the total number of transcripts that meet that criteria is ~42,000 as opposed to the 123,591 filtered transcripts assembled total.