Functional characterization of differentially expressed miRNAs in *Trematomus bernacchii* acclimated to acute and chronic thermal stress

by

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Abstract

The Antarctic notothenioids have evolved for millions of years in a cold stable environment and are found to have a narrow window for thermal tolerance. Global climate change is predicted to bring warmer ocean temperatures in polar regions thereby challenging the physiological capacity of these fish to environmental perturbations. Despite the lack of an inducible heat shock response (HSR), notothenioids have displayed remarkable physiological plasticity and an ability to at least partially compensate for the effects of thermal stress. Both physiological and transcriptomic studies have signaled these fish can mitigate the effects of acute heat stress by employing other aspects of the cellular stress response (CSR) that help confer thermotolerance as well as drive homeostatic mechanisms during long term thermal acclimations. However, the regulatory mechanisms that determine temperature-induced changes in gene expression remain largely unknown. Therefore, this study utilized next generation sequencing coupled with a bioinformatics *in silico* approach to explore the regulatory role of microRNAs in governing the transcriptomic level response observed in notothenioids with respect to the CSR. MicroRNAs (miRNAs) are small (~22 nucleotides) evolutionarily conserved, non-coding RNAs that predominantly downregulate gene expression in a sequence specific manner. Using RNAseq, this study characterized the global expression of 125 distinct miRNAs in *Trematomus bernacchii* gill tissue. Additionally, I examined the functional role of 10 differentially expressed (DE) miRNAs in *T. bernacchii* given an acute (7 days) and long-term thermal acclimation to +4 °C. Using various bioinformatics pipelines, this study determined the gene targets of DE miRNA that are enriched in particular biological pathways and examined the impact of these enriched miRNA pathways with respect to the CSR in *T. bernacchii*. The miRNA enriched pathways identified in these analyses were found to be involved in regulating diverse aspects of the CSR during acute and chronic heat stress in *T. bernacchii* such as inflammatory response, antioxidant activity, lipid metabolism, tissue and cytoskeletal remodeling, membrane maintenance, cell proliferation and apoptosis. While most miRNA enriched pathway effects coincided with the acute and chronic CSR observed in the transcriptome of thermally stressed *T bernacchii*, other miRNA pathways appear to potentially exert a contradictory effect.

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Introduction

The notothenioids are a diverse suborder of Antarctic fish that comprise more than ninety percent of the fish biomass found in Antarctic waters (Eastman, 2005). During the last 14-25 million years, these endemic fauna have evolved in an extremely cold, oxygen rich and thermally stable environment where the mean annual seawater temperature is -1.86 °C (Eastman, 1993). Consequently, the region harbors a distinct lineage of fish that possess a unique array of evolutionary adaptations that include antifreeze glycoproteins, stable micro-tube assembly, and increased polyunsaturated fatty acids in plasma membranes (DeVries & Cheng, 2005; Near *et al*., 2012). Additionally, notothenioids also possess unique metabolic adaptations such as increased mitochondrial density and preference for lipid oxidation as a primary energy source for fueling metabolism (Johnston *et al*., 1998; Pörtner *et al*., 2005). Given these unique adaptations, notothenioids serve as unique model systems to investigate genomic mechanisms that contribute to loss or gain of function phenotypes that arise from extreme stenothermy. The molecular mechanism underlying one such occurrence, the inability to mount a functional heat shock response (HSR), has yet to be elucidated (Buckley *et al*., 2004; Place *et al*., 2004; Hoffman *et al*., 2005; Place & Hofmann, 2005; Petricorena & Somero, 2007). The lack of HSR in these fish is characterized by constitutive expression of inducible heat shock proteins that under normal conditions would be upregulated during stress in an effort to restore the functions of damaged proteins denatured by environmental stressors (Feder & Hofmann, 1999; Frydman, 2001). Although these unique adaptations may be suitable for life in subzero waters, the advent of climate change is predicted to bring warmer ocean temperatures that can potentially affect the physiological mechanisms of these fish given their narrow window for thermotolerance (Perry *et al*., 2005; Turner *et al*., 2009; Near *et al*., 2012). In fact, the region around the Western Antarctic Peninsula is one of the fastest warming on Earth where sea surface temperatures have risen by 1

°C over the last 50 years (Meredith & King, 2005). It is imperative that we gain a better understanding of the molecular regulatory response of these fish to thermal stress since their physiological susceptibility is exacerbated by the absence of a functional heat shock response.

Cellular stress has many detrimental effects characterized by disruption of protein homeostasis, cytoskeletal and ribosomal assembly defects, macromolecule damage and cytotoxic aggregation of proteins in general (Morimoto, 1998; Feder & Hofmann, 1999; Frydman, 2001; Kültz, 2005). The CSR mitigates stress by employing homeostatic mechanisms that include molecular chaperoning by heat shock proteins, redistributing metabolic resources away from house-keeping functions such as cell growth to more stress responsive functions such as DNA repair, promoting reversible arrest of the cell cycle and in cases of profound stress, apoptosis is induced (Kültz, 2005). As a result, cells respond to stress by reprogramming their gene expression patterns to maintain a physiology necessary to support survival. The physiological and molecular effects of thermal stress on Antarctic fish have been well documented by numerous studies, albeit few transcriptomic studies are published.

Most Antarctic notothenioids like *Trematomus bernacchii* are characterized by the unique absence of the HSR to elevated temperatures, which is an important hallmark of the CSR that is functional in nearly all organisms. It is hypothesized that the constitutive expression of inducible HSPs may be due to the cold denaturation of proteins that require constant chaperoning function in notothenioid fish (Place *et al*., 2004; Todgham *et al*., 2007). Despite the absence of the HSR in these fish, transcriptomic studies have further characterized the genome wide cellular stress response which also correlate with physiological effects reported in notothenioids exposed to acute and chronic heat stress (Buckley & Somero, 2009; Chen *et al*., 2008; Shin *et al*., 2012; Bilyk & Cheng, 2013, 2014; Huth & Place, 2013, 2016a, 2016b). In most cases, true acclimation

may be occurring as seen in notothenioids like *T. bernacchii* and *Pagothenia borchgrevinki* that display an acute response to short term thermal stress followed by a return to basal state in long term thermal acclimation experiments. In fact numerous studies have established that notothenioids have the capacity to acclimate to long-term heat exposure (Enzor & Place, 2005; Franklin *et al*., 2007; Bilyk & DeVries, 2011; Bilyk *et al*., 2012; Huth & Place, 2013, 2016a, 2016b).

Notothenioids primarily utilize lipids as energy sources under non-stressed conditions (Crocket *et al.*, 1990; Eastman, 1993; Sidell *et al.*, 1995). However, under acute thermal stress, they increase their lipid metabolism and resting metabolic rate (Chen *et al*., 2008; Enzor *et al*., 2013; Huth & Place, 2016a). Chen *et al*. demonstrated that under thermal stress, *Dissostichus mawsoni* exhibited increased lipid metabolism as evidenced by high levels of apoliporoteins and cytochrome oxidase in liver and gill tissues respectively. These findings also correlate with an acute increase in resting metabolic rate observed in thermally stressed Antarctic species like *P. borchgrevinki* and *T. bernacchii* (Wilson *et al*., 2002; Robinson & Davison, 2008a, 2008b; Enzor *et al*., 2013). An increase in metabolism at higher temperatures coupled with high mitochondrial densities has increased oxidative stress in notothenioids (O'Brien & Sidell, 2000; Enzor & Place, 2014; Almroth *et al*., 2015) and consequently an upregulation of reductases, redoxins and dehydrogenases has been observed in response to short term oxidative stress (Huth & Place, 2016a). However, for notothenioids, these antioxidant defenses return to basal levels correlated with reduced oxidative damage following long term acclimation to thermal stress (Bilyk & Cheng, 2014; Enzor & Place, 2014; Huth & Place, 2016a, 2016b; Almroth *et al.*, 2015). For instance, in long term thermal stress, *T. bernacchii, P. borchgrevinki* and *Trematomus newnesi* displayed no change in antioxidant capacity, and a significant decrease in oxidative stress which

is also reported to coincide with reduced levels of constitutive heat shock proteins in long term thermal acclimation (Biyvek & Cheng, 2014; Enzor & Place, 2014). This would suggest that there is less demand for protein folding capacity in the cells during longer thermal acclimation after 56 days of thermal acclimation (Huth & Place, 2013; Enzor & Place 2014).

Taken together, these studies demonstrate that notothenioids have remarkable physiological plasticity to cope with thermal stress in various ways despite the lack of an inducible HSR. Furthermore, their ability to alter levels of other stress response proteins during short-versus long-term heat stress further demonstrates their capacity to drive homeostatic mechanisms during chronic thermal acclimations. However, there is limited knowledge on the regulatory mechanisms responsible for CSR during acute and chronic thermal acclimation in these fish, including the absence of the HSR in these fish. For instance, the disruption of the HSR in these fish may have occurred through a loss of regulatory control of gene expression. It still remains to be determined whether the disruption of the HSR in notothenioids like *T. bernacchii* is due to genetic aberrations in cis- or trans- regulatory controls of the heat shock protein (HSP) genes themselves, or disruption of post-transcriptional regulatory pathways that impact mRNA or protein half-life (Hofmann *et al.*, 2000; Place & Hofmann, 2005). Although transcriptomic and proteonomic studies have characterized temporal changes in the CSR to thermal acclimation in notothenioids, post-transcriptional regulation of mRNA half-life by small non-coding RNAs called microRNAs (miRNAs) has not been explored. In the last decade, miRNAs have emerged as key regulatory elements in controlling the differential expression of genes (Bartel & Chen, 2004; Bartel, 2004, 2009). For non-model organisms, miRNA research is still in its infancy; however, we do know that they are evolutionarily conserved and ubiquitous across all taxa. Given the limited knowledge surrounding the molecular mechanisms involved in the CSR of

Antarctic stenotherms, this study will make the first attempt to examine the regulatory role of miRNAs in *T. bernacchii* exposed to thermal stress. Therefore, investigating the differential expression of miRNAs in *T. bernacchii* during short and long term thermal acclimation may reveal underlying mechanisms that regulate the transcriptomic response seen in *T. bernacchii* with respect to the CSR. Additionally, this study may shed light on possible mechanisms that explain the loss of the heat shock response in these Antarctic notothenioids, and atypical CSR seen in this fish such as the sustained immune response and muted antioxidant response observed during chronic stress in *T. bernacchii*.

MicroRNAs have emerged as important players in post-transcriptional gene regulation in a broad range of animals, plants, protozoa and viruses. They are evolutionarily conserved, small (~21-24 nucleotides long) endogenous, non-coding RNAs that negatively regulate gene expression through sequence-specific interactions with the 3'untranslated regions (UTRs) of mRNAs and thereby cause mRNA destabilization and/or translation inhibition. These tiny molecules are intricately involved in regulating many cellular pathways that involve cell differentiation, proliferation, immunity, apoptosis and metabolism (Bartel, 2004, 2009; Bartel & Chen, 2004; He & Hannon, 2004; Yates *et al*., 2013; Moreno-Moya *et al*., 2014). In order to facilitate the understanding of how miRNAs negatively regulate gene expression, it is helpful to review miRNA biogenesis and structure. The biogenesis of miRNA is a multistage process (Figure 1), which begins in the nucleus with transcription by RNA polymerase II/III from intergenic or intragenic regions of the DNA to form a long primary miRNA (pri-miRNA) transcript containing secondary hairpin structure with length of 1-3 kb (Bartel 2004, 2009; Yates *et al*., 2013; Moreno-Moya *et al*., 2014). The pri-miRNA is then processed through a series of enzymatic cleavage steps that leads to the formation of a 60-70 nt double stranded pre-miRNA.

The pre-miRNA is exported from the nucleus and is further cleaved by the enzyme Dicer to form a 20-24 nucleotide (nt) double stranded miRNA duplex. One strand of the duplex is degraded, and the other strand (mature miRNA*) is incorporated into a complex of ribonucleic proteins to form the miRISC complex (miRNA induced silencing complex). However, it is important to note that the miRNA maturation process can produce distinct mature miRNAs from the 3′ and 5′ arms of the pre-miRNA duplex. Hence, miRNA nomenclature assigns a 3p or 5p suffix at the end of the name (Figure 1). The miRISC complex facilitates the complementarily binding of the 5' "seed" sequence of the mature miRNA to the 3' UTR region of target messenger RNA. This miRISC:mRNA interaction promotes mRNA degradation and/or induces translation inhibition which ultimately affects gene expression in the cell at the post transcriptional level.

MicroRNAs are implicated in regulating cellular stress response pathways in many organisms. For instance, gene deletions or inactivation of specific miRNAs reveal detrimental effects to the organism's phenotype during environmental stress. One such study subjected mir-7 knockout flies to temperature stress during development that resulted in malformed eyes (Li *et al.*, 2009); another study found that inactivation of mir-8 in zebrafish incapacitated their response to osmotic stress (Flynt *et al*., 2009). In plants, the expression of mir-393 was strongly upregulated in Arabidopsis exposed to chronic cold, desiccation and salinity treatments (Sankar & Zhu, 2004). Another study by Yin *et al* demonstrated how miRNAs can confer a cytoprotective role against tissue damage in mammals. Pre-treatment of mice hearts with exogenous miRNAs like mir-1, mir-21 and mir-24 protected cardiac tissue from injury brought on by ischemic stress. Moreover, the study also found corresponding increase in levels of Hsf1 and Hsp70 suggesting miRNAs can directly regulate the activation of Hsf1 and thereby the expression of Hsp70 (Yin *et al.,* 2009). Bioinformatic analysis predicts numerous miRNA target

sites in heat shock gene transcripts like Hsf1 (master regulator of the heat shock response) and many of the downstream proteins in the pathway (Place & Noonan, 2014). For instance, one study elucidating the cause of cardiac hypertrophy verified that mir-378 directly targets Hsf1 and represses its expression in murine cardiomyocytes (Yuan *et al*., 2010). The growing body of literature in miRNA regulation of cellular stress response suggests the characterization of thermally regulated miRNAs and investigating their potential role in cellular stress response of nototheniods is rather timely.

MiRNAs can regulate different biological processes through their interaction with their target genes that are involved in multiple signaling networks and metabolic pathways. Investigating the functional role miRNA differential expression can be challenging since a single miRNA can bind to hundreds of different mRNAs and thus, control the expression of many genes that participate in multiple biological pathways. Furthermore, a single gene can be under the control of many different miRNAs leading to regulatory complexity that rivals trans activation of transcription; a perturbation of a single miRNA can affect many biological functions (Bartel & Chen, 2004; Bartel, 2004, 2009; He & Hannon, 2004; Dong *et al*., 2013; Yates *et al*., 2013; Moreno-Moya *et al*. 2014,). Given the extremely large number of potential target sites that exists for any given miRNA, the process of experimentally validating a combinatorial matrix of potential miRNAs-mRNA interactions in the lab is time consuming and costly. Fortunately, computational tools have been employed successfully by miRNA researchers to quickly and accurately predict miRNA gene targets and determine their functional characteristics through their target genes. Once the miRNA gene targets are identified, their functional annotation can be determined using bioinformatic tools that perform useful integration and meta-analysis of curated pathway databases like GO and KEGG. This *in silico* approach

facilitates the investigative process by producing statistically significant results through gene enrichment analyses and thus narrowing down potential target sites for experimental validation. MiRNA research is still in its infancy for non-model organisms and the issue is further exacerbated by the lack of genomic data of Antarctic fish. Since miRNAs are conserved among species, I can utilize the genomic resource of the more established and curated zebrafish genome (*Danio rerio*) to perform the miRNA functional analysis in *T. bernacchii*. This conservation feature based on the highly conserved seed sequence shared by the two species will facilitate the computational search for miRNAs target genes based on homology to *D. rerio* miRNAs and gene targets (Bartel, 2009; Mott & Mohr, 2016).

In this study, I used next generation sequencing (NGS) to detect differentially expressed miRNAs across experimental conditions in *T. bernacchii* exposed to control and heat stress treatments over 7-day, 28-day and 56-day time points. I have also used a bioinformatic approach to characterize the gene targets of miRNA and their biological functions to gain insight into the cellular processes that are being differentially regulated in heat stressed and control fish by miRNAs. The aim of this study is to generate a complete miRNA transcriptome profile in *T. bernacchii* gill tissue and functionally characterize miRNAs that are differentially expressed (DE miRNAs) following heat stress at 7-day, 28-day and 56-day acclimations. In doing so, the study will explore the potential influence of miRNAs in various cellular pathways that exert early and late phase changes in the CSR and potentially gain more insight into the mechanisms affecting the alteration of the heat shock response in *T. bernacchii*.

Methods

Collection of fish

T. bernacchii were collected in McMurdo Sound, Antarctica in Fall 2012. They were caught using hook and line through holes drilled in the sea ice. Fish were then transported back to the laboratory and housed in flow through seawater aquaria maintained at ambient temperature (-1.5 °C). Fish were transferred to one of two experimental conditions, a control tank which was held near ambient temperature (-1.5 °C) and a high temperature treatment at 4 °C (n=15 fish per treatment). Fish were acclimated at aforementioned temperatures for a total of 56 days. Five fish from each treatment were removed after 7d, 28d, and 56d of acclimation to the thermal stress and humanely euthanized in MS-222. Gill tissues were then excised and immediately flash frozen in liquid nitrogen. All samples were stored at -80 °C until use. All procedures were approved by the Sonoma State University IACUC.

Total RNA extraction

For preparation of sequencing libraries, I extracted total RNA from n=4 fish per treatment per time point. Approximately 50 mg of frozen gill tissue was excised and homogenized in Trizol using automated tissue lyser according to manufacturer's recommended protocol. The quality of RNA extracts was performed by gel electrophoresis and peak analysis on an Agilent 2100 bioanalyzer. Only RNA samples with RIN (RNA integrity number) scores greater than 8 were submitted for sequencing.

Small Library Prep & Illumina Sequencing

The Illumina Truseq Small RNA library prep kit and size selection with the BluePippin automated gel extraction system was carried out to create small cDNA libraries by UC Berkeley Genome sequencing facility. The 50 bp SE small RNA libraries were indexed and then pooled prior to sequencing on 2 lanes of an Ilumina 4000 HiSeq platform which yielded approximately 10 million reads per sample.

Processing and Mapping Sequence Data

Raw sequencing reads were imported into the comprehensive CLC Genomics Workbench software for bioinformatic analysis and annotation of miRNAs. The data were first filtered by discarding low quality reads and reads <15 bases in length after adapter trimming. The filtered reads were mapped to known mature miRNAs and miRNA precursors in miRBase (v21.0) using default parameters to identify putative homologous miRNAs originating from the gill tissue of *T. bernacchii*. Read mapping was restricted to miRNA precursors in other fish species and prioritized based on phylogenetic relatedness. Species included in the read mapping were *Salmo salar* (ssa), *Ictalurus punctatus* (ipu), *Fugu rubripes* (fru), *Tetraodon nigroviridis* (tni), *Danio rerio* (dre), and *Oryzias latipes* (ola).

Mfold Secondary Structure: The putative microRNA sequences for *T. bernacchii* were further assessed using Mfold software to predict the secondary hairpin structure and evaluate its thermodynamic stability (Zucker, 2003). As the genome references of *T. bernacchii* are not available, this was accomplished by mapping the predicted phylogenetically conserved miRNA precursor sequence to genomic regions of *Notothenia corriceps*, the closest related species with an existing genome available at the time of this analysis. Putative pre-miRNAs with a folding threshold ≥-15 kcal/mol were considered unstable and removed from further analyses (Zhang *et al*., 2013).

Differential Expression of miRNAs: In addition to annotation of putative miRNAs, we performed a differential expression (DE) analysis of these miRNAs in control and heat stressed fish by first normalizing the reads in each sample denoted by tags per million (TPM). After normalization, we determined the DE of miRNAs between two experimental conditions, heat stress and control at each time point 7d, 28d, and 56d. CLC genomics workbench program uses a

weighted t-test (Baggerley's test) to identify differentially expressed genes (Baggerley *et al*., 2003). False discovery rate (FDR) corrected *p*-values were calculated using the Benjamini-Hochberg correction for multiple testing to determine the statistical significance of the changes in miRNA expression (FDR ≤ 0.05). Differentially expressed miRNAs were identified as upregulated or downregulated using fold change values to profile differences in miRNA levels between samples.

Prediction of miRNA target genes and functional analysis

To gain insight into the potential biological consequences of the underlying changes in miRNA expression identified in the DE analysis described above, I used a two-step approach commonly applied in similar studies (Nie *et al*., 2013; Jing et al., 2015; Das *et al*., 2015). First, I generated a gene set targeted by the DE miRNAs identified at each time point in the *T. bernacchii* gill samples. Next, I performed a functional enrichment analysis using Fisher's exact test for over-representation analyses.

Target gene prediction: The comprehensive miRNA target prediction tool DIANA-microT-CDS using annotated zebrafish miRNAs was applied to predict the target genes for newly sequenced miRNAs. The software incorporates multiple prediction algorithms and utilizes several stringent parameters to predict miRNA target genes based on target site conservation, degree of seed match complementarity in 3'UTR or non-canonical target sites, site accessibility of UTR and thermodynamic stability of miRNA:mRNA duplex. These parameters are individually calculated for each miRNA and the algorithm combines conserved and non-conserved microRNA recognition elements (MRE) into a final prediction score "miTG". The algorithm scores the strength of the predicted duplex interaction by assigning a miTG score between 0 and 1. Higher miTG scores correspond to higher precision with increased confidence

level of a correct prediction. The default cutoff of the program is using 0.8 score as a default threshold, which provides an average of 350 targets per miRNA. Furthermore, DIANA-microT is integrated into the miRNA pathway enrichment program DIANA- mirPath (Vlachos *et al*., 2015).

Enrichment Analysis of DE miRNA Target Genes: In order to gain an in-depth understanding of the potential impacts the changes in miRNA expression patterns could have in the gill cells of *T. bernacchii*, I used two separate enrichment pipelines: 1) Gene Ontology (GO) Functional Enrichment of DE miRNAs using PANTHER, and 2), the Kyoto Encyclopedia of Genes and Genomes (KEGG) Functional Enrichment of miRNA pathways using DIANA-mirPath v3.0 (Vlachos et al, 2015).

The Gene Ontology (http://www.geneontology.org/) is a database of known curated genes whose functional properties are represented by ontology of hierarchical controlled terms that fall under three broad domains, molecular function, biological process, and cellular component (GO Consortium, 2017). Molecular function describes the molecular activity of the gene such as binding or catalysis; the cellular component describes where the gene is located intra- or extracellularly; and the biological process encompasses the higher level functions of the gene with respect to its interaction network with other genes or molecules. GO is a widely accepted standard for annotation of genes. For this study, I am using GO-Slim which is a cut-down version of the GO ontologies as it provides a broad overview of GO content, rather than the full detail of highly resolved terms. The GO-Slim analysis is especially useful for summarizing the processes or functions mediated by groups of genes targeted by miRNAs. Therefore, GO-slim pipeline is useful for preliminary functional analysis of miRNA target genes.

In order to determine if the gene targets of our DE miRNAs are enriched in certain biological categories, we mapped these genes to known functional GO categories and pathways using the PANTHER Classification System that is currently part of GO interface (Huang *et al*., 2017). GO enrichment analysis was performed by mapping these target genes to the zebrafish reference genome. Statistical overrepresentation analysis was carried out using Fisher's exact test with FDR multiple test correction. GO is used here to obtain a comprehensive knowledge about genes targeted by DE miRNAs.

The KEGG pathway database is a comprehensive collection of manually drawn reference pathway maps representing experimental knowledge of gene interaction networks captured from literature (Kanehisa et al., 2017). Basically, genes are grouped based on their participation in the same biological process. Unlike the GO-Slim annotation analysis, KEGG pathway analysis allows for examining higher level systemic functions of the cell and organism as a whole. These high level cellular functions are represented by specific networks of gene interactions, and biochemical reactions related to cellular processes in the form of KEGG pathway maps. KEGG pathway analysis is used in my study to systematically understand the miRNA targeted pathways involved in cellular processes or organismal systems.

As such, I used DIANA- mirPath v3.0 program that utilizes KEGG database for pathway enrichment analyses of DE microRNAs; this indirect approach uses miRNA gene targets and compares them to pathways of protein coding genes (Vlachos et al, 2015). The miRNA target genes are cross-referenced with annotated zebrafish genes in curated KEGG pathways. More importantly, the KEGG pathways are tailored to only keep genes targeted by at least one miRNA in a given set of DE miRNAs. Through the miRNA-targeted pathway union analysis we can find significantly targeted pathways by our DE miRNAs of interest (Fisher Exact Probability Test,

$p < 0.05$). This analysis allows us to look for the functions or pathways on which miRNAs converge. The program enables the filtering of miRNA–gene interactions based on p-value < 0.5 for pathway enrichment analyses. The resulting p-value shows that the pathway is significantly enriched with gene targets from at least one selected miRNA in the list. Through sophisticated meta-analysis and clustering algorithms, DIANA- mirPath selects pathways that are significantly targeted concurrently by multiple miRNAs. Moreover, DIANA-mirPath offers visual representations of such functional characterization of miRNAs in the form of miRNA versus KEGG pathway heat map that characterize miRNAs which share similar enriched KEGG pathways. Note that we could only utilize dre-mir-21in the program and not its specific members (mir-21a/mir-21b) as DIANA- mirPath did not contain the known variants for the mir-21 family in zebrafish. Similarly, dre-mir-30b was not available in the program at the time of this study; therefore I substituted it for dre-mir-30a. It is well known that miRNA family members share either the full sequence conservation of mature miRNA or partial conservation of only the seed sequences at nucleotide positions 2-8 that primarily determine their biological function through complementarity binding with 3' UTR of mRNA; thus sequence variants within miRNA families are thought to regulate the same cellular processes since they share majority of their gene targets (Brennecke *et al*., 2005; Mott & Mohr, 2016).

Results

Identification of global and DE miRNAs in Trematomus bernacchii gill

Total RNA was extracted from 24 samples of *T. bernacchii* gill tissues excised from -1.5 °C control group and 4 °C heat stressed group of fish at various time points and subjected to next generation sequencing (see Methods). Next generation sequencing (NGS) resulted in ~725M raw reads that were generated from 24 samples using Illumina Hi Seq 4000. After adaptor trimming

and discarding low quality reads, the number of reads ranged from 11.9M to 22.3M reads per sample, corresponding to ~3.25M unique small RNA reads. Mapping the filtered dataset to the miRBase sequence database (v 21.0) resulted in 1,101 unique small RNA sequences mapping to known miRNAs.

Small RNA-sequencing resulted in a total of 125 distinct miRNAs in gill tissue. (Table 1.) These global miRNA expressions were further binned into broader miRNA families to assess their relative abundance in gill tissue. The relative distribution of these miRNA families showed that mir-146 (19%), mir-200 (14%), mir-21 (13%) are highly expressed in gill tissue followed by let-7 (7%), mir-203 (6%), mir-22 and mir-143 (4% each); (Figure 2). The remaining miRNAs (~30%) were subdivided between 49 additional miRNAs whose average relative abundance fell below 4%. In all, a total of 56 unique miRNA families are identified for the first time in *T. bernacchii* gill tissue. In addition to identifying the distribution of miRNAs in control samples of *T. bernacchii* gill tissue, I also identified DE miRNAs between control and heat stressed samples (Fig. 3). Most of these were mapped to *Salmo salar*, the closest available relative to notothenioids represented in the miRBase database. I identified a total of 11 miRNAs that were differentially expressed between control and heat stressed treatments at 7d and 28d time points (Table 2). There were no differentially expressed miRNAs at the 56d time point. During acute heat stress at the 7d time point, 9 miRNAs were down-regulated and 1 miRNA, mir-146a-3-5p, was up-regulated. We found only one miRNA family (mir-21) upregulated at 28d treatment (Table 2).

Thermodynamic stability of DE miRNAs using Mfold

In an attempt to further validate the sequenced *T. bernacchii* miRNAs were in-fact functional, I modeled the secondary structures of DE miRNAs using a RNA folding program

called Mfold. I found all of the DE precursor miRNA structures possessed thermodynamically stable hairpin loop structures characteristic of functionally expressed miRNAs, even at the experimental temperature of 4℃ (Fig. 3). The DE miRNAs exhibited very low minimum free energy (MFE) values that ranged from -68.9 kcal/mol to -35.5 kcal/mol. Table 3 lists the MFE values with corresponding mature miRNA sequence found in their hairpin structure: (i) mir-725 = -32.2 kcal/mol; (ii) mir-21 = -60.73 kcal/mol; (iii) let-7g = -62.5 kcal/mol; (iv) mir-22a = -68.9 kcal/mol; (v) mir-200a = -35.5 kcal/mol; (vi) mir-30b = -41.3 kcal/mol. (viii) mir-146a = -37.5 kcal/mol; (ix) mir-26a = -43.7 kcal/mol; (x) let-7a = -45.6 kcal/mol. Additionally, all 10 DE mature miRNA sequences are found within the stem region of the hairpin structure. The location of these mature miRNA sequences are highlighted in yellow corresponding to the correct 5p or 3p arm of the miRNA hairpin duplex (Figure 4a-4e).

Functional Enrichment of MiRNA target genes

In order to understand the biological functions of the differentially expressed miRNAs identified, I first predicted the potential target genes of these miRNAs using DIANA-microT-CDS. This returned a total of 1,627 miRNA target genes for our DE miRNAs filtered by default threshold of 0.8 < MiTG. I then performed an over-representation analysis of GO classification terms on this gene set to determine general cellular pathways targeted for regulation by DE miRNAs (Table 4). This was followed by an in-depth functional enrichment analysis of miRNA pathways using DIANA-mirPath to obtain a higher-level understanding of systemic functions of the cell and organism as a whole (Table 5).

GO Functional Enrichment of DE miRNAs - GO annotations for parent categories (molecular, cellular and biological) are organized at multiple hierarchical levels with smaller and more specific gene lists in lower levels of the hierarchy. We report the specific GO-Slim terms

that provides a broad functional overview of miRNA target genes in general categories relating to molecular, cellular and biological processes. Enrichment results are represented in tables for each GO parent category (Table 4). GO enrichment highlighted processes related to cellular stress response and were largely reflective of the higher order functions also identified in the KEGG analysis.

Molecular functions are highly enriched in GTPase, pyrophosphatase and kinase activity that drives many important cellular processes such as protein synthesis and translocation, intracellular trafficking, cytoskeleton organization, lipid metabolism, signal transduction, immune response and cell cycle (Table 4). Most of these GO biological processes such as intracellular signal transduction (1.5 fold enrichment), lipid metabolism (1.7 fold enrichment), cellular component morphogenesis (1.72 fold enrichment), cytoskeleton organization (1.85 fold enrichment) and response to stimulus (1.28 fold enrichment) are highly enriched in *T. bernacchii* transcriptome. In addition, GO enrichment identified key cellular processes involved in cellular remodeling activities (cytoskeleton organization, angiogenesis and cellular component morphogenesis) upregulated in *T. bernacchii* transcriptome during acute thermal stress (Table 4).

KEGG pathway enrichment analysis of DE miRNAs - Using the miRNA-targeted pathway union analysis in DIANA-mirPath, I found a total of 10 distinct KEGG pathways targeted by DE miRNAs and the corresponding miRNA target genes embedded within these pathways (Table 5). These significantly targeted pathways regulate changes in the *T. bernacchii* transcriptome such as lipid metabolism, cell cycle regulation, cellular remodeling, and immune response.

7d downregulated miRNAs- There are 6 KEGG pathways significantly (Fisher Exact Probability Test, $p < 0.05$) enriched with genes targeted by downregulated miRNAs for 7-day

acclimation period (Table 5a, Figure 5). The enriched miRNA pathways in this group include ECM-receptor interaction, mucin type O-glycan biosynthesis, Tgf-β signaling pathway, steroid hormone biosynthesis, SNARE interactions in vesicular transport and metabolism of xenobiotics. Figures 6, 7 & 8 illustrate the highlighted gene targets in the Toll-like receptor signaling pathway, Tgf-β signaling pathway and SNARE mediated vesicular transport, respectively. To further illustrate the functional relationship of differentially expressed miRNAs and their respective biological pathways, a heat map is generated showing the hierarchical clustering of DE miRNAs and their target pathways (Fig 5). MiRNAs are clustered based on shared pathway functions and pathways are clustered together by related miRNAs. For instance, mir-26a is shown to be involved in multiple pathways through its enriched target genes and it shares these pathways with other miRNAs like mir-30a and mir-203b (Table 5a).

Upregulated miRNAs: mir-146 & mir-21, at 7 day and 28 day respectively- The only upregulated miRNA at the 7d time point, mir-146a is enriched in 2 KEGG pathways: toll-like receptor signaling pathway (Fig. 6) and mucin type O-glycan biosynthesis (Table 5b). The 28d mir-21 upregulated miRNA is enriched in 3 KEGG pathways: FoxO signaling pathway (Fig. 9), gap junction and phosphatidylinositol signaling system pathways (Table 5c).

Despite using two distinct bioinformatic analyses to gain insight into the functional implications for the DE miRNAs identified in the heat stressed fish, there is remarkable overlap between the two with respect to the cellular processes being targeted for regulation by DE miRNAs in *T. bernacchii*. Table 6 summarizes the overlap between the biological functions identified in the GO classification analysis and the cellular functions identified in the KEGG enrichment analysis of pathways targeted by the DE miRNAs.

Discussion

MiRNAs are evolutionary conserved and their seed sequence motifs are highly identical even among distant species. This study represents the first characterization of the miRNA transcriptome from the cold stenotherm, *T. bernacchii*. A total of 125 homologous miRNAs representing 56 distinct families were identified in *T. bernacchii* gill tissue. In addition to the characterization of the global miRNAome, I also identified 12 miRNAs that displayed differential expression between heat stressed and control treatments at 7d and 28d time points. This suggests that miRNAs play an important role in temporally regulating the CSR of *T. bernacchii* during acute and chronic heat stress. My study found 8 DE miRNAs at the 7d time point and only 3 DE miRNAs (mainly mir-21 family members) at the 28d time point (Table 2). Interestingly, no DE miRNAs were observed at the 56d thermal acclimation time point. The absence of DE miRNA after 56d of acclimation suggests that the fish may have fully acclimated to the elevated temperatures and thus returned to basal level gene expression. These data coincide with gene expression data, biochemical analyses, and whole animal physiological measures that show notothenioids including, *T. bernacchii*, can re-establish cellular homeostasis after prolonged thermal acclimation (Enzor & Place, 2005; Franklin *et al*., 2007; Bilyk & DeVries, 2011; Bilyk *et al*., 2012; Almroth *et al.*, 2015; Huth & Place, 2016a). Unlike the largely distinct DE mRNA changes seen between 7d and 28d acclimated fish in previous transcriptomic studies, the highly expressed miRNA families mir-146, mir-200, mir-21, let-7 and mir-203 are differentially expressed both in the acute (7d) and chronic (28d) heat stress.

MiRNA precursors fold into a thermodynamically stable secondary structure that is important for maturation process by Dicer and ultimate incorporation of the mature miRNA strand into argonaute macromolecule complex (Bonnet *et al*., 2004). Since possessing a stable secondary structure is vital for subsequent miRNA function, I determined the minimum free

energy (MFE) values to evaluate the thermodynamic property of DE miRNA precursor structures at 4℃, the temperature at which they are differentially expressed. The MFE values for all ten miRNAs were measured between -68.9 kcal/mol and -35.5 kcal/mol, well below the -15 kcal/mol MFE threshold commonly used to differentiate stable and unstable miRNAs (Table 3, Figure 4a-4e). These low MFE values suggest that these putative miRNAs are thermodynamically stable and functional for regulating CSR at 4℃ acclimation in *T. bernacchii*. Furthermore, support for the validity of the annotation is provided by the predicted models that place the ~22 nt mature miRNA sequences on the same arm (either the 5p arm or 3p) of the stem region in the overall hairpin structure as was originally predicted by the annotation (Figure 4a-4e). Taken together, these results provide solid evidence to suggest the identified small RNA sequences obtained in this study characterize the first set of miRNA homologs described in an Antarctic notothenioid fish.

Since the current study is focused on the examination of miRNA regulation of the cellular stress response in *T. bernacchii*, it is worth reviewing the results generated from the complimentary Huth & Place (2016a) transcriptomic study of *T. bernachhii* gill tissue that examined the synergistic effects of high temperature and low pH on gene expression. Acute exposure to heat stress after 7 days showed expression patterns that supported increased lipid peroxidation, robust inflammatory response and a down-regulation in cell growth and proliferation-associated genes, an increase in redox response and induction of apoptosis. However, over a longer acclimation period (28d and 56d), these responses were diminished or muted as the expression pattern returned to basal levels. This study not only demonstrated that Antarctic fish are capable of acclimation to increased temperatures but also show how the cellular response of *T. bernacchii* to thermal stress is manifested in two phases, an acute cellular

stress response lasting between 7 and 14 days followed by a longer-term, more chronic response occurring around 28 days of acclimation. These responses largely track the acute cellular stress response (CSR) and cellular homeostatic response (CHR) described by Kültz (2005) and thus I will refer to these responses as such from here on out.

Acute Phase CSR

As noted previously, *T. bernacchii* showed a robust and coordinated CSR to an acute (7d) cellular stress. The acute CSR is characterized by increase in antioxidant enzymes and pro-inflammatory cytokines, apoptosis, cell-cycle arrest, tissue repair and remodeling. In this study, the short term CSR appears to be predominantly mediated by the downregulation of several miRNAs and only one upregulated miRNA, mir-146 in 7d acclimated fish (Table 2). These DE miRNAs are enriched in pathways that functionally overlap to mediate the CSR in several biological processes such as cell proliferation, cell differentiation, cell cycle regulation, apoptosis, immune function, signal transduction, cellular trafficking, cytoskeletal restructuring, and extracellular matrix (ECM) interaction and remodeling.

Fine tuning the immune response:

MiRNA regulation of the immune system has been extensively studied in higher vertebrates such as mammals but little is known about their function in teleost immune response (O'Connell *et al*., 2013; Han *et al.*, 2016; Andreassen & Hoyheim, 2017). However, few studies have confirmed a subset of miRNAs that are consistently involved in other teleost immune system following bacterial and viral challenges (Zhang *et al.*, 2014; Schyth *et al.*, 2015; Lie *et al.*, 2016; Andreassen & Hoyheim, 2017). At the level of the transcript, it is well characterized that Antarctic fish launch a sustained cellular inflammation response when exposed to elevated temperatures that damage cells and tissues (Chen *et al.*, 2008; Thorne *et al.*, 2010; Huth & Place,

2013; Windisch *et al.*, 2014; Huth & Place, 2016a, 2016b). Transcriptomic analysis of *T. bernacchii* acclimated to stressful conditions for 7d showed that these fish strongly activate inflammation pathways by upregulating pro-inflammatory signaling molecules like the CXCL family of chemokines that recruit immune cells and mediate inflammation (Huth & Place, 2016a). Functional enrichment analysis found the set of genes targeted by miRNAs DE at 7d were significantly enriched for toll-like receptor and mucin type O-Glycan biosynthesis pathways which are involved in regulation of the immune response (Table 4b).

Toll-like receptor (TLR) pathway- The DE miRNA-146a is functionally enriched for the Toll-like receptor (TLR) pathway and has been shown to regulate the immune response in other fish species such zebra fish, Japanese flounder, rainbow trout, and snakehead fish (Ordas *et al.*, 2013; Zhang *et al.*, 2014; Schyth *et al.*, 2015; Liu *et al.*, 2016). Furthermore, studies have documented the negative regulation of immune response by upregulation of mir-146a, which targets immune gene activators in the TLR pathway (Bi Y *et al.*, 2009). Toll-like receptors are expressed on diverse immune cells and function as primary sensors of cellular stress in form of pathogen infection or cellular damage caused by abiotic stressors (Han *et al.*, 2016). They activate downstream signaling effectors that elicit an innate immune response by producing inflammatory cytokines like type I interferon (IFN), and other immune system mediators (Moresco *et al.*, 2011; Han *et al.*, 2016). In this study, traf6 (TNF receptor-associated factor 6) was found to be an enriched gene target for mir-146a (Figure 6, Table 5b). Studies in higher vertebrates have shown that miRNA-146a participates in the negative regulation of the toll-like receptor pathway. The miRNA is shown to downregulate its target genes like TRAF6 and IRAK1, which ultimately leads to decreased expression of pro-inflammatory cytokines and an

attenuated immune response in human and murine cells (Hou *et al.*, 2009; Bi Y *et al.*, 2009; Saba *et al.*, 2014).

Another enriched gene target of mir-146a is pik3c (phosphatidylinositol 3-kinase), member of the pi3k family that participates in multiple signaling networks that control diverse cellular processes such as cell growth, differentiation, proliferation, survival, immunity, and intracellular trafficking (Koyasu, 2003). Phosphatidylinositol 3-kinase is implicated in three different pathways, toll-like receptor, phosphatidylinositol signaling system and FoxO signaling pathway and is targeted by both mir-146a and mir-21, albeit at different time points (Table 5). In the toll-like receptor pathway, pik3c activates Akt, an intermediary kinase that induces expression of inflammatory cytokines (Koyasu, 2003). Similar to traf6 regulation, upregulation of mir-146 during acute heat stress would inhibit expression of pik3c thereby decreasing the inflammatory response. Figure 6 illustrates the pathway involving Traf6 and pik3c as potent activators for pro-inflammatory cytokine expression in toll like receptor signaling pathway. In addition, the overall immune suppression by upregulated mir-146a coincides with the documented upregulation of immune cytokine suppressors such as members of SOCS (suppressors of cytokine signaling) family in *T. bernacchii* exposed to cellular stress for 7d (Huth & Place, 2016a). This gene family is known to suppress immune responses in fish by inhibiting the JAK/STAT pathway (Phillip & Vijayan, 2015). Given its previously established role, it is reasonable to assume that the upregulation of mir-146a observed in this study plays a temporal role in buffering the heightened immune response previously observed during an acute stress as means to titer the inflammatory response which can be harmful to the host if left unchecked (Bi Y *et al.*, 2009; Saba *et al.*, 2014; Andreassen & Hoyheim, 2017).

Mucin type O-Glycan biosynthesis pathway- In eukaryotes, the two major forms of protein glycosylation occurring on secretory and membrane bound proteins are N-linked and O-linked; of which the most abundant, ubiquitous and highly conserved is mucin type O-glycosylation. Mucin type O-glycans modulate cellular recognition, adhesion, migration and extracellular communication between cells and the extracellular matrix in mostly every biological system including immune system, digestive, circulatory and respiratory system (Yang & Liu, 2003; Tian & Hagen, 2009; Herzog *et al.*, 2014; Staudacher, 2015). For instance, O-glycans are well characterized in angiogenesis and vascular function. This may explain why angiogenesis is highly enriched GO-Slim term in biological process (Table 4). Endothelial cells of vascular networks express high levels of O-glycans to facilitate adhesion and trafficking of leukocyte during inflammation (von Andrian, 2003; Herzog *et al.*, 2014).

Downregulation of mir-26a and mir-30a in 7d acclimation positively impacts the mucin-type O-glycan synthesis pathway by elevating the role of specific members of their enriched target genes called galectins that can be found in the nucleus, cytoplasm, outer plasma membrane, and extracellular matrix. Gelactins are a large family of glycan binding proteins that function intracellularly or extracellularly (Yang & Liu, 2003). In the latter, these proteins interact and form complexes with cell-surface and extracellular matrix glycan conjugates (glycoproteins and glycolipids) which allows them to induce a broad variety of cellular responses such as proliferation, cell adhesion, migration, cell motility, and apoptosis. One of the major functions of galectins is to regulate immune and inflammatory responses in mammalian systems (Kuwabara *et al.*, 2002; Liu & Rabinovich, 2010). The sheer number and types of gelactins can induce either pro or anti-inflammatory responses depending on immune cell type and its cell surface glycoproteins. In this study, specific members of galectin superfamily gelactin2 and

gelactin7 are predicted gene targets of mir-26 and mir-30. The downregulation of these miRNAs intensifies the functional role of gelactin2 and gelactin7 as negative regulators of the immune response. Unfortunately, there is limited knowledge of these specific galectins in teleost species. However, they have been implicated in p53 mediated apoptosis and cellular repair in higher vertebrates (Kuwabara *et al.*, 2002). Galectin2 induces T-cell and neutrophil apoptosis as well as decreases interferons and tumor necrosis factors while at the same time increases interleukins 5 & 10 which are anti-inflammatory cytokines. Galectin7 is shown to induce apoptosis of tumor cells and inhibit cellular growth through p53 activation (Kuwabara *et al.*, 2002). In fact, the role of pro-apoptotic galectins in this pathway coincides with the *T. bernacchii* transcriptome results that showed elevated apoptotic levels at 7d thermal acclimation (Huth & Place, 2016a).

Interestingly, the overall induction of the anti-inflammatory response by mir-26 and mir-30 plays a synergistic role with mir-146 to dampen the immune response during 7d acute stress. At first glance, these miRNA pathway effects do not appear to coincide with the robust immune response documented for *T. bernacchii* during acute thermal stress (Huth & Place, 2016a). Although *T. bernacchii* sustains a heightened immune response throughout longer acclimation periods, maintaining an elevated immune response over time can be potentially harmful and energetically costly to an organism coping with chronic stress (Bi Y *et al.*, 2009; Saba *et al.*, 2014; Andreassen & Hoyheim, 2017). Thus, it is necessary to modulate the immune response by having regulatory mechanisms to offset the response in terms of bio-energetic costs and preventing cellular damage. Hence, miRNAs (like mir-146, mir-30 and mir-26) antagonize the expression of immune gene activators and in doing so, act as fine-tuning knobs to control a robust immune response in order to buffer against the damaging effect of pathological inflammation (He & Hannon, 2004; Franceschetti *et al.*, 2014; Saba *et al.*, 2014; Gulyaeva &

Kushlinskiy, 2016; Bracken *et al.*, 2016; Andreassen & Hoyheim, 2017). In all, upregulated mir-146 and downregulated mir-26 & mir-30 can concurrently attenuate the inflammatory response by targeting positive and negative regulators of the immune system pathway. Moreover, the hallmark of the CSR is evident here since miRNA modulation of the energetically costly immune response can help the cell distribute the energy budget towards essential pathways necessary for cellular maintenance and survival.

Lipid Metabolism:

Previous studies have shown lipid metabolism plays a critical role in the cellular stress response of notothenioid fish during acute heat stress (Chen *et al.*, 2008; Buckley & Somero, 2009; Huth & Place, 2013; Huth & Place, 2016a, 2016b). I also found target genes associated with lipid metabolism and were enriched in the gene set associated with DE miRNAs in this study (Table 5a). In particular, the GO sub-class of lipid metabolism appears to be heavily represented by two significantly enriched KEGG pathways targeted by downregulated miRNAs in the 7d +4°C acclimated fish: steroid hormone biosynthesis and xenobiotic metabolism which are associated with genes targeted by mir-26a, mir-30a & mir-203b (Table 4a).

Metabolism of xenobiotics pathway- The miRNAs mir-26a and mir-203b, downregulated in 7d +4 °C acclimated fish, specifically target glutathione S-transferases (GST) and UDP-glucuronosyltransferases (UGT) that play an important role in detoxification of exogenous compounds such as drugs, environmental pollutants and in metabolism of endogenous compounds such as steroid hormones, fatty acids, vitamins, and bilirubin (Nebert *et al*., 2004; Rowland *et al*., 2013). Both GSTs and UGTs function by conjugating glutathione or glucuronic acid, respectively, with toxic substances like reactive oxygen species (ROS) that results in these substances being less reactive, thus facilitating their excretion from cells and the organism. For

instance, GSTs are directly involved in protecting cells against oxidative stress (Sies, 1999). They help detoxify highly reactive lipoperoxidation generated by products through conjugation of GSH with lipid peroxides, thus facilitating their excretion. Previous enzymatic studies have documented the antioxidant systems in Antarctic fish (Ansaldo *et al.*, 2000; Chen *et al.*, 2008; Mueller *et al.*, 2011; Machado *et al.*, 2014; Almroth *et al.*, 2015; Enzor & Place, 2015; Klein *et al.*, 2017). Two relevant studies in notothenioids specifically measured GST activity level in *Notothenia coriiceps* and *Notothenia rossii* (Machado *et al.*, 2014; Klein *et al.*, 2017) and found GST acting to reduce ROS in these fish; however antioxidant enzymes like superoxide dismutase (SOD), catalase and GST transcript levels remained unchanged in response to acute heat stress after 6 days (Machado *et al.*, 2014). Contrary to these findings, Almroth *et al* (2015) showed elevated levels of GST and antioxidant activity in *Pagothenia borchgrevinki* exposed to acute heat stress. Additionally, GST transcript levels were also shown to be elevated in response to oxidative stress in the Antarctic eelpout, *Pachycara brachycephalum* (Windisch *et al.*, 2014). Previous studies also show that *T. bernacchii* accrues significant oxidative damage after a 7d acclimation to +4 °C (Enzor *et al.*, 2014) and like other notothenioids, *T. bernacchii* mounts an effective antioxidant response after an acute thermal insult (Almroth *et al.*, 2015; Enzor & Place, 2015; Huth & Place, 2016a; Klein *et al.*, 2017; Pierson & Place, in prep). GSTs provide an effective protection against cellular stress by combatting harmful xenobiotics or endogenously generated toxic metabolites like ROS that can damage DNA and other cell constituents (Josephy & Mannervik, 2006). Thus, downregulation of mir-26 and mir-30 in these fish likely acts synergistically with other regulatory changes in gene expression to strengthen the cytoprotective effect of GSTs and UGTs against oxidative stress and toxic metabolites during acute thermal acclimation of *T. bernacchii* at 7d.

Steroid hormone biosynthesis- The xenobiotic metabolic pathways described earlier are also linked to steroid hormone anabolic pathways as they share similar enzymes involved in lipid metabolism. The enriched gene targets of mir-26, mir-30 & mir-203 miRNAs are mostly steroidogenic enzymes such as cytochrome P450 enzymes (CYP family), steroid-5-alpha-reductase, and 17-beta-hydroxysteroid dehydrogenase (17-HSD) (Table 4a). UGT, 17-HSDs and steroid-5-alpha-reductase participate in steroid metabolism and control concentrations of steroids by catabolism and removal of steroids in tissues; thus affecting intracellular concentrations of steroids as a means to modulate hormone response (Hum *et al*., 1999; Mindnich *et al.*, 2004). The CYP super family of enzymes are monooxygenases involved in diverse functions that mainly include xenobiotic and lipid metabolism. The specific members of the CYP super family (CYP11A and CYP19A) targeted by DE miRNAs identified in this study are mainly involved in steroid hormone synthesis. For instance, CYP11A is involved in catalyzing the first step for steroidogenesis that is converting cholesterol into pregnenolone, a precursor of all other steroid hormones including progestogens, androgens, estrogens, glucocorticoids, and mineralocorticoids (Nebert & Dalton, 2006). In fish, glucocorticoids like cortisol serve important functions in osmoregulation, growth, and metabolism (McCormick, 1995, 2001; Takahashi & Sakamoto, 2013). For instance, *P. borchgrevinki* showed elevated levels of cortisol to mitigate effects of temperature stress (Ryan, 1995). In osmoregulation, gills are the main organ for exchanging ions between the extracellular fluid and the environment (McCormick, 2001). Cortisol has been shown to stimulate activity of Na^+/K^+ ATPase in gills of multiple species of teleost such as tilapia and Atlantic salmon (McCormick, 1995; Pelis & McCormick, 2001). Cortisol is also shown to increase Na^+/K^+ pumps in chloride cells of tilapia and salmon gills (Dang *et al.*, 2000). Interestingly, *T. bernacchii* does not exhibit elevated cortisol levels when exposed to chronic

thermal stress for 28 days (Hudson *et al.*, 2008), however they demonstrated a robust gene response associated with maintaining acid–base homeostasis (Huth & Place, 2016a). While *T. bernacchii* does appear to increase Na^+/K^+ pump activity, this appears to be achieved through isoform switching of the γ-subunit and not increases in the number of Na^+/K^+ pumps (Enzor & Place, in prep). With regards to temperature insensitivity of cortisol levels reported in *T. bernacchii*, I postulate that since cortisol is known to suppress immune functions in many species including fish (Phillip & Vijayan, 2015), maintaining low levels of cortisol help sustain an elevated immune response in *T. bernacchii* during chronic heat stress as previously described (Huth & Place, 2016a).

Besides serving as hormonal mediators in osmoregulation and immune system, certain steroids are helpful in "homeoviscous adaptation" that requires adjustment to membrane fluidity and structure of cell membrane (Hazel, 1995). Hence fatty acids like steroids are crucial for maintaining the structure and function of cell membranes. For instance, Antarctic fish, *Harpagifer antarcticus* exposed to acute heat stress showed upregulation of steroidogenic enzyme C-4 methylsterol oxygenase involved in ergosterol biosynthesis and ergosterol (Thorne *et al.*, 2010). In their study, Thorne *et al* discussed the importance of ergosterol in regulating the fluidity and permeability of membrane in hyperthermia. Given the cellular functions of these target genes for mir-26, mir-30 and mir-203, it appears as though the downregulation of these miRNAs are promoting broad endocrine functions in the fish by driving steroidogenic activity to regulate hormone and steroid levels that affect osmoregulation, immune response, reproduction, lipid metabolism and plasma membrane homeostasis.

Cellular remodeling & transport:

ECM-receptor Interaction Pathway- Cell adherence to the extracellular matrix (ECM) is required for normal cell growth, proliferation and survival and is known to be an important component of the CSR as cells maintain and constantly remodel ECM in response to stress or injury (Stamenkovic, 2003; Kültz, 2005; Takai *et al.*, 2011). Accordingly, a transcriptomic study in thermally stressed *Gillichthys mirabilis* showed upregulation of genes like integrin binding protein that are important in mediating signals from cell–cell and cell–matrix interactions to intracellular signaling cascades (Buckely *et al.*, 2006). In this study I found miRNAs from the let-7 superfamily are greatly downregulated and likely exert a potent effect on the ECM by intensifying the role of its gene targets that belong to the collagen family of proteins as well as thrombospondins found in the ECM. Collagen fibers provide structural integrity and mechanical strength to connective tissues. Thrombospondins guide ECM synthesis and tissue remodeling by facilitating proper assembly of collagen fibers (Kemin & Lawler, 2009). This coincides with Antarctic fish transcriptomic studies that show extensive tissue remodeling that occurs during acute thermal stress (Buckley & Somero, 2009; Thorne *et al.*, 2010; Huth & Place, 2016a) For instance, Thorne *et al* (2010) discussed that collagen and actin genes are upregulated in acutely heat stressed transcription profile of *H. antarcticus* in an effort to repair structural damage to cells and tissues caused by high temperature stress. In another study, transcriptomic analysis of notothenioid, *D. mawsoni* showed the overexpression of matrix metalloproteinases MMP9 and nephrosinare involved in extracellular matrix remodeling in these cold adapted fish (Chen *et al.*, 2008; Stamenkovic, 2003). In a non-polar system, *Cyprinus carpio* transcriptome exhibited a coordinated expression of sarcomere structural proteins (i.e. myosin, actin, tropomyosin) to promote extensive muscle tissue remodeling in this fish (Gracey *et al.*, 2004). Therefore, the GO

enrichment analysis shows a 1.7 fold in cellular component morphogenesis which is likely attributed to modulation of the ECM interaction pathway (Table 5).

Vesicular trafficking: Two of the DE miRNAs identified in this study, miR-203b and mir-725, are involved in the regulation of mRNAs associated with SNARE complex machinery that facilitates vesicular transport and fusion; vesicular transport was also found to be enriched in the GO functional analysis (Table 5a, Fig. 7). SNAREs play important roles in mediating vesicle fusion in a plethora of important cellular processes such mitosis, synaptic transmission in neurons, cell signaling and apoptosis (Söllner *et al*., 1993; Rothman, 1994; Chen *et al.*, 1999; Chen & Scheller, 2000; Jahn & Scheller, 2006). The process of fusing membranes is energetically costly as it requires bringing together two membranes to overcome the repulsive electrostatic forces between the membranes. This membrane fusion is mediated by Rab-GTPases that also initiate signaling pathway that leads to formation of the SNARE complex (Jahn & Scheller, 2006). In fact, the GO function analysis performed on the gene targets from DE miRNAs in this study revealed enrichment of GTPase activity and Rab genes were shown to be upregulated in *Gillichthys mirabilis* gill tissue during acute heat stress (Buckley *et al.*, 2006).

The gene targets of miR-203b, syntaxins stx1 and stx6, facilitate correct interaction of a membrane with one or more SNAREs on another membrane to mediate membrane fusion (Chen & Scheller, 2000; Jahn & Scheller, 2006). Meanwhile, the mir-725 target, Sec22 homolog B, is a vesicle trafficking protein that complexes with SNARE and participates in trafficking proteins between the ER and Golgi (Figure 7). The gene Vti1b mediates vesicle transport through interaction with t-SNAREs. BNIP1 participates in the formation and maintenance of ER membrane through membrane fusion and budding events, but not in membrane trafficking between the ER and Golgi (Nakajima *et al*., 2004). Taken together, these miRNA target genes

enhance cellular trafficking process through membrane fusion and budding; processes that are vital for all facets of protein function during CSR. In a transcriptomic study of rainbow trout gill tissue showed increased expression of cluster of genes related to vesicle-mediated transport during heat stress (Rebl *et al.*, 2013).

Cell cycle Regulation & Apoptosis:

Tgf-β signaling pathway- The transforming growth factor (Tgf-β) signaling pathway exerts control over a broad spectrum of cellular processes through its interaction with multiple signaling pathways. As such, Tgf-β pathway regulates fundamental cellular processes, including cell growth, cell proliferation, differentiation, apoptosis and myriad of other cellular functions (Lutz & Knaus, 2002; Huang & Chen, 2012). The overall biological effect of Tgf-β signaling pathway results in prevention of cell growth and proliferation by promoting cell cycle arrest and apoptosis. In this study, the Tgf-β signaling pathway is exclusively enriched by mir-26 target genes (Table 5a). Most of the mir-26 enriched target genes are members of the Bambi (BMP and activin membrane-bound inhibitor) and Inhibin gene family. Both gene families antagonize the function of Tgf-β signaling pathway resulting in cell growth and proliferation (Fig. 8). Bambi functions as a decoy or pseudo receptor that prevents Tgf-β growth factors from forming active receptor complexes upon ligand binding (Onichtchouk *et al*., 1999). Inhibins, as the name suggests, antagonizes activin and Bmp activation of Tgf-β through competitive inhibition (Fig. 5).

As a result of antagonistic impact of downregulated mir-26 on Tgf-β signaling pathway, it appears that cell growth and proliferation is promoted during acute heat stress in *T. bernacchii*. This inference is contrary to the cumulative evidence that show notothenioid generally exhibit cell cycle arrest that inhibited cell growth and proliferation and promoted apoptosis during acute

thermal stress (Chen *et al.*, 2006; Buckley & Somero, 2009; Slead & Buckley, 2013; Bilyk & Cheng, 2014; Huth & Place, 2016a). For instance, cell cycle genes were shown to be downregulated by second day of thermal stress in *P. borchgrevinki*, suggesting slowing of cellular proliferation and cell cycle arrest in this bald notothen (Bilyk & Cheng, 2014). Furthermore, during acute heat stress, *T. bernacchii* promoted cell cycle arrest and apoptosis as evidenced by upregulation of anti-proliferative transcription factor C/EBP-δ (Slead & Buckley, 2013). Interestingly, *T. bernacchii* transcriptomic responses show strong downregulation in bmp, and Tgf-β (Huth & Place, 2016a) that would promote for cell proliferation. This further coincides with upregulation of PCNA (cell proliferative marker) transcript levels in the gill tissue of *T. bernacchii* after a 7d acclimation to thermal stress (Huth & Place, 2016a). Apparently, there is a contradictory outcome of seeing upregulation of anti-proliferative C/EBP-δ (Slead & Buckley, 2013) and upregulation of PCNA (Huth & Place, 2016a). However, due to the pleiotropic effects of PCNA, it could be acting in another capacity such as DNA repair (Huth & Place, 2016a). Further investigation is warranted to elucidate precise mechanisms that are responsible for the heat induced PCNA and C/EBP-δ expression seen in Antarctic fishes.

In regards to miRNA regulatory role, I can only postulate that since gill tissues are directly exposed to environmental perturbations, mir-26 probably exerts a more positive influence on the Tgf-β pathway as an extensive effort to mitigate tissue damage by favoring growth and cell proliferative pathways during acute thermal stress. Immune relevant genes might also trigger cell proliferation of immune and endothelial cells as shown in transcriptome of gill tissue of rainbow trout exposed to heat stress (Rebl *et al.*, 2013). Additionally, the transcriptome profiling of gill tissue in *Gillichthys mirabilis* revealed that cell proliferation is favored during acute heat stress (Buckley *et al.*, 2006). The study showed upregulation of cell cycle promoters

such as F-box protein, SCF boxes, and cullin-3 work together to promote cell cycle progression through cyclin degradation. Taken together, the role of downregulated mir-26 in this pathway suggests that cell growth and proliferation may still be promoted at some level to counteract the extensive temperature induced apoptotic pathways that would impede tissue growth, repair and remodeling.

The aforementioned conflicting outcomes are further confounded by the paradoxical role of mir-26 in regulating this pathway through the opposing effects of its target genes. Mir-26 also targets a Smad protein that positively regulates the Tgf-β signaling pathway (Figure 8). To explain the contradictory nature of mir-26, I hypothesize that the miRNA probably exerts control of Smad concentration in cells as a way to fine tune the overall signaling strength in the pathway with respect to its overlapping role in other pathway interactions. In fact, nuclear concentration of Smad is shown to affect the specificity of the transcriptional outcome in Tgf-β pathway (Wran, 2000; Huang & Chen, 2012; Chris L *et al.*, 2017). Therefore, it is possible that mir-26 is controlling the levels of Smad proteins to influence the biological outcome of the Tgf-b pathway. In all, the contradictory outcomes of mir-26 target genes complicate the analysis of the regulatory role of mir-26 in Tgf-β signaling pathway. As a result of the overwhelmingly complex pathway interactions and the limited knowledge of molecular regulation of Tgf-β, it is challenging to tease apart the precise functional role of miRNAs in such pathways without further investigation. Due to the broad and complex nature of miRNA signaling pathways where a given miRNA can target both activators and antagonists in a pathway, miRNA pathway effects in my study may not be easily reconcilable with phenotypic effects seen in literature cited herein. Therefore, further experimental based studies are warranted to elucidate the precise miRNA regulation of pathways.

Chronic Phase CHR

Gap Junction Pathway- Gap junctions are a family of proteins called connexins that form a channel between adjacent cell membranes to allow the intercellular transfer of many small molecules like ions, secondary messengers and metabolites (Musil *et al*., 2000; Salameh, 2006). They have many pleotropic cellular functions that include cell-to-cell communication in autocrine and paracrine signaling pathways, propagation of ions in excitable cells among others. Due to their structural domains, connexins also exhibit channel independent functions that include cell adhesion, cytoskeleton interactions and activation of intracellular signal transduction pathways (Martin *et al*., 2001) Cellular stress can affect the abundance of functional gap junctions which in turn can effect intercellular communication, nerve conduction, ion exchange and other related processes that depend on functional gap junction channels (Laing *et al.*, 1998; Berthoud *et al.*, 2004). As a result, the CSR pathways can regulate the abundance of gap junctions through the regulation of connexin synthesis or degradation as means to achieve homeostasis (Musil *et al*., 2000; Salameh, 2006). In my study, mir-21 upregulation at 28 days negatively impacts the expression of connexin proteins which may act to reduce the abundance of gap junction channels in cell membranes. This antagonistic effect of mir-21 on gap junction connexins may mark the transition from the acute response discussed above and a return to homeostasis as indicated by the return to a basal level of expression seen in *T. bernacchii* at 28d acclimation (Huth & Place, 2016a).

Similarly, upregulation of mir-21 may also be acting to attenuate the phosphatidylinositol signaling system that control diverse cellular processes such as cell cycle regulation, immunity, cytoskeleton organization, membrane restructuring and intracellular trafficking (Berridge, 1995; Di Paolo & De Camilli, 2006; Liu & Bankaitis, 2010). Cytoskeleton organization and membrane

restructuring has been particularly well documented in fish acclimated to elevated temperatures. For instance, rainbow trout transcriptome of gill tissue showed upregulation of several cytoskeletal genes such as actin, tropomyosin, calmodulin during heat acute heat stress (Rebl *et al.*, 2013). Furthermore, transcriptomic studies in *T. bernacchii* show the fish also undergoes cytoskeleton restructuring and reorganization after acute stress (Buckley & Somero, 2009; Huth & Place, 2013, 2016a). However, the upregulation of miR-21 during chronic heat stress would in effect dampen the cellular effects of phosphatidylinositol signaling system, attenuating intracellular trafficking and remodeling activities. These activities were shown to be positively regulated by DE miRNAs during acute CSR in 7d acclimated specimens of *T. bernacchii* (Huth & Place, 2016a). However, at longer acclimation periods, cellular trafficking and remodeling activities drop off as these fish transition from the CSR to the CHR after 28d of acclimation.

FoxO signaling pathway- It is well documented that cellular stress elicits apoptotic pathways during environmental stress in many organisms (Kültz, 2005). One of the enriched pathway for mir-21 is the FoxO signaling pathway (Fig. 9) that involves a family of forkhead transcription factors (FoxO) which play a crucial role in many cellular processes regulating cell fate such as apoptosis, cell cycle progression, cell differentiation, DNA repair, oxidative stress and glucose metabolism (Accili & Arden, 2004; Greer & Brunet, 2005). The transcriptional activity of FoxO activates expression of other genes that induce cell cycle arrest and promote autophagy as well as confer oxidative stress resistance by inducing expression of antioxidants like SOD and catalase. For instance, FoxO serves as an important pro-apoptotic signal that activates transcription of genes such as FasL, a death ligand for the cell death pathway in response to damaged cells. As a result, FoxOs are also well established tumor suppressors in cancer biology by inhibiting cell proliferation processes and promoting apoptosis. Similarly,

cancer research has also established mir-21 as an anti-apoptotic oncogene that targets a variety of apoptotic factors and is evidently found to be overexpressed in many cancers (Pfeffer *et al*., 2015).

Relative to this study, the upregulation of miR-21, which implies that FoxO expression should be repressed in *T. bernacchii* after 28 days of thermal acclimation, suggests the gill tissue of *T. bernacchii* is returning to a more basal level of growth and proliferation. This data substantiate much of the transcriptomic data collected from prior studies. During acute heat stress, notothenioids generally exhibit cell cycle arrest and promote pro-apoptotic pathways (Chen *et al.*, 2006; Buckley & Somero, 2009; Slead & Buckley, 2013; Slead *et al.*, 2014; Bilyk & Cheng, 2014; Huth & Place, 2016a). However, during long-term chronic stress, these apoptotic pathways as well as antioxidant activity are downregulated as the organism transitions from an acute CSR to a more basal level of expression associated with the CHR (Bilyk & Cheng, 2014; Enzor & Place, 2014; Huth & Place, 2016a; Almroth *et al.*, 2015). Consequently, the upregulation of miR-21 observed in this study provides a potential regulatory link between the transition in cellular fates observed in *T. bernacchii* after homeostasis is reacquired. With the inclusion of these data, there is large agreement across multiple levels of biological organization that suggests *T. bernacchii* requires upwards of 28 days to attain a new state of relative homeostasis after acclimation to elevated temperatures.

In addition to controlling cellular trafficking and remodeling, mir-21 gene targets in the phosphatidylinositol signaling system also produce certain phosphoinositides that are involved in cell cycle regulation such as cell proliferation, growth, and survival (Berridge, 1995). For instance, phospholipase C, an enriched gene target for mir-21 (Table 5c), induces cell proliferation through hydrolysis of phosphatidylinositol bisphosphate (PIP2) which produces

agonists of cell proliferation and growth. Another enriched gene target of mir-21, phosphatidylinositol 3-kinase (previously discussed in toll-like receptor pathway) also activates PIP2 that ultimately results in promotion of cell survival. In the context of this study, upregulation of mir-21 would impede these mitogenic activities during long-term thermal acclimations. However, mir-21 appears to promote cell cycle progression and survival by inihibiting the expression of FoxO.

Similar to the paradoxical role of mir-26 in the Tgf-β pathway, mir-21 regulation exerts opposing effects in the FoxO signaling pathway by simultaneously targeting pathway agonist (FoxO) and pathway antagonist pik3c (previously described). Pik3c inhibits FoxO transcriptional activity through activation of intermediate kinase Akt (Zhang *et al*., 2011). However, upregulation of mir-21 would decrease the expression of its target gene pik3c and counteract mir-21 inhibition of FoxO activity (Figure 9). Similar to mir-26 role in the Tgf-β pathway; it is possible that mir-21 is modulating the relative levels of FoxO and pik3c in order to attain an optimal signaling strength for the FoxO signaling pathway that results in a more balanced biological outcome. The most interesting revelation about the paradoxical roles of mir-21 and mir-26 is that they both target transcription factors that are master regulators of aforementioned cellular processes. Therefore, these miRNAs can help fine tune signaling fluctuations in a pathway by broadly controlling the levels of transcription factors that regulate gene expression and consequently, help buffer against adverse biological outcomes.

Conclusion

Even in the absence of a heat shock response, notothenioids like *T. bernacchii* appear to mitigate the effects of heat stress by employing other aspects of the CSR that help confer thermotolerance. The functional characterization of the enriched pathways targeted by the

miRNAs differentially expressed during an acute thermal stress provides the first insight into the regulatory role of thermally induced miRNAs in the stress response of *T. bernacchii*. However, our finding highlight the regulatory complexity of the stress response by exposing the potential pleiotropic effects of miRNAs through their target genes. While a majority of miRNA pathway effects coincided in a temporal manner with heat-induced mRNA changes seen in *T. bernacchii* during acute and chronic thermal acclimations, it also identified the potential existence of antagonistic effects on important biological pathways such as toll-like receptor, Tgf-β and FoxO signaling pathways.

It is important to realize that miRNAs, and signaling pathways in general, do not function as isolated signaling events in cells. Combining the growing body of literature available to date has established that there is no single miRNA-pathway effect currently known in context of miRNA regulation. MiRNAs are highly interwoven into a vast integrated cell-signaling network that spans multiple cell processes. As observed in this study, multiple pathways are targeted concurrently by multiple miRNAs allowing for co-regulation during cellular stress response. Therefore, miRNAs can broadly influence cross-talk between various signaling pathways which can lead to synergistic or antagonistic effects that ultimately determine biological outcomes.

Lastly, there is limited knowledge concerning the mechanistic roles of miRNAs in stress responses and how stress modulates miRNA activities. Only a handful of studies attempted to describe how miRNAs function in the stress response based on available observations in mammalian studies of miRNA regulation. To this end, this study expands our understanding of the role of post-transcriptional controls of the CSR and provides important connections between a non-model organism's transcriptome and its environment. Due to the bioinformatic approach of this study, future work will require experimental validation of the functional role of these

miRNAs to verify their role in modulating the stress response pathways discussed herein. Moreover, experimental work will potentially help elucidate the true impact changes in miRNA levels have on signal transduction networks in-which target genes have opposing effects such as the Tgf-β signaling pathway and FoxO signaling pathway. Perhaps most importantly, this study, identified miRNA enriched pathways that are involved in regulating several aspects of the CSR unrelated to the heat shock response that manifest during acute and chronic heat stress in *T. bernacchii.* As such, these data reinforce the growing body of literature that suggest notothenioid fish can mount an effective CSR and return to a relative state of cellular homeostasis when acclimated to temperatures as high a $^{+}$6 °C above ambient seawater.

APPENDIX A

Table 1. Global expression of miRNAs in *Trematomus bernacchii* gill tissue.

MiRNA	Mature sequence 5' to 3'	Species	Arm
let-7a-1	TGAGGTAGTAGGTTGTATAGT	Oryzias latipes	Mature 5'
let-7a-1//let-7a-3//let-7a-2//let-7a-4//let-7a-5	TGAGGTAGTAGGTTGTATAGTT	Salmo salar	Mature 5'
let-7b-1//let-7b-2	CTGTACAACCTACTGCCTTCCC	Salmo salar	Mature 3'
let-7b-1//let-7b-2	TGAGGTAGTAGGTTGTGTGGTT	Salmo salar	Mature 5'
let-7d-1//let-7d-2	TGAGGTAGTTGGTTGTATGGTT	Salmo salar	Mature 5'
let-7e	TGAGGTAGTAGATTGAAT	Oryzias latipes	Mature 5'
let-7e-1//let-7e-2	TGAGGTAGTAGATTGAATAGTT	Salmo salar	Mature 5'
let-7g	TGAGGTAGTAGTTTGTATAG	Oryzias latipes	Mature 5'
let-7g-1//let-7g-2	TGAGGTAGTAGTTTGTATAGTT	Danio rerio//Salmo salar	Mature 5'
let-7h	TGAGGTAGTAAGTTGTGTTGTT	Salmo salar	Mature 5'
let-7h	CTATACAACTTACTGCCTTCCT	Salmo salar	Mature 3'
let-7i	TGAGGTAGTAGTTTGTGCTGT	Cyprinus carpio	Mature 5'
let-7i-1//let-7i-2	TGAGGTAGTAGTTTGTGCTGTT	Salmo salar	Mature 5'
let-7j	TGAGGTAGTTGTTTGTACAGTT	Hippoglossus hippoglossus	Mature 5'
mir-100	AACCCGTAGATCCGAACTTGT	Cyprinus carpio	Mature 5'
mir-100//mir-100a-1//mir-100a-2	AACCCGTAGATCCGAACTTGTG	Fugu rubripes//Salmo salar	Mature 5'
mir-100-1//mir-100-2	AACCCGTAGATCCGAACTT	Oryzias latipes	Mature 5'
mir-101a//mir-101a-1//mir-101a-2	TACAGTACTGTGATAACTGAAG	Fugu rubripes//Salmo salar	Mature 3'
mir-103	AGCAGCATTGTACAGGGCTATGA	Salmo salar	Mature 3'
mir-10a-1//mir-10a-2	TACCCTGTAGATCCGGATTTGT	Salmo salar	Mature 5'
mir-10b-1//mir-10b-2	TACCCTGTAGAACCGAATTTGTG	Fugu rubripes	Mature 5'
mir-10b-1//mir-10b-2//mir-10b-3//mir-10b-4	TACCCTGTAGAACCGAATTTGT	Salmo salar	Mature 5'
mir-10c	TACCCTGTAGATCCGGATTTGTG	Cyprinus carpio	Mature 5'
mir-10d	TACCCTGTAGAACCGAATGTGT	Cyprinus carpio	Mature 5'
mir-10d	TACCCTGTAGAACCGAATGTGTG	Danio rerio	Mature 5'
mir-10d	TACCCTGTAGAACCGAATGTGTGTG	Fugu rubripes	Mature 5'
mir-125a	TCCCTGAGACCCTTAACCTGTG	Fugu rubripes//Oryzias latipes	Mature 5'
mir-125a-1//mir-125a-2	TCCCTGAGACCCTAACTTGTGA	Salmo salar	Mature 5'
mir-125b-1//mir-125b-2//mir-125b-3	TCCCTGAGACCCTTAACCTGTGA	Salmo salar	Mature 5'
mir-125c	TCCCTGAGACCCTAACTTGTGAC	Oryzias latipes	Mature 5'
mir-126	TCGTACCGTGAGTAATAATGC	Fugu rubripes	Mature 3'
mir-126-1//mir-126-2//mir-126-3//mir-126-4	TCGTACCGTGAGTAATAATGCA	Salmo salar	Mature 3'
mir-128-1//mir-128-2	TCACAGTGAACCGGTCTCTTTT	Danio rerio//Fugu rubripes//Tetraodon nigroviridis	Mature 3'
mir-128-1//mir-128-2//mir-128-3//mir-128-4	TCACAGTGAACCGGTCTCTTT	Salmo salar	Mature 3'
mir-1-3//mir-1-2//mir-1-1//mir-1-4	TGGAATGTAAAGAAGTATGTAT	Salmo salar	Mature 3'
mir-1338	AGGACTGTCCAACCTGAGAAT	Salmo salar	Mature 5'
mir-140	TACCACAGGGTAGAACCACGGAC	Danio rerio	Mature 3'
mir-140	ACCACAGGGTAGAACCACGGAC	Ictalurus punctatus	Mature 3'
mir-140	TACCACAGGGTAGAACCACGGA	Salmo salar	Mature 3'

MiRNA	Mature sequence 5' to 3'	Species	Arm
mir-141	TAACACTGTCTGGTAACGATGC	Danio rerio	Mature 3'
mir-143	TGAGATGAAGCACTGTAGCT	Cyprinus carpio	Mature 3'
mir-143-1//mir-143-2//mir-143	TGAGATGAAGCACTGTAGCTC	Danio rerio//Salmo salar	Mature 3'
mir-146a	TGAGAACTGAATTCCATAGATGGTA	Oryzias latipes	Mature 5'
mir-146a-1//mir-146a-2//mir-146a-3	TGAGAACTGAATTCCATAGATGG	Salmo salar	Mature 5'
mir-148	TCAGTGCATTACAGAACTTT	Oryzias latipes	Mature 3'
mir-148a	TCAGTGCATTACAGAACTTTGT	Salmo salar	Mature 3'
mir-150	ACTCCCAATCCTTGTACCAGT	Oryzias latipes	Mature 3'
mir-150	TCTCCCAATCCTTGTACCAGT	Salmo salar	Mature 5'
mir-152	TCAGTGCATAACAGAACTTTGTC	Oryzias latipes	Mature 3'
mir-152	TCAGTGCATAACAGAACTTTG	Salmo salar	Mature 3'
mir-16a-1//mir-16a-2	TAGCAGCACGTAAATATTGGAG	Salmo salar	Mature 5'
mir-181a	AACATTCAACGCTGTCGGTGA	Cyprinus carpio	Mature 5'
mir-181a-1	ACCATCGACCGTTGATTGTACC	Fugu rubripes	Mature 3'
mir-181a-1//mir-181a-2	AACATTCAACGCTGTCGGT	Oryzias latipes	Mature 5'
mir-181a-1//mir-181a-2//mir-181a-3//mir-181a-4//mir-181a-5	AACATTCAACGCTGTCGGTGAG	Ictalurus punctatus	Mature 5'
mir-181a-1//mir-181a-2//mir-181a-3//mir-181a-4//mir-181a-5	AACATTCAACGCTGTCGGTGAGT	Salmo salar	Mature 5'
mir-181a-5	ACCATCGACCGTTGACTGTGCC	Salmo salar	Mature 3'
mir-181c	AACATTCATTGCTGTCGGTGGG	Salmo salar	Mature 5'
mir-184-1//mir-184-2	TGGACGGAGAACTGATAAGGGC	Danio rerio	Mature 3'
mir-199a-1//mir-199a-2//mir-199a-3//mir-199a-4	CCCAGTGTTCAGACTACCTGTT	Oryzias latipes	Mature 5'
mir-199a-1//mir-199a-2//mir-199a-3//mir-199a-4	CCCAGTGTTCAGACTACCTGTTC	Salmo salar	Mature 5'
mir-200a	TAACACTGTCTGGTAACGATG	Cyprinus carpio	Mature 3'
mir-200a	TAACACTGTCTGGTAACGATGT	Fugu rubripes	Mature 3'
mir-200a	AACACTGTCTGGTAACGA	Oryzias latipes	Mature 3'
mir-200a-1//mir-200a-2//mir-200a-3	TAATACTGCCTGGTAATGATGAT	Salmo salar	Mature 3'
mir-200b	TAATACTGCCTGGTAATGATGA	Fugu rubripes	Mature 3'
mir-200b	TAATACTGCCTGGTAATGATG	Oryzias latipes	Mature 3'
mir-200b-1//mir-200b-2	TAACACTGTCTGGTAACGATGTT	Salmo salar	Mature 3'
mir-203	AGTGGTTCTCAACAGTTCAACAG	Paralichthys olivaceus	Mature 5'
mir-203//mir-203a//mir-203a-1//mir-203a-2	GTGAAATGTTTAGGACCACTTG	Fugu rubripes//Cyprinus carpio//Salmo salar	Mature 3'
mir-203b	TGAAATGTTTAGGACCACTCG	Salmo salar	Mature 3'
mir-205	TCCTTCATTCCACCGGAGTCTGT	Ictalurus punctatus	Mature 5'
mir-205//mir-205b-1//mir-205b-2//mir-205b-3//mir-205b-4	TCCTTCATTCCACCGGAGTCTG	Fugu rubripes//Salmo salar	Mature 5'
mir-21	TAGCTTATCAGACTGGTGTTGG	Paralichthys olivaceus	Mature 5'
mir-21-1//mir-21-2	TAGCTTATCAGACTGGTGTTG	Oryzias latipes	Mature 5'
mir-21-2//mir-21b	TAGCTTATCAGACTGGTGTTGGC	Danio rerio//Salmo salar	Mature 5'
mir-2188	GCTGTGTGAGGTCAGACCTATC	Salmo salar	Mature 3'
mir-21a-1//mir-21a-2	TAGCTTATCAGACTGGTGTTGACT	Salmo salar	Mature 5'
mir-221	ACCTGGCATACAATGTAGATTTCTGT	Danio rerio	Mature 5'
mir-22-1//mir-22-2	AAGCTGCCAGCTGAAGAACTG	Oryzias latipes	Mature 3'
mir-222//mir-222a-1//mir-222a-2	AGCTACATCTGGCTACTGGGTCTC	Fugu rubripes//Salmo salar	Mature 3'
mir-22a	AAGCTGCCAGCTGAAGAACTGT	Fugu rubripes//Salmo salar	Mature 3'

MiRNA	Mature sequence 5' to 3'	Species	Arm
mir-23a-2//mir-23a-3	ATCACATTGCCAGGGATTTCCA	Fugu rubripes	Mature 3'
mir-23a-3//mir-23a-4//mir-23a-1//mir-23a-2	ATCACATTGCCAGGGATTTCC	Salmo salar	Mature 3'
mir-24-2//mir-24-1//mir-24-3	TGGCTCAGTTCAGCAGGAACAGG	Ictalurus punctatus	Mature 3'
mir-25-1//mir-25-2//mir-25-3	CATTGCACTTGTCTCGGTCTGA	Salmo salar	Mature 3'
mir-26-1//mir-26-2	TTCAAGTAATCCAGGATAGGC	Hippoglossus hippoglossus	Mature 5'
mir-26a-1//mir-26a-2//mir-26a-3//mir-26a-4//mir-26a-5//mir-26a-6	TTCAAGTAATCCAGGATAGGCT	Salmo salar	Mature 5'
mir-26b	TTCAAGTAATCCAGGATAGGTT	Danio rerio//Salmo salar	Mature 5'
mir-27a	TTCACAGTGGCTAAGTTCCG	Oryzias latipes	Mature 3'
mir-27b	TTCACAGTGGCTAAGTTCTGCA	Danio rerio//Fugu rubripes	Mature 3'
mir-27b-1//mir-27b-2	TTCACAGTGGCTAAGTTCTGC	Salmo salar	Mature 3'
mir-27c	TTCACAGTGGTTAAGTTCTGCC	Cyprinus carpio	Mature 3'
mir-27c	TTCACAGTGGTTAAGTTCTGC	Fugu rubripes	Mature 3'
mir-27c	AGGACTTAGCCCACATGTGAAC	Oryzias latipes	Mature 5'
mir-27d	TTCACAGTGGCTAAGTTC	Oryzias latipes	Mature 3'
mir-30a	CTTTCAGTCGGATGTTTGC	Oryzias latipes	Mature 3'
mir-30a-3//mir-30a-4//mir-30a-1//mir-30a-2	TGTAAACATCCTACACTCTCAGC	Salmo salar	Mature 5'
mir-30b	TGTAAACATCCCCGACTGGAAGCT	Salmo salar	Mature 5'
mir-30c	TGTAAACATCCTACACTCTCGG	Fugu rubripes	Mature 5'
mir-30c	TGTAAACATCCTACACTCTCAGCT	Ictalurus punctatus	Mature 5'
mir-30d	TGTAAACATCCCCGACTGGAAGC	Cyprinus carpio	Mature 5'
mir-30d	TGTAAACATCCCCGACTGGAAG	Fugu rubripes	Mature 5'
mir-30e-2	TGTAAACATCCTTGACTGGAAG	Danio rerio	Mature 5'
mir-30e-2//mir-30c-1//mir-30c-2//mir-30d-1	CTTTCAGTCGGATGTTTGCAGC	Danio rerio//Salmo salar	Mature 3'
mir-31	GGGCAAGATGTTGGCATAGCTGT	Oryzias latipes	Mature 5'
mir-375-1//mir-375-2	TTTGTTCGTTCGGCTCGCGTT	Ictalurus punctatus	Mature 3'
mir-375-1//mir-375-2//mir-375-3	TTTGTTCGTTCGGCTCGCGTTA	Danio rerio//Fugu rubripes//Salmo salar	Mature 3'
mir-429	TAATACTGTCTGGTAATGCCG	Salmo salar	Mature 3'
mir-451	AAACCGTTACCATTACTGAGTT	Danio rerio	Mature 5'
mir-455	TATGTGCCCTTGGACTACATC	Oryzias latipes	Mature 5'
mir-455	TATGTGCCCTTGGACTACATCG	Salmo salar	Mature 5'
mir-458	ATAGCTCTTTAAATGGTACTGC	Fugu rubripes	Mature 3'
mir-462	TAACGGAACCCATAATGCAGCT	Danio rerio	Mature 5'
mir-462a	TAACGGAACCCATAATGCAGCTG	Salmo salar	Mature 5'
mir-7132b	TGAGGCGTTTAGAACAAGTTCA	Salmo salar	Mature 3'
mir-725-1//mir-725-2	TTCAGTCATTGTTTCTGGTAGT	Salmo salar	Mature 3'
mir-731	AATGACACGTTTTCTCCCGGATCG	Danio rerio	Mature 5'
mir-731	AATGACACGTTTTCTCCC	Oryzias latipes	Mature 5'
mir-731	AATGACACGTTTTCTCCCGGATT	Salmo salar	Mature 5'
mir-92a-1	TATTGCACTTGTCCCGGCCTG	Oryzias latipes	Mature 3'
mir-92a-1//mir-92-2//mir-92-1//mir-92a-2//mir-92a-3//mir-92a-4	TATTGCACTTGTCCCGGCCTGT	Danio rerio//Fugu rubripes//Salmo salar	Mature 3'
mir-99	AACCCGTAGATCCGATCTTGT	Cyprinus carpio	Mature 5'
mir-99-1//mir-99-2	AACCCGTAGATCCGATCTTGTG	Danio rerio//Salmo salar	Mature 5'
mir-99b	AACCCGTAGATCCGATCTTGTGA	Ictalurus punctatus	Mature 5'

Table 2. Fold change values (FDR ≤ 0.05) of differentially expressed miRNAs during acute and chronic heat stress.

7-day DE mature miRNA	Fold Change	Mature Sequence 5' to 3'	Species	Arm
let-7a	-1.25	TGAGGTAGTAGGTTGTATAGTT	Salmo salar	Mature 5'
let-7g	-5.618537676	TGAGGTAGTAGTTTGTATAGTT	Danio rerio/Salmo salar	Mature 5'
mir-22a	-2.524349786	AAGCTGCCAGCTGAAGAACTGT	Fugu rubripes//Salmo salar	Mature 3'
mir-26a	-3.234380218	TTCAAGTAATCCAGGATAGGCT	Salmo salar	Mature 5'
mir-30b	-4.587915787	TGTAAACATCCCCGACTGGAAGCT	Salmo salar	Mature 5'
mir-146a	2.727891618	TGAGAACTGAATTCCATAGATGG	Salmo salar	Mature 5'
mir-200a	-6.034307743	TAATACTGCCTGGTAATGATGAT	Salmo salar	Mature 3'
mir-203b	-2.938460598	TGAAATGTTTAGGACCACTCG	Salmo salar	Mature 3'
mir-725	-1.625	TTCAGTCATTGTTTCTGGTAGT	Salmo salar	Mature 3'
28-day DE mature miRNA	**Fold Change**	**Mature Sequence 5' to 3'**	**Species**	**Arm**
mir-21	2.89296747	TAGCTTATCAGACTGGTGTTG	Oryzias latipes	Mature 5'
mir-21a	2.380589435	TAGCTTATCAGACTGGTGTTGACT	Salmo salar	Mature 5'
mir-21b	2.490684932	TAGCTTATCAGACTGGTGTTGGC	Danio rerio//Salmo salar	Mature 5'

Table 3. Thermodynamic stability of pre-cursor miRNA structures as determined by minimum fold energies (kcal/mol).

Mature miRNA	Arm	MFE (kcal/mol)	Mature Sequence 5' to 3'
mir-22a	3'	-68.9	AAGCUGCCAGCUGAAGAACUGU
let-7g	5'	-62.5	UGAGGUAGUAGUUUGUAUAGUU
mir-21	5'	-60.73	UAGCUUAUCAGACUGGUGUUG
let-7a	5'	-45.6	UGAGGUAGUAGGUUGUAUAGUU
mir-26a	5'	-43.7	UUCAAGUAAUCCAGGAUAGGCU
mir-30b	5'	-41.3	UGUAAACAUCCCCGACUGGAAGCU
mir-146a	5'	-37.5	UGAGAACUGAAUUCCAUAGAUGG
mir-203b	3'	-36.9	UGAAAUGUUUAGGACCACUCG
mir-200a	3'	-35.5	UAAUACUGCCUGGUAAUGAUGAU
mir-725	3'	-32.2	UUCAGUCAUUGUUUCUGGUAGU

Table 4. GO-Slim Enrichment Analysis (FDR ≤ 0.05) for DE miRNAs.

GO-Slim Molecular Function	Fold Enrichment	FDR
GTPase activity	1.77	2.52E-02
catalytic activity	1.29	2.75E-04
pyrophosphatase activity	1.71	7.48E-03
kinase activity	1.57	1.93E-02
protein binding	1.47	2.41E-05

GO-Slim Cellular Component	Fold Enrichment	FDR
plasma membrane	1.38	2.24E-02
protein complex	1.35	2.11E-02
macromolecular complex	1.29	3.59E-02
intracellular	1.21	4.06E-03

GO-Slim Biological Process	Fold Enrichment	FDR
angiogenesis	5.58	0.0344
developmental process	1.46	0.000293
cytoskeleton organization	1.85	0.0106
cellular component movement	1.85	0.00611
locomotion	1.83	0.0416
regulation of phosphate metabolic process	1.83	0.00621
cellular component morphogenesis	1.72	0.0314
lipid metabolic process	1.67	0.0188
intracellular signal transduction	1.49	0.00697
single-multicellular organism process	1.4	0.0193
transport	1.32	0.0491
response to stimulus	1.28	0.0217

Table 5. KEGG Pathway Enrichment Analysis by DIANA-mirPath. (A) MiRNA enriched pathways for 7-day downregulated miRNAs. (B) MiRNA enriched pathways for 7-day upregulated mir-146. (C) MiRNA enriched pathways for 28-day upregulated mir-21.

A.

Enriched KEGG pathway	p-value < 0.05	Target Genes	miRNAs
ECM-receptor interaction	0	col1a2, col1a1g, thbs1b	let-7a, let-7g
Mucin type O-Glycan biosynthesis	1.95E-05	galnt7	mir-26a
		galnt2, galnt7	mir-30a
Metabolism of xenobiotics by cytochrome P450	0.001100709	gsto1, ugt1u7	mir-26a
		gstm	mir-203b
TGF-beta signaling pathway	0.01135606	inhbab,inhbb, smad, bambia, bambib	mir-26a
SNARE interactions in vesicular transport	0.01797762	snap23, sec22ba, stx16, stx6	mir-203b
		stx6, bnip1a, vti1b	mir-725
Steroid hormone biosynthesis	0.01801988	ugt1a7	mir-26a
		cyp19a1b, srd5a1	mir-30a
		cyp11a1, hsd17b12b	mir-203b

B.

Enriched KEGG pathway	p-value < 0.05	Target Genes	miRNAs
Mucin type O-Glycan biosynthesis	1.74E-10	galectin17	mir-146
Toll-like receptor signaling pathway	0.000966204	traf6, pik3c	mir-146

C.

Enriched KEGG pathway	p-value < 0.05	Target Genes	miRNAs
Phosphatidylinositol signaling system	0.0104262	plcd3a, pik3c	mir-21
Gap junction	0.0104262	connexin 35b	mir-21
FoxO signaling pathway	0.0202337	foxo3a, pik3c	mir-21

Table 6. MiRNA enriched pathways attributed to GO-Slim Biological Process

GO-Slim Biological Process	Description	Fold Change	FDR ≤ 0.05	DE miRNA enriched pathways GO representation
Angiogenesis	Blood vascular formation	5.58	3.44E-02	Mucin type-O glycan biosynthesis, TGF-beta signaling pathway, Toll-like receptor pathway, FoxO signaling pathway
Embryo development	Process that modulates cell proliferation, growth, differentiation and apoptosis during embryogenesis	2.54	2.52E-02	TGF-beta signaling pathway
Cell Differentiation	Cell fate determination and subsequent maturation	1.96	1.26E-03	TGF-beta signaling pathway, Toll-like receptor pathway, FoxO signaling pathway
Cytoskeleton organization	Assembly, arrangement of constituent parts, or disassembly of cytoskeletal structures	1.85	1.06E-02	phosphatidylinositol signaling, gap junction
Cellular component movement	The directed, self-propelled movement of a cellular component without the involvement of transporter channel or a pore."	1.85	6.11E-03	SNARE interactions in vesicular transport
Locomotion	Cell migration or whole organism movement from one location to another.	1.83	4.16E-02	Gap juntions, Fox-O signaling, Tgf beta signaling
Regulation of phosphate metabolic process	Process that modulates cellular pathways involving phosphates	1.83	6.21E-03	TGF-beta signaling pathway,, Phosphatidylinositol signaling , Toll-like receptor pathway, FoxO signaling pathway
Cellular component morphagenesis	Prcoess by which cellular structures or whole cells are generated and organized.	1.72	3.14E-02	ECM-receptor interaction, SNARE interactions in vesicular transport, phosphatidylinositol signaling
Lipid metabolic process	Chemical reactions and pathways involving lipids	1.67	1.88E-02	Metabolism of xenobiotics by cytochrome P450, Steroid hormone biosynthesis
Intracellluar signal transduction	Intracellular ropagation of signals to downstream effectors that trigger functional changes in the cell	1.49	6.97E-03	TGF-beta signaling pathway,, Phosphatidylinositol signaling , Toll-like receptor pathway, FoxO signaling pathway
Single-multicellular organism process	Process occurring within a single, multicellular organism	1.4	1.93E-02	Steroid hormone biosynthesis
Transport	Inter-Intracellular directed movement of macromolecules such as ions and metabolites by means of transporter channel or pore	1.32	4.91E-02	Gap juntions
Response to stimili	Any process that changes the state or activity of a cell or an organism in response to stimulus.	1.28	2.17E-02	ECM-receptor interaction, Mucin type O-Glycan biosynthesis, Metabolism of xenobiotics by cytochrome P450, TGF-beta signaling pathway, SNARE interactions in vesicular transport, Steroid hormone biosynthesis, Gap juntions, Phosphatidylinositol signaling , Toll-like receptor pathway, FoxO signaling pathway

APPENDIX B

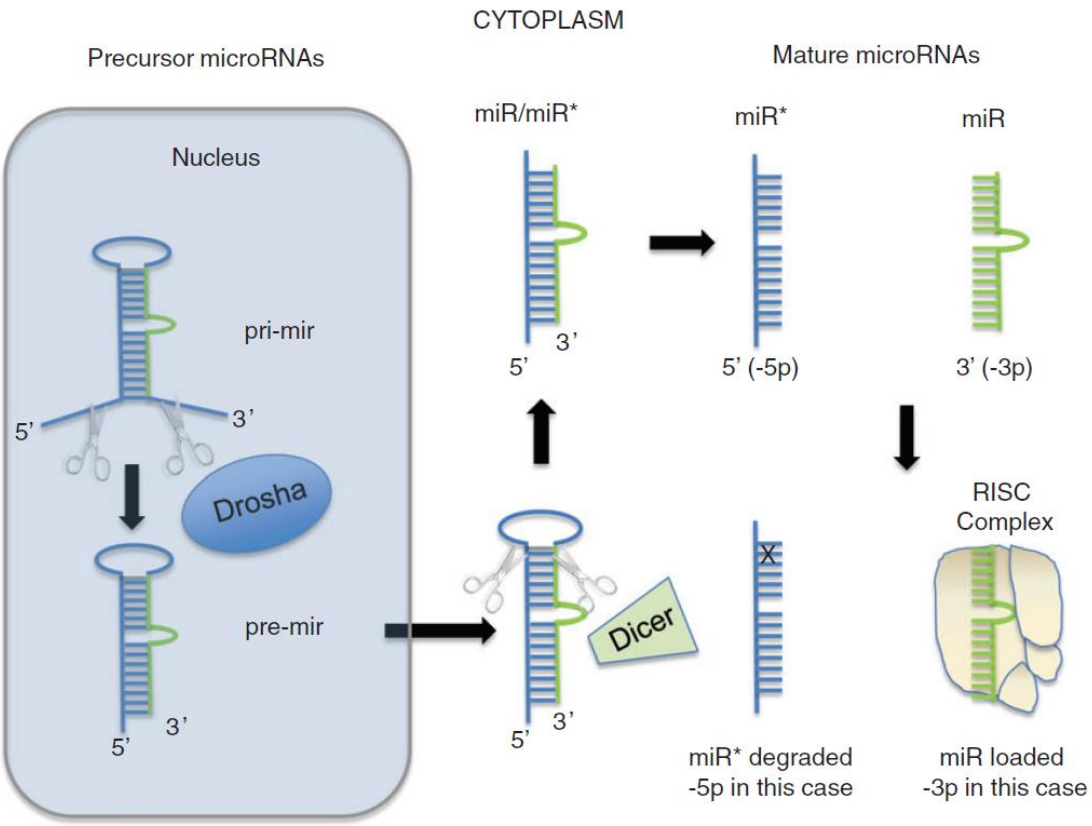

Figure 1. MicroRNA biogenesis: MiRNA is transcribed from DNA to form a long primary miRNA (pri-miRNA) transcript containing secondary hairpin structure with length of 1-3 kb. The pri-miRNA is then processed by Drosha that leads to the formation of a 60-70 nt double stranded pre-miRNA. The pre-miRNA is exported from the nucleus and is further cleaved by the Dicer to form a 20-24 nucleotide (nt) double stranded miRNA duplex. The miRNA maturation process can produce distinct mature miRNAs from the 3′ and 5′ arms of the pre-miRNA duplex. One strand of the duplex is degraded*, and the other strand (mature miRNA) is incorporated into a complex of ribonucleic proteins to form the miRISC complex (miRNA induced silencing complex). The miRISC complex facilitates the complementarily binding of the 5' "seed" sequence of the mature miRNA to the 3' UTR region of target messenger RNA.

(Figure reprinted with permission from corresponding author & journal: Nair, V. S., Pritchard, C. C., Tewari, M., & Ioannidis, J. P. (2014). Design and analysis for studying microRNAs in human disease: a primer on-omic technologies. *American journal of epidemiology*, 180(2), 140-152.)

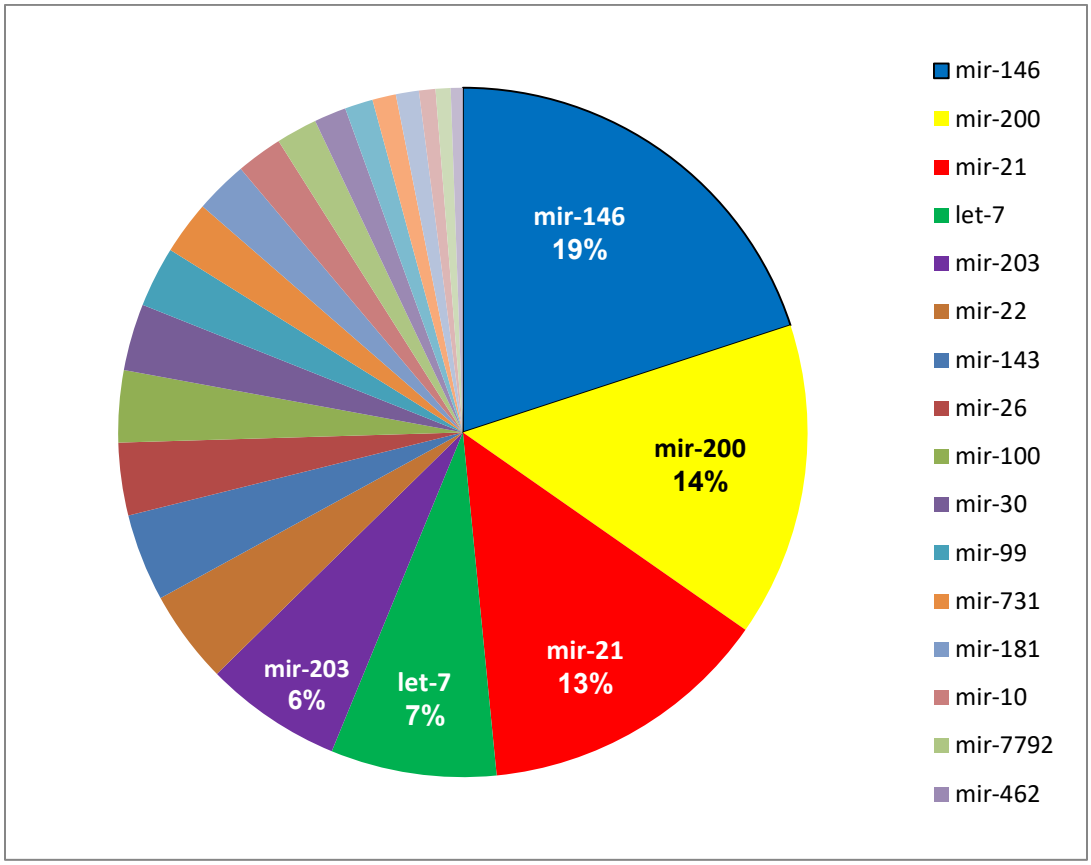

Figure 2. Pie chart showing distribution of 56 miRNA families by abundance. The top 6 miRNA families represent 63 % of the expressed miRNAs. All the others (miRNAs with less than 3 % of the total abundance) represent 37 % of the overall expressed miRNA in gill tissue.

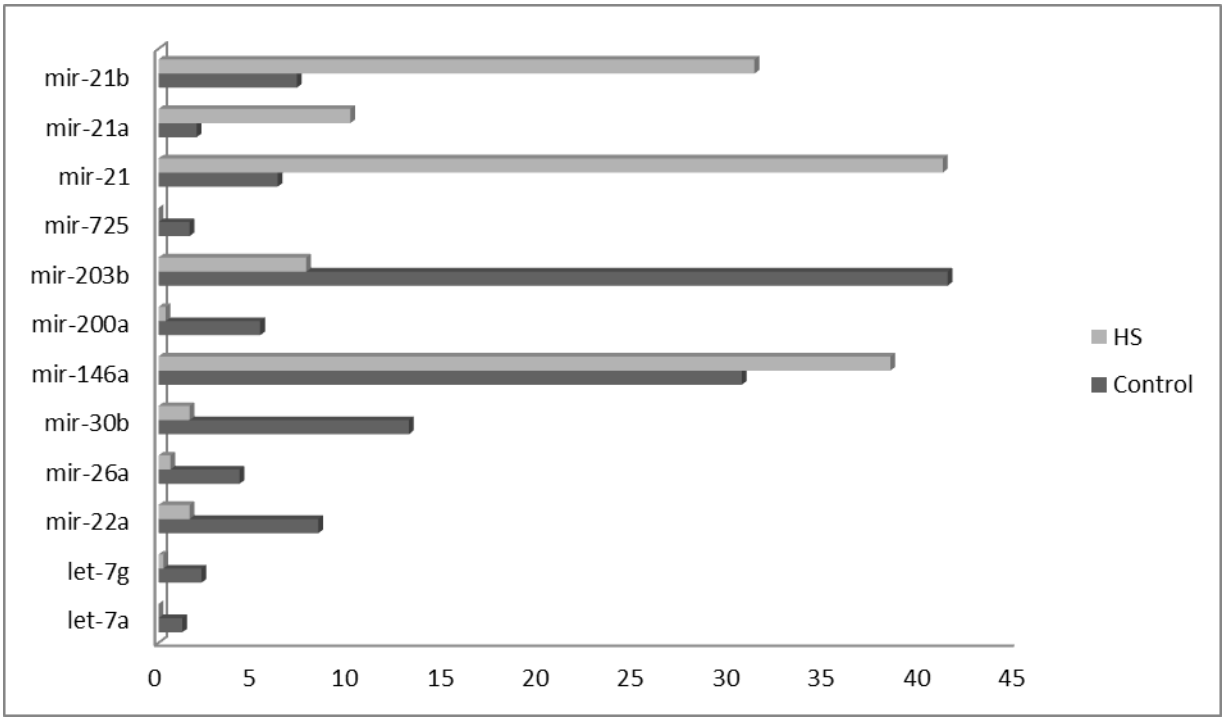

Figure 3. Fold change values (FDR ≤ 0.05) of differentially expressed miRNAs between heat stress (4 °C) and control treatments (-1.5 °C).

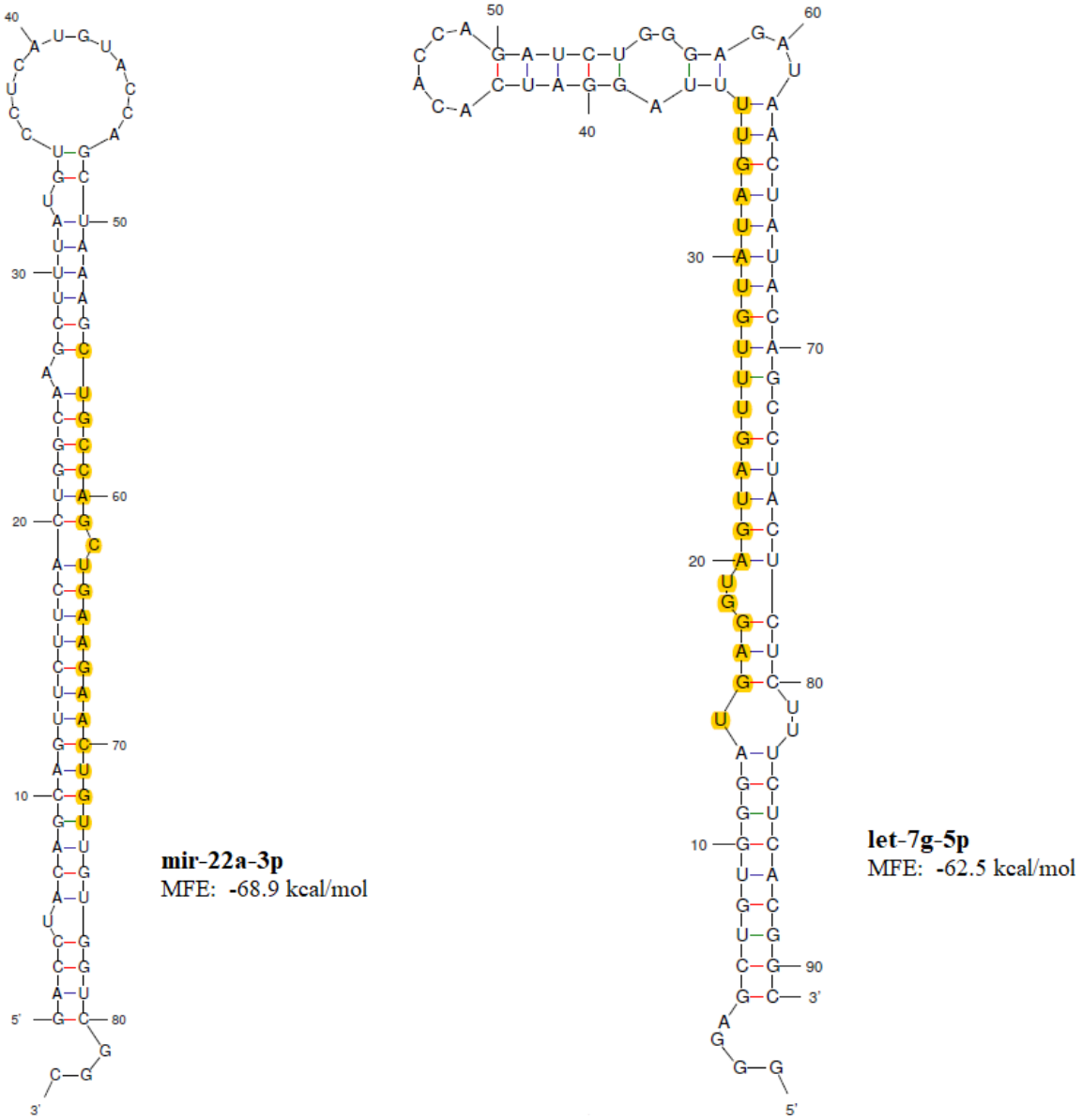

Figure 4a. Minimum free energy (MFE) of pre-cursor miRNA secondary structures at 4 °C using Mfold.

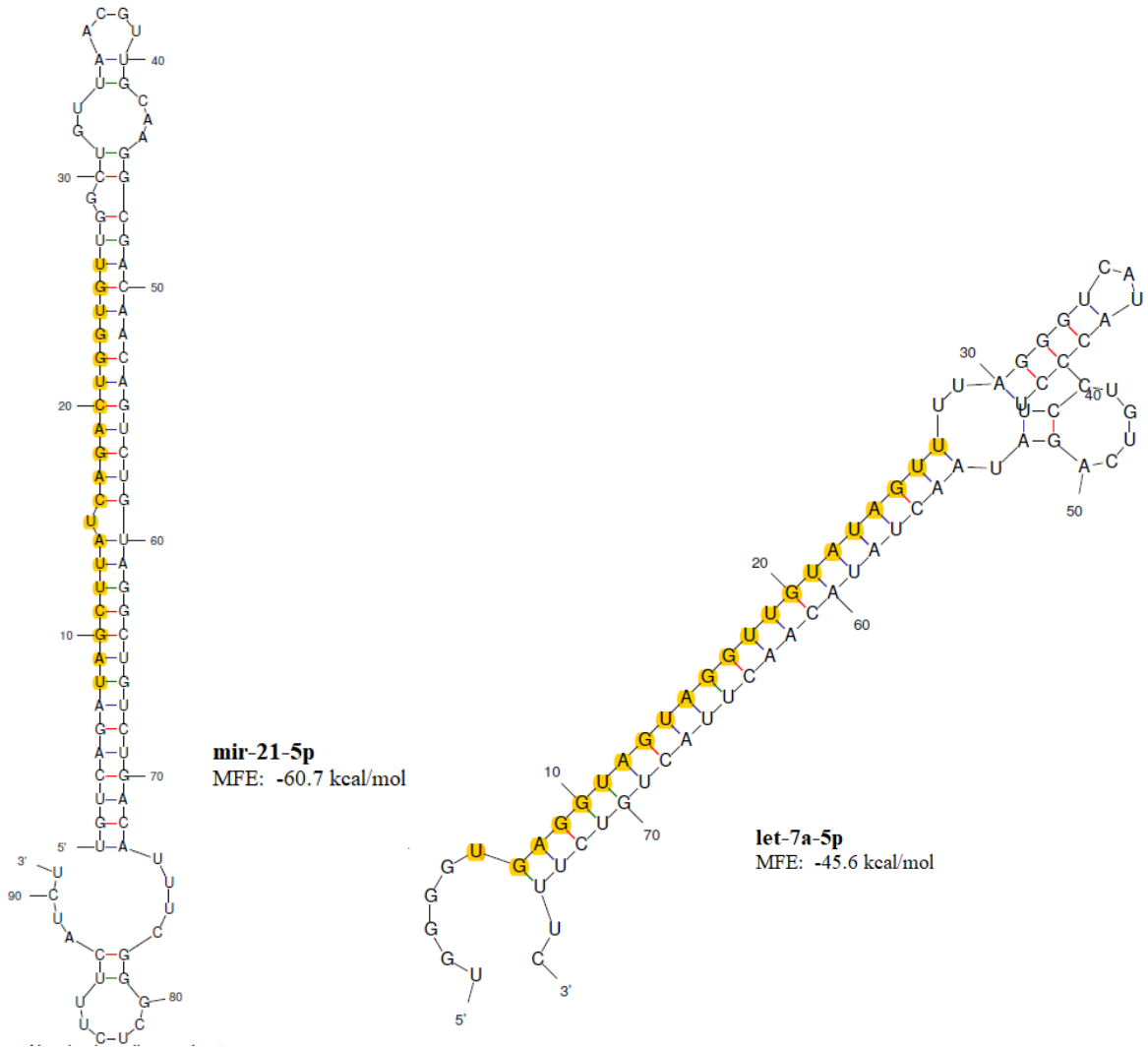

Figure 4b. Minimum free energy (MFE) of pre-cursor miRNA secondary structures at 4 °C using Mfold.

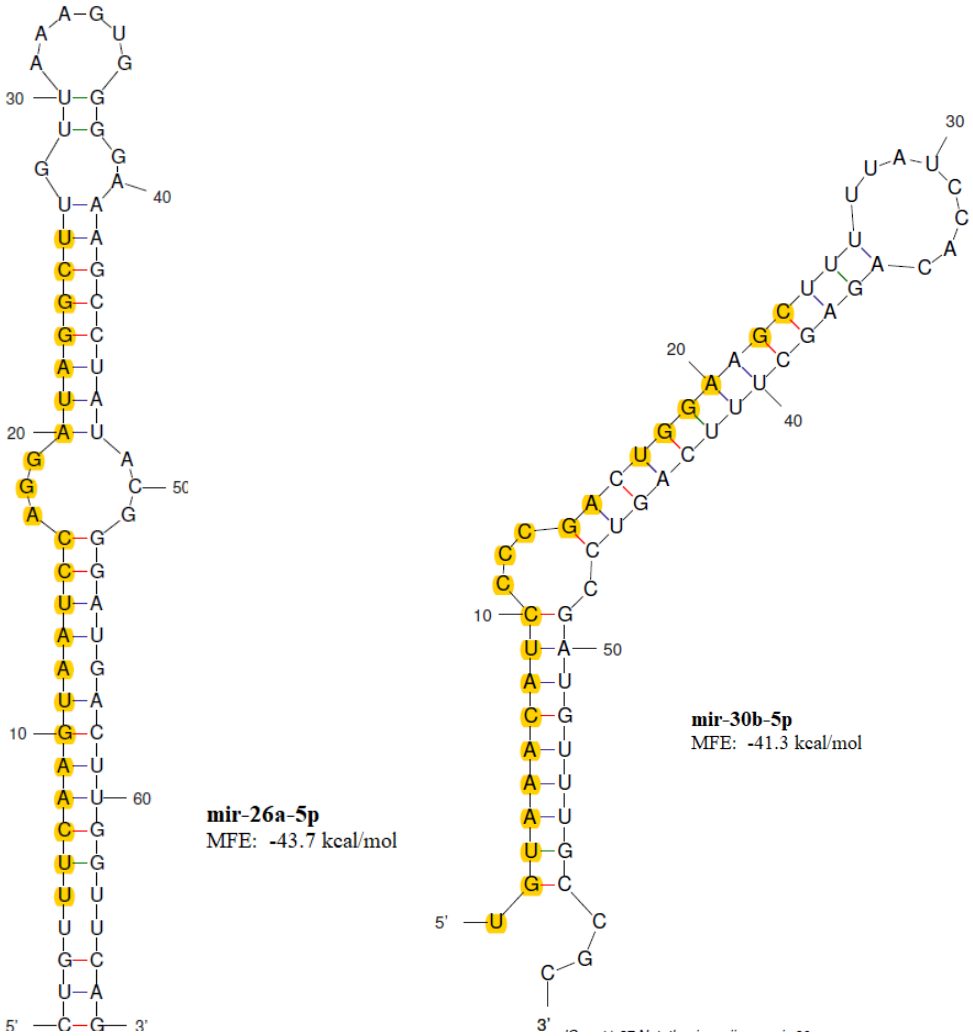

Figure 4c. Minimum free energy (MFE) of pre-cursor miRNA secondary structures at 4 °C using Mfold.

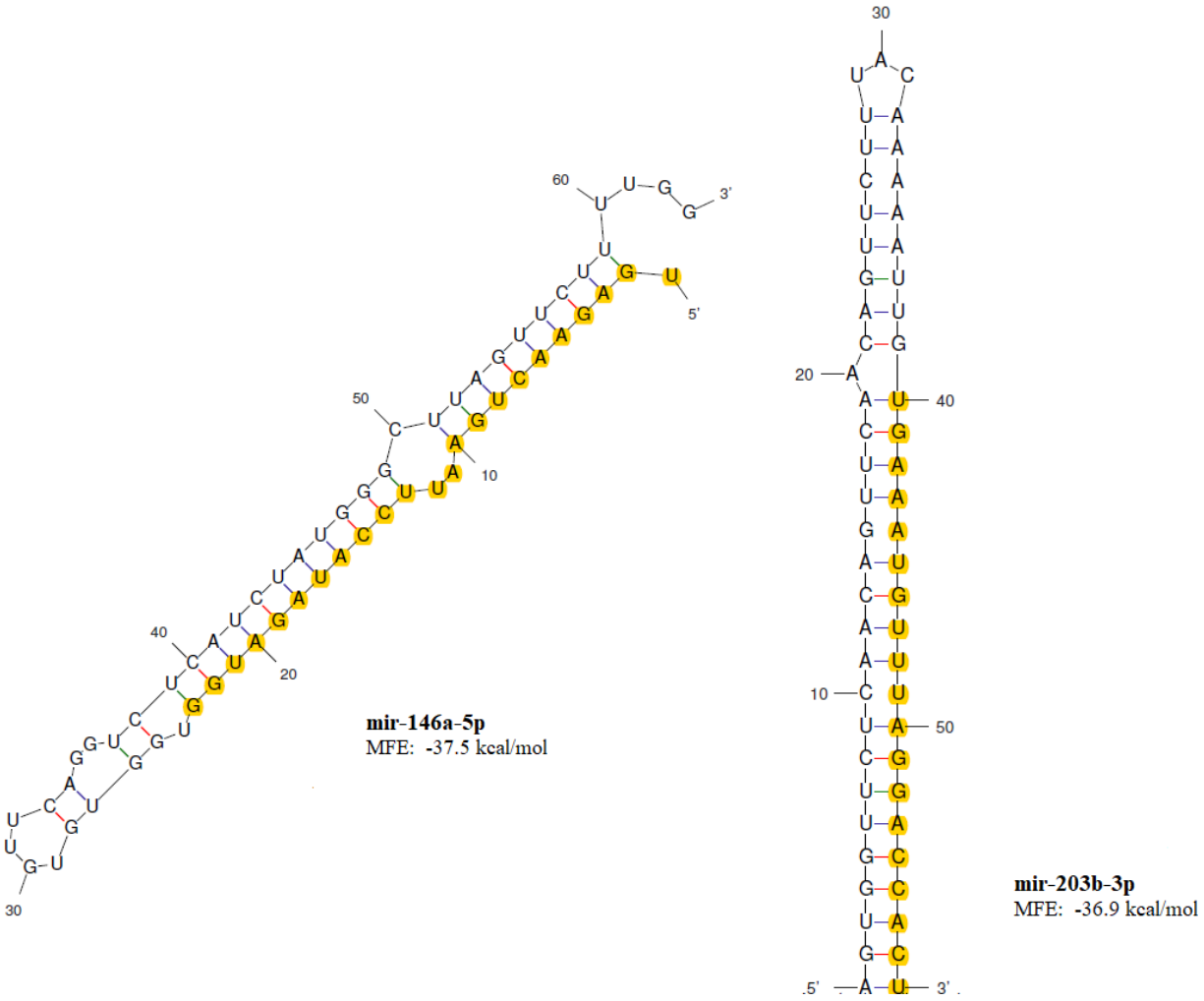

Figure 4d. Minimum free energy (MFE) of pre-cursor miRNA secondary structures at 4 °C using Mfold.

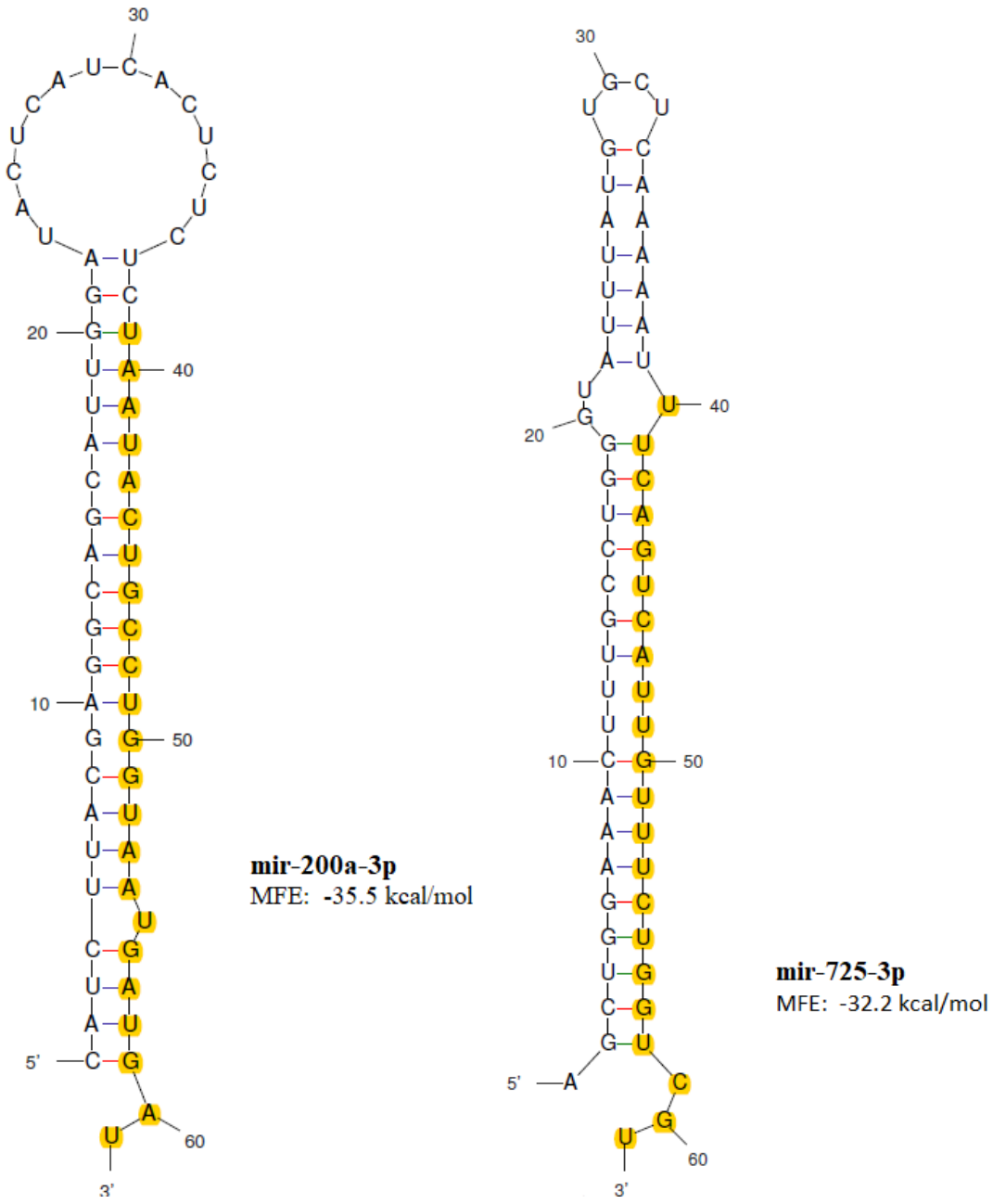

Figure 4e. Minimum free energy (MFE) of pre-cursor miRNA secondary structures at 4 °C using Mfold.

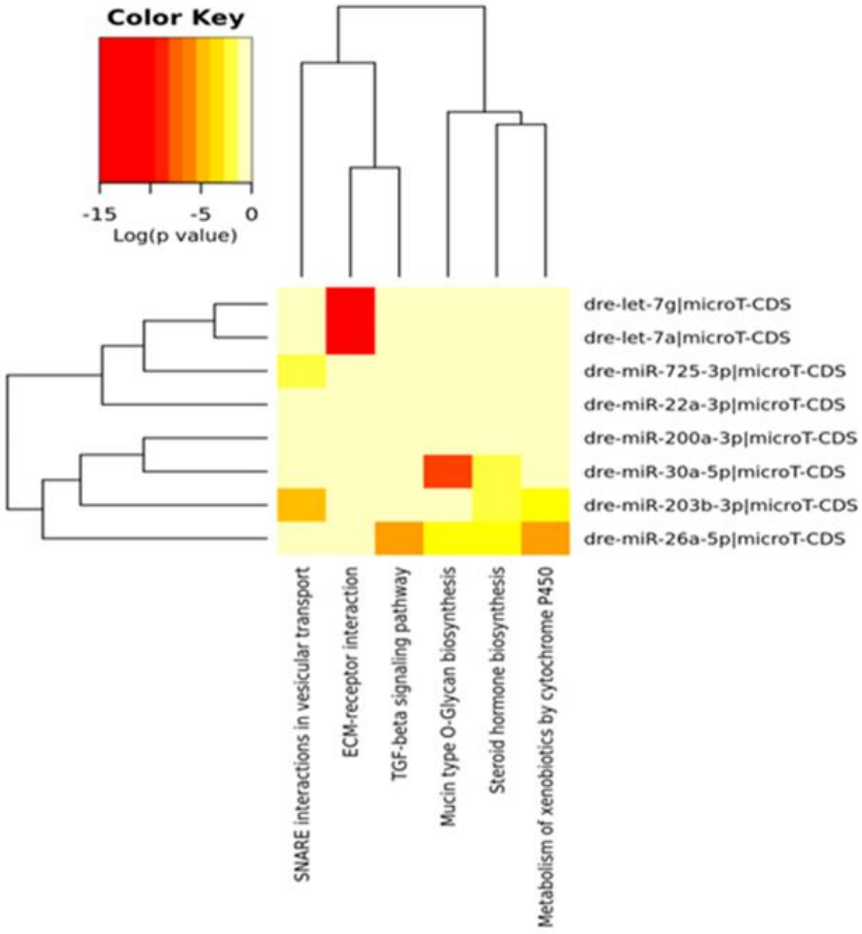

Figure 5. P-value heatmap showing significantly enriched KEGG pathways for 7-day downregulated miRNAs. Heat map shows enriched pathways targeted by at least one miRNA.

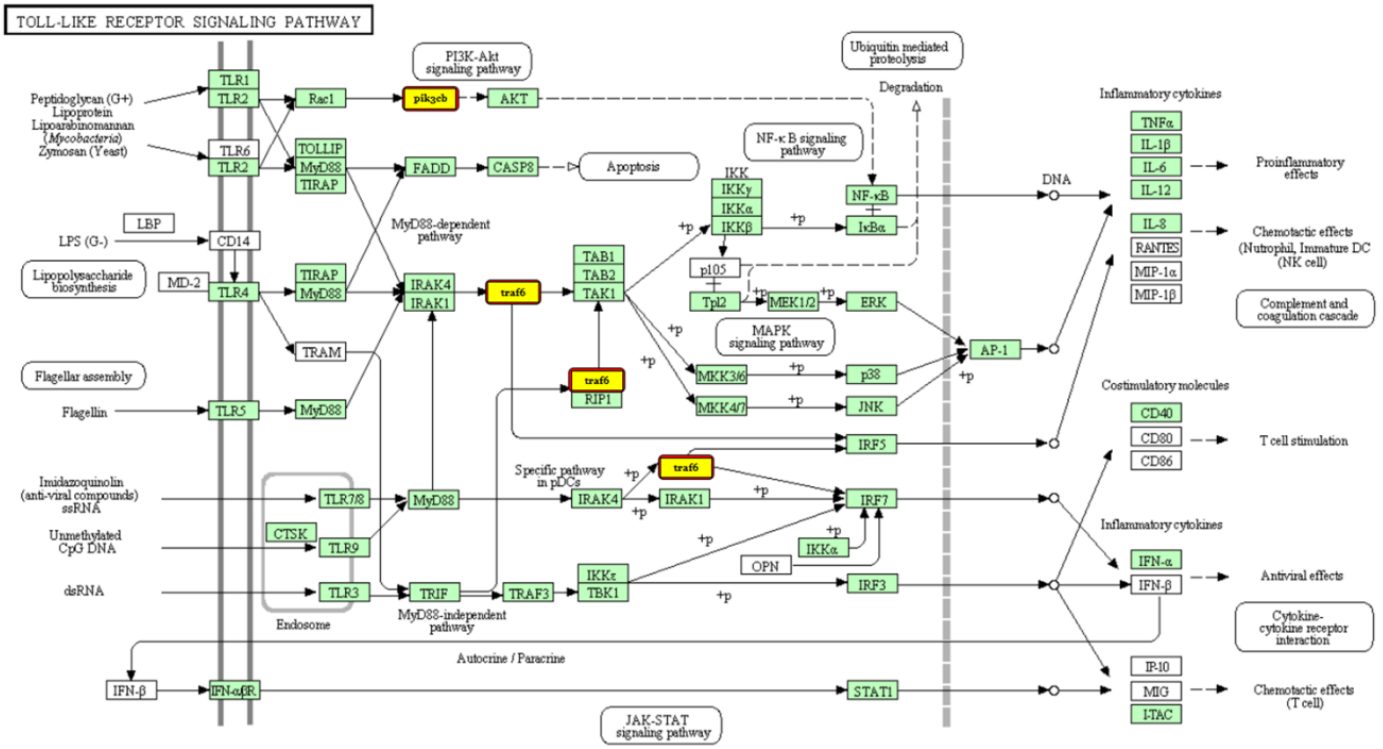

Figure 6. Toll-like receptor pathway for 7-day upregulated mir-146a. The enriched target genes for mir-146a are highlighted in yellow.

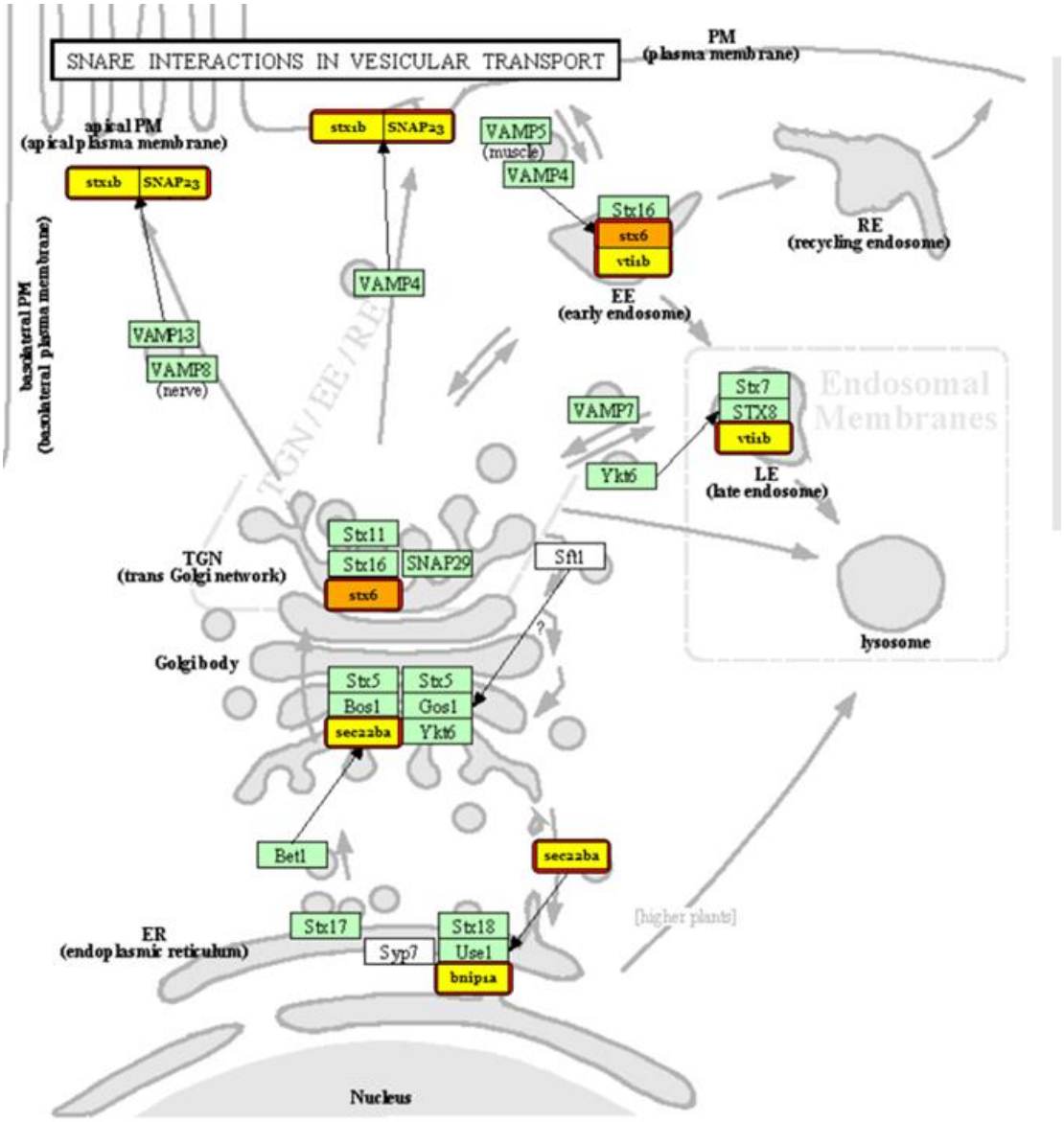

Figure 7. SNARE interaction in vesicular transport pathway of 7-day downregulated mir-203b & mir-725. The enriched target genes for mir-725 are highlighted in yellow

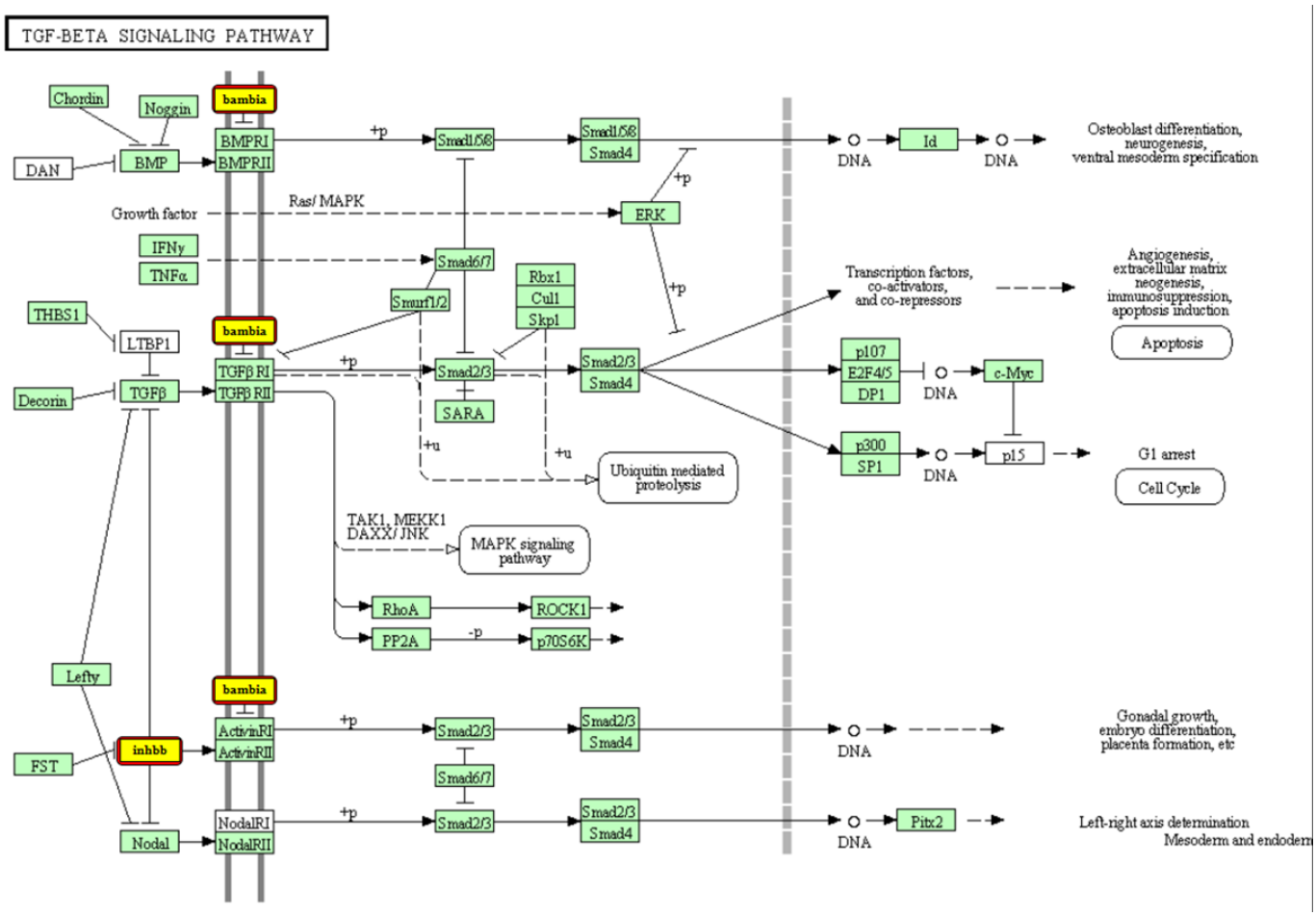

Figure 8. Tgf-β signaling pathway for 7-day downregulated mir-26a. The enriched target genes for mir-26a are highlighted in yellow.

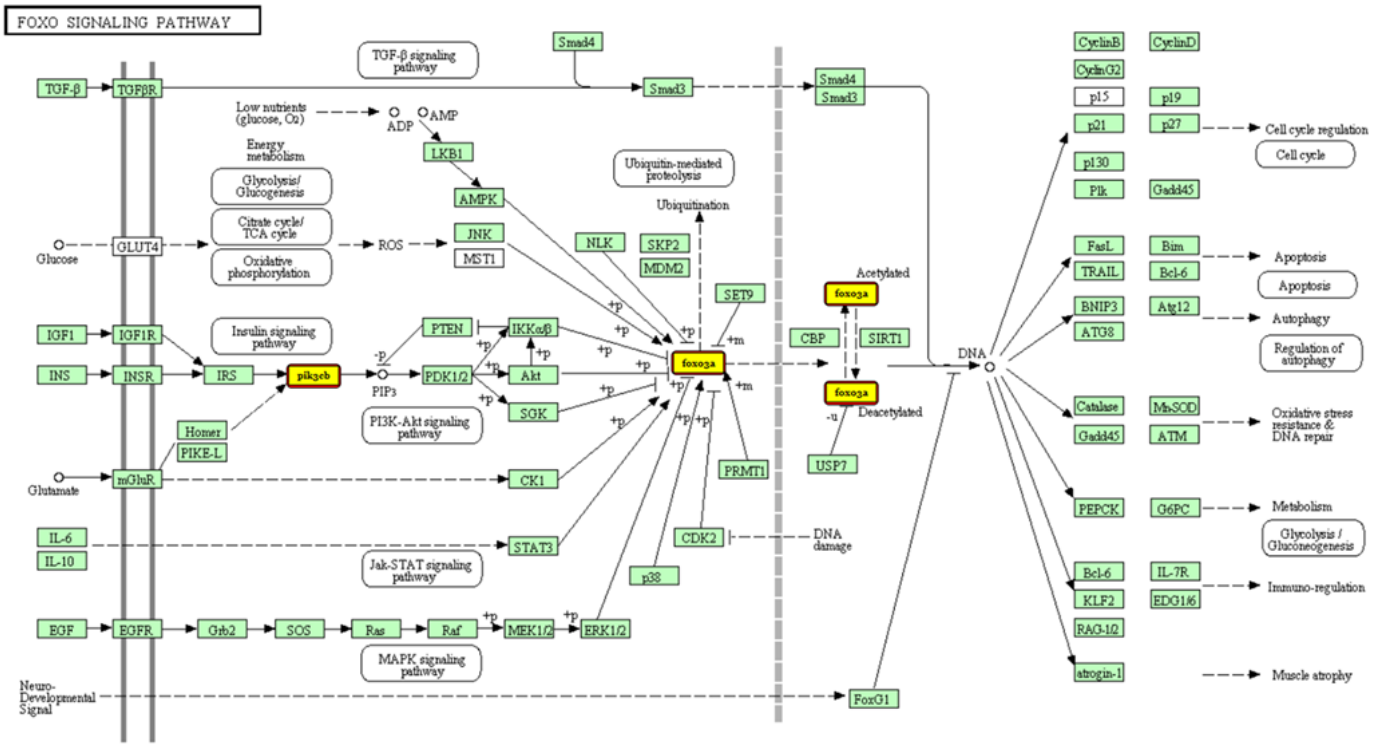

Figure 9. FoxO signaling pathway for 28-day upregulated mir-21. The enriched target genes for mir-21 are highlighted in yellow.

References

Accili, D., & Arden, K. C. (2004). FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell*, *117*(4), 421-426.

Ahmed, H., Du, S. J., O'Leary, N., & Vasta, G. R. (2003). Biochemical and molecular characterization of galectins from zebrafish (Danio rerio): notochord-specific expression of a prototype galectin during early embryogenesis. *Glycobiology*, *14*(3), 219-232.

Ameres, S. L., & Zamore, P. D. (2013). Diversifying microRNA sequence and function. *Nature reviews Molecular cell biology*, *14*(8), 475-488.

Almroth, B. C., Asker, N., Wassmur, B., Rosengren, M., Jutfelt, F., Gräns, A & Sturve, J. (2015). Warmer water temperature results in oxidative damage in an Antarctic fish, the bald notothen. *Journal of Experimental Marine Biology and Ecology*, *468*, 130-137.

Andreassen, R., Worren, M.M., Hoyheim, B. (2013). Discovery and characterization of miRNA genes in Atlantic salmon (Salmo salar) by use of a deep sequencing approach. *BMC Genomics*, 14, 482.

Ansaldo, M., Luquet, C.M., Evelson, P.A., Polo, J.M., Llesuy, S. (2000). Antioxidant levels from different Antarctic fish caught around South Georgia Island and Shag Rocks. *Polar Biol*, 23, 160–165.

Baggerly, K. A., Deng, L., Morris, J. S., & Aldaz, C. M. (2003). Differential expression in SAGE: accounting for normal between-library variation. *Bioinformatics*, *19*(12), 1477-1483.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell*, *136*(2), 215-233.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, *116*(2), 281-297.

Bartel, D. P., & Chen, C. Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nature Reviews Genetics*, *5*(5), 396-400.

Brennecke, J., Stark, A., Russell, R. B., & Cohen, S. M. (2005). Principles of microRNA–target recognition. *PLoS biology*, 3(3), e85.

Berridge, M. J. (1995). Calcium signalling and cell proliferation. *Bioessays*, *17*(6), 491-500.

Berthoud, V. M., Minogue, P. J., Laing, J. G., & Beyer, E. C. (2004). Pathways for degradation of connexins and gap junctions. *Cardiovascular research*, 62(2), 256-267.

Bi Y., Liu G., Yang R. (2009). MicroRNAs: Novel Regulators during the Immune Response. *J. Cell. Physiol,* 218, 467–472.

Bilyk, K. T., & Cheng, C. C. (2013). Model of gene expression in extreme cold-reference transcriptome for the high-Antarctic cryopelagic notothenioid fish Pagothenia borchgrevinki. *BMC genomics*, *14*(1), 634.

Bilyk, K. T. and Cheng, C.-H. C. (2014). RNA-seq analyses of cellular responses to elevated body temperature in the high Antarctic cryopelagic notothenioid fish Pagothenia borchgrevinki. *Mar. Genomics,* 18, 163-171.

Bilyk KT, DeVries AL. (2011) Heat tolerance and its plasticity in Antarctic fishes. *Comp Biochem Physiol,* 158A, 382–390.

Bilyk KT, Evans CW, DeVries AL. (2012) Heat hardening in Antarctic notothenioid fishes. *Polar Biol,* 35, 1447–1451.

Bonnet, E., Wuyts, J., Rouzé, P., & Van de Peer, Y. (2004). Evidence that microRNA precursors, unlike other non-coding RNAs, have lower folding free energies than random sequences. *Bioinformatics*, *20*(17), 2911-2917.

Buckley, B. A., Place, S. P., & Hofmann, G. E. (2004). Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, Trematomus bernacchii. *Journal of Experimental Biology*, *207*(21), 3649-3656.

Buckley, B. A. and Somero, G. N. (2009). cDNA microarray analysis reveals the capacity of the cold-adapted Antarctic fish Trematomus bernacchii to alter gene expression in response to heat stress. *Polar Biol,* 32, 403-415.

Buckley BA, Gracey AY, Somero GN. (2006). The cellular response to heat stress in the goby Gillichthys mirabilis: a cDNA microarray and protein-level analysis. *J Exp Biol*, 209, 2660–2677.

Chen YA, Scales SJ, Patel SM, Doung YC, Scheller RH. (1999). SNARE complex formation is triggered by Ca^{2+} and drives membrane fusion. *Cell*, 97, 165–174.

Chen, Z., Cheng, C.-H. C., Zhang, J., Cao, L., Chen, L., Zhou, L., Jin, Y., Ye, H., Deng, C., Dai, Z. et al. (2008). Transcriptomic and genomic evolution under constant cold in Antarctic notothenioid fish. *Proc. Natl. Acad. Sci,* 105, 12944-12949.

Dang Z, Balm PH, Flik G, Wendelaar Bonga SE, Lock RA. (2000). Cortisol increases Na^+/K^+-ATPase density in plasma membranes of gill chloride cells in the freshwater tilapia Oreochromis mossabicus. *J. Exp. Biol,* 203, 2349–2355.

Das, A., Chaudhury, S., Kalita, M. C., & Mondal, T. K. (2015). In silico identification, characterization and expression analysis of miRNAs in Cannabis sativa L. *Plant Gene*, *2*, 17-24.

DeVries, A.L., Cheng, C.H.C. (2005). Antifreeze proteins and organismal freezing avoidance in polar fishes. *Physiology of Polar Fishes,* 22, 152-201.

Di Paolo, G., & De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature*, 443(7112), 651-657.

Dittmar, J., Janssen, H., Kuske, A., Kurtz, J., & Scharsack, J. P. (2014). Heat and immunity: an experimental heat wave alters immune functions in three-spined sticklebacks (Gasterosteus aculeatus). *Journal of Animal Ecology*, 83(4), 744-757.

Dobrowolny, G., Bernardini, C., Martini, M., Baranzini, M., Barba, M., & Musarò, A. (2015). Muscle expression of SOD1G93A modulates microrna and mRNA transcription pattern associated with the myelination process in the spinal cord of transgenic mice. *Frontiers in cellular neuroscience*, *9*.

Dong, H., Lei H, Ding, L, Yongqiang Wen, Huangxian J, Zhang X. (2013). MicroRNA: Function, Detection, and Bioanalysis. *Chemical Reviews,* 113, 6207-6233.

Eastman, J. T. (1993). *Antarctic fish biology: evolution in a unique environment*. Academic Press.

Eastman, J.T. (2005). The nature of the diversity of Antarctic fishes. *Polar Biol*, 28, 94–107.

Enzor, L. A., Zippay, M. L. and Place, S. P. (2013). High latitude fish in a high CO2 world: synergistic effects of elevated temperature and carbon dioxide on the metabolic rates of Antarctic notothenioids. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol*, 164, 154-161.

Enzor, L. A., & Place, S. P. (2014). Is warmer better? Decreased oxidative damage in notothenioid fish after long-term acclimation to multiple stressors. *Journal of Experimental Biology*, *217*(18), 3301-3310.

Feder ME and Hofmann GE. (1999). Heat-shock proteins, molecular chaperones, and the stress response: *Evolutionary and ecological physiology*, *Annu Rev Physiol*, 61, 243–82.

Ferjani, A., & Maeshima, M. (2016). Multiple facets of H+-pyrophosphatase and related enzymes. *Frontiers in plant science*, 7.

Franklin, C. E., Davison, W. and Seebacher, F. (2007). Antarctic fish can compensate for rising temperatures: thermal acclimation of cardiac performance in Pagothenia borchgrevinki. *J. Exp. Biol*, 210, 3068-3074.

Flynt A, Thatcher E, Burkewitz K, Li N, Liu Y, Patton J. (2009). miR-8 microRNAs regulate the response to osmotic stress in zebrafish embryos. *Journal of Cell Biology*, 185, 115-127.

Frick, C. L., Yarka, C., Nunns, H., & Goentoro, L. (2017). Sensing relative signal in the Tgf-β/Smad pathway. *Proceedings of the National Academy of Sciences*, 201611428.

Frydman J. (2001). Folding of Newly Translated Proteins In Vivo: The Role of Molecular Chaperones. *Annu. Rev. Biochem*, 70, 603–47.

Gene Ontology Consortium. (2017). Expansion of the Gene Ontology knowledgebase and resources. *Nucleic acids research*, 45(D1), D331-D338.

Gracey, A. Y., Fraser, E. J., Li, W., Fang, Y., Taylor, R. R., Rogers, J., ... & Cossins, A. R. (2004). Coping with cold: an integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(48), 16970-16975.

Greer, E. L., & Brunet, A. (2005). FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene*, *24*(50), 7410-7425.

Gulyaeva, L. F., & Kushlinskiy, N. E. (2016). Regulatory mechanisms of microRNA expression. *Journal of Translational Medicine*, 14, 143.

Guo, X., & Wang, X. F. (2009). Signaling cross-talk between TGF-β/BMP and other pathways. *Cell research*, 19(1), 71-88.

Gusev, Y., Schmittgen, T. D., Lerner, M., Postier, R., & Brackett, D. (2007). Computational analysis of biological functions and pathways collectively targeted by co-expressed microRNAs in cancer. *BMC bioinformatics*, *8*(7), S16.

Hazel J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu. Rev. Physiol*, 57 19–42.

Hazel J. R., Williams E. E. (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog. Lipid Res*, 29 167–227.

He L and Hannon G.J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet*, 5, 522–531.

Herzog, B. H., Fu, J., & Xia, L. (2014). Mucin-type O-glycosylation is critical for vascular integrity. *Glycobiology*, *24*(12), 1237–1241.

Hofmann, G.E., Lund, S.G., Place, S.P., Whitmer, A.C. (2005). Some like it hot, some like it cold: the heat shock response is found in New Zealand but not Antarctic notothenioid fishes. *J. Exp. Mar. Biol. Ecol*, 316, 79–89.

Hou, J., Wang, P., Lin, L., Liu, X., Ma, F., An, H., ... & Cao, X. (2009). MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. *The Journal of Immunology*, *183*(3), 2150-2158.

Huang, H., & Tindall, D. J. (2007). Dynamic FoxO transcription factors. *J Cell Sci*, *120*(15), 2479-2487.

Huang, F., & Chen, Y. G. (2012). Regulation of TGF-β receptor activity. *Cell & bioscience*, *2*(1), 9.

Hudson, H. A., Brauer, P. R., Scofield, M. A. and Petzel, D. H. (2008). Effects of warm acclimation on serum osmolality, cortisol and hematocrit levels in the Antarctic fish, Trematomus bernacchii. *Polar Biol,* 31, 991-997.

Huth, T. J. and Place, S. P. (2013). De novo assembly and characterization of tissue specific transcriptomes in the emerald notothen, Trematomus bernacchii. *BMC Genomics,* 14, 805.

Huth, T. J. and Place, S. P. (2016a). Transcriptome wide analyses reveal a sustained cellular stress response in the gill tissue of Trematomus bernacchii after acclimation to multiple stressors. *BMC Genomics,* 17, 127.

Huth, T. J., & Place, S. P. (2016). RNA-seq reveals a diminished acclimation response to the combined effects of ocean acidification and elevated seawater temperature in Pagothenia borchgrevinki. *Marine genomics*, 28, 87-97.

Hum, D. W., Bélanger, A., Lévesque, É., Barbier, O., Beaulieu, M., Albert, C. & Dubois, S. (1999). Characterization of UDP-glucuronosyltransferases active on steroid hormones. *The Journal of steroid biochemistry and molecular biology*, *69*(1), 413-423.

Jing, L., Hou, Y., Wu, H., Miao, Y., Li, X., Cao, J., ... & Zhao, S. (2015). Transcriptome analysis of mRNA and miRNA in skeletal muscle indicates an important network for differential Residual Feed Intake in pigs. *Scientific reports*, *5*.

Johnston, I., Calvo, J., Guderley, Y, H., Fernandez, D. and Palmer, L. (1998). Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. *J. Exp. Biol*, 201, 1-12.

Josephy, P. D., Mannervik, B., & De Montellano, P. O. (1997). Hydrogen peroxide, catalase and peroxidases. *Molecular toxicology*, 81-89.

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., & Morishima, K. (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic acids research*, *45*(D1), D353-D361.

Klein, R. D., Borges, V. D., Rosa, C. E., Colares, E. P., Robaldo, R. B., Martinez, P. E., & Bianchini, A. (2017). Effects of increasing temperature on antioxidant defense system and oxidative stress parameters in the Antarctic fish Notothenia coriiceps and Notothenia rossii. *Journal of Thermal Biology*.

Koyasu, S. (2003). The role of PI3K in immune cells. *Nature immunology*, *4*(4), 313-319.

Kultz, D. (2005). Molecular and Evolutionary Basis of the Cellular Stress Response. *Annual Reviews Physiology*, 67, 225-257.

Kuwabara I, Kuwabara Y, Yang R, Schuler M, Green D, Zuraw B, Hsu D, Liu F.(2002). Galectin-7 (PIG1) exhibits pro-apoptotic function through JNK activation and mitochondrial cytochrome c release. *J Biol Chem*, 277, 3487–3497.

Laing, J. G., Tadros, P. N., Green, K., Saffitz, J. E., & Beyer, E. C. (1998). Proteolysis of connexin43-containing gap junctions in normal and heat-stressed cardiac myocytes. *Cardiovascular research*, *38*(3), 711-718.

Leung A. K., Sharp P. A. (2010). MicroRNA functions in stress responses. *Mol. Cell*, 40, 205–215.

Li X, Cassidy JJ, Reinke CA, Fischboeck S, Carthew RW. (2009). A microRNA imparts robustness against environmental fluctuation during development. *Cell*, 137, 273–282.

Li, Y., & Shi, X. (2013). MicroRNAs in the regulation of TLR and RIG-I pathways. *Cellular & molecular immunology*, *10*(1), 65-71.

Liu F. T., Rabinovich G. A. (2010). Galectins: regulators of acute and chronic inflammation. *Annals of the New York Academy of Sciences*, 1183, 158–182.

Liu, Y., & Bankaitis, V. A. (2010). Phosphoinositide phosphatases in cell biology and disease. *Progress in lipid research*, *49*(3), 201-217.

Liu, X., Tu, J., Liu, X., Yuan, J., Liu, X., Zhao, L., Dawar, F.U., Khattak, M.N.K., Hegazy, A.M. (2016). Identification and characterization of MicroRNAs in Snakehead Fish Cell line upon Snakehead Fish Vesiculovirus Infection. *Int. J. Mol Sci*, 17, 154.

Lu, P., Takai, K., Weaver, V. M., & Werb, Z. (2011). Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harbor perspectives in biology*, *3*(12), a005058.

Lutz, M., & Knaus, P. (2002). Integration of the TGF-β pathway into the cellular signalling network. *Cellular signalling*, *14*(12), 977-988.

Machado, C., Zaleski, T., Rodrigues, E., Carvalho, C., dos, S., Cadena, S. M., Gozzi, G. J., Krebsbach, P., Rios, F. S. and Donatti, L. (2014). Effect of temperature acclimation on the liver antioxidant defence system of the Antarctic notothenioids Notothenia coriiceps and Notothenia rossii. *Comp. Biochem. Physiol*, 172-173B, 21-28

Maragkakis, M., Reczko, M., Simossis, V. A., Alexiou, P., Papadopoulos, G. L., Dalamagas, T., ... & Vergoulis, T. (2009). DIANA-microT web server: elucidating microRNA functions through target prediction. *Nucleic acids research*, *37*(suppl_2), W273-W276.

Martin, P. E., Errington, R. J., & Evans, W. H. (2001). Gap junction assembly: multiple connexin fluorophores identify complex trafficking pathways. *Cell communication & adhesion*, 8(4-6), 243-248.

McCormick SD. (1995). Hormonal control of gill Na1, K1-ATPase and chloride cell function. Fish Physiology, 285–315. Academic Press.

McCormick, S. D. (2001). Endocrine control of osmoregulation in teleost fish. *American zoologist*, *41*(4), 781-794.

Meredith, M. P., & King, J. C. (2005). Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century. *Geophysical Research Letters*, *32*(19).

Mi, H., Huang, X., Muruganujan, A., Tang, H., Mills, C., Kang, D., & Thomas, P. D. (2017). PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic acids research*, *45*(D1), D183-D189.

Mindnich R, Moller G & Adamski J. (2004). The role of 17 betahydroxysteroid dehydrogenases. *Molecular and Cellular Endocrinology*, 218, 7–20.

Moresco, E. M. Y., LaVine, D., & Beutler, B. (2011). Toll-like receptors. *Current Biology*, *21*(13), R488-R493.

Morimoto RI. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & Development*, 12, 3788–3796.

Mueller, I. A., Grim, J. M., Beers, J. M., Crockett, E. L. and O'Brien, K. M. (2011). Inter-relationship between mitochondrial function and susceptibility to oxidative stress in red- and white-blooded Antarctic notothenioid fishes. *J. Exp. Biol*, 214, 3732-3741.

Musil, L. S., Le, A. C. N., VanSlyke, J. K., & Roberts, L. M. (2000). Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *Journal of Biological Chemistry*, 275(33), 25207-25215.

Nakajima, K. I., Hirose, H., Taniguchi, M., Kurashina, H., Arasaki, K., Nagahama, M. & Tagaya, M. (2004). Involvement of BNIP1 in apoptosis and endoplasmic reticulum membrane fusion. *The EMBO journal*, *23*(16), 3216-3226.

Near T.J, Dornburg A, Kuhn K, Eastman, J, Pennington J, Patarnello T, Zane L, Fernandez D and Jones C.D. (2012). Ancient climate change, antifreeze, and the evolutionary diversification of Antarctic fishes**.** *PNAS,* 109 (9), 3434-3439.

Nebert, D. W., & Dalton, T. P. (2006). The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. *Nature Reviews Cancer*, *6*(12), 947-960.

Nebert, D. W., & Vasiliou, V. (2004). Analysis of the glutathione S-transferase (GST) gene family. *Human genomics*, *1*(6), 460.

Nie, W., Jin, L., Wang, Y., Wang, Z., & Guan, X. (2013). The bioinformatics analysis of miRNAs signatures differentially expressed in HER2 (+) versus HER2 (−) breast cancers. *Cancer Biotherapy and Radiopharmaceuticals*, *28*(1), 71-76.

O'Brien, K. M. and Sidell, B. D. (2000). The interplay among cardiac ultrastructure, metabolism and the expression of oxygen-binding proteins in Antarctic fishes. *J. Exp. Biol,* 203, 1287-1297.

O'Connell RM, Rao DS, Baltimore D. (2012). microRNA regulation of inflammatory responses. *Annual review of immunology*, 30, 295–312.

Onichtchouk, D., Chen, Y. G., Dosch, R., Gawantka, V., Delius, H., Massague, J., & Niehrs, C. (1999). Silencing of TGF-β signalling by the pseudoreceptor BAMBI. *Nature*, *401*(6752), 480-485.

Ordas, A., Kanwal, Z., Lindenberg, V., Rougeot, J., Mink, M., Spaink, H.P., Meijer, A.H. (2013). MicroRNA-146 function in the innate immune transcriptome response of zebrafish embryos to Salmonella typhimurium infection. *BMC Genomics*, 14, 696.

Pelis, R. M., & McCormick, S. D. (2001). Effects of growth hormone and cortisol on Na+–K+–2Cl− cotransporter localization and abundance in the gills of Atlantic salmon. *General and comparative endocrinology*, *124*(2), 134-143.

Perry, A.L., Low, P.J., Ellis, J.R. & Reynolds, J.D. (2005). Climate change and distribution shifts in marine species. *Science*, 308, 1912–1915.

Petricorena, Z.C., Somero, G.N. (2007). Biochemical adaptations of notothenioid fishes: Comparisons between cold temperate South American and New Zealand species and Antarctic species. *Comparative Biochemistry and Physiology*, 147, 799-807.

Pfeffer, S. R., Yang, C. H., & Pfeffer, L. M. (2015). The Role of miR-21 in Cancer. *Drug development research*, *76*(6), 270-277.

Philip, A. M., & Vijayan, M. M. (2015). Stress-immune-growth interactions: cortisol modulates suppressors of cytokine signaling and JAK/STAT pathway in rainbow trout liver. *PloS one*, *10*(6), e0129299.

Place RF, Noonan EJ. (2014). Non-coding RNAs turn up the heat, an emerging layer of novel regulators in the mammalian heat shock response. *Cell Stress Chaperones*, 19(2), 159–172.

Place SP, Hofmann GE. (2005). Constitutive expression of a stress-inducible heat shock protein gene, hsp70, in phylogenetically distant Antarctic fish. *Polar Biology*, 28, 261-267.

Place, S.P., Zippay, M.L., Hofmann, G.E. (2004). Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible hsp70 gene in Antarctic notothenioid species. *Am. J. Physiol*, 287, 429–436.

Pörtner, H. O., Lucassen, M., & Storch, D. (2005). Metabolic biochemistry: its role in thermal tolerance and in the capacities of physiological and ecological function. *Fish physiology*, *22*, 79-154.

Rebl, A., Verleih, M., Köbis, J. M., Kühn, C., Wimmers, K., Köllner, B., & Goldammer, T. (2013). Transcriptome profiling of gill tissue in regionally bred and globally farmed rainbow trout strains reveals different strategies for coping with thermal stress. *Marine biotechnology*, *15*(4), 445-460.

Robinson E, Davison W. (2008. a) Antarctic fish can survive prolonged exposure to elevated temperatures. *J Fish Biol*, 73, 1676–1689.

Robinson E, Davison W. (2008. b) The Antarctic notothenioid fish Pagothenia borchgrevinki is thermally flexible: acclimation changes oxygen consumption. *Polar Biol*, 31, 317–326.

Rothman, J. E. (1994). Mechanisms of intracellular protein transport. *Nature*, *372*(6501), 55-63.

Jahn, R., & Scheller, R. H. (2006). SNAREs—engines for membrane fusion. *Nature reviews Molecular cell biology*, *7*(9), 631-643.

Rowland A., Miners J. O., Mackenzie P. I. (2013). The UDP-glucuronosyltransferases: their role in drug metabolism and detoxification. Int. J. Biochem. *Cell Biol*. 45, 1121–1132.

Russell, R. G. G. (1976). Metabolism of inorganic pyrophosphate (PPi). *Arthritis & Rheumatology*, *19*(S3), 465-478.

Ryan, S. N. (1995). The effect of chronic heat stress on cortisol levels in the antarctic fishPagothenia borchgrevinki. *Experientia*, *51*(8), 768-774.

Saba, R., Sorensen, D. L., & Booth, S. A. (2014). MicroRNA-146a: a dominant, negative regulator of the innate immune response. *Frontiers in immunology*, *5*.

Salameh, A. (2006). Life cycle of connexins: regulation of connexin synthesis and degradation. In *Cardiovascular Gap Junctions* (Vol. 42, pp. 57-70). Karger Publishers.

Schardt, A., Brinkmann, B. G., Mitkovski, M., Sereda, M. W., Werner, H. B., & Nave, K. A. (2009). The SNARE protein SNAP-29 interacts with the GTPase Rab3A: Implications for membrane trafficking in myelinating glia. *Journal of neuroscience research*, *87*(15), 3465-3479.

Schyth, B. D., Bela-ong, D. B., Jalali, S. A. H., Kristensen, L. B. J., Einer-Jensen, K., Pedersen, F. S., & Lorenzen, N. (2015). Two virus-induced microRNAs known only from teleost fishes are orthologues of micrornas involved in cell cycle control in humans. *PloS one*, *10*(7), e0132434.

Shin, S. C., Kim, S. J., Lee, J. K., Ahn, D. H., Kim, M. G., Lee, H., Lee, J., Kim, B.-K. and Park, H. (2012). Transcriptomics and comparative analysis of three Antarctic notothenioid fishes. PLoS ONE 7, e43762.

Sidell, B.D., O'Brien, K.M. (2006). When bad things happen to good fish: the loss of hemoglobin and myoglobin in Antarctic icefishes. *J. Exp. Biol*, 209, 1791–1802.

Sidell, B. D., Crockett, E. L. and Driedzic, W. R. (1995). Antarctic fish tissues preferentially catabolize monoenoic fatty acids. *J. Exp. Zool*, 271, 73-81.

Sies, H. (1999). Glutathione and its role in cellular functions. *Free Rad. Biol Med*, 27, 916–921.

Sleadd, I. M., & Buckley, B. A. (2013). The CCAAT/enhancer-binding protein δ (C/EBP-δ) transcription factor is heat inducible in the cold-adapted antarctic fish Trematomus bernacchii. *Polar biology*, *36*(3), 335-342.

Söllner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., & Rothman, J. E. (1993). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell*, *75*(3), 409-418.

Sonkoly, E., Ståhle, M., & Pivarcsi, A. (2008, April). MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. In *Seminars in cancer biology* (Vol. 18, No. 2, pp. 131-140). Academic Press.

Stamenkovic I. (2003). Extracellular matrix remodelling: the role of matrix metalloproteinases. *Journal of Pathology*, 200,448–464.

Staudacher, E. (2015). Mucin-type O-glycosylation in invertebrates. *Molecules*, *20*(6), 10622-10640.

Sunkar R., Kapoor A., Zhu J. K. (2006). Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by down-regulation of miR398 and important for oxidative stress tolerance. *Plant Cell*,18, 2051–2065.

Tan, K., & Lawler, J. (2009). The interaction of Thrombospondins with extracellular matrix proteins. *Journal of Cell Communication and Signaling*, *3*(3-4), 177–187.

Taganov, K. D., Boldin, M. P., Chang, K. J., & Baltimore, D. (2006). NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proceedings of the National Academy of Sciences*, *103*(33), 12481-12486.

Takahashi, H., & Sakamoto, T. (2013). The role of 'mineralocorticoids' in teleost fish: relative importance of glucocorticoid signaling in the osmoregulation and 'central'actions of mineralocorticoid receptor. *General and comparative endocrinology*, *181*, 223-228.

Tian E, Ten Hagen KG. (2009). Recent insights into the biological roles of mucin-type O-glycosylation. *Glycoconj J*, 26(3), 325–334.

Todgham, A.E., Hoaglund, E.A. and Hofmann, G.E. (2007). Is cold the new hot?: Elevated ubiquitin conjugated protein levels in tissues of Antarctic fish as evidence for cold-denaturation of proteins in vivo. *J. Comp. Physiol*, 177, 857-866.

Vasta, G. R., Ahmed, H., Du, S. J., & Henrikson, D. (2004). Galectins in teleost fish: Zebrafish (Danio rerio) as a model species to address their biological roles in development and innate immunity. *Glycoconjugate journal*, *21*(8), 503-521.

Vlachos, I. S., Kostoulas, N., Vergoulis, T., Georgakilas, G., Reczko, M., Maragkakis & Hatzigeorgiou, A. G. (2012). DIANA miRPath v. 2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic acids research*, *40*(W1), W498-W504.

Vlachos, I. S., Zagganas, K., Paraskevopoulou, M. D., Georgakilas, G., Karagkouni, D., Vergoulis, T & Hatzigeorgiou, A. G. (2015). DIANA- mirPath v3. 0: deciphering microRNA function with experimental support. *Nucleic acids research*, *43*(W1), W460-W466.

von Andrian UH, Mempel TR. (2003). Homing and cellular traffic in lymph nodes. *Nat Rev Immunol*, 3:867–78.

Wiater, E., & Vale, W. (2003). Inhibin is an antagonist of bone morphogenetic protein signaling. *Journal of Biological Chemistry*, 278(10), 7934-7941.

Wilson, R. S., Franklin, C. E., Davison, W., & Kraft, P. (2001). Stenotherms at sub-zero temperatures: thermal dependence of swimming performance in Antarctic fish. *Journal of Comparative Physiology B*, *171*(4), 263-269.

Windisch HS, Kathöver R, Pörtner HO, Frickenhaus S, Lucacssen M. (2011). Thermal acclimation in Antarctic fish: transcriptomic profiling of metabolic pathways. *Am J Phys Reg, Integr Comp Phys*, 301(5), 1453–1466.

Wrana, J. L. (2000). Regulation of Smad activity. *Cell*, 100(2), 189-192.

Xie, Y. F., Shu, R., Jiang, S. Y., Song, Z. C., Guo, Q. M., Dong, J. C., & Lin, Z. K. (2014). miRNA-146 negatively regulates the production of pro-inflammatory cytokines via NF-κB signalling in human gingival fibroblasts. *Journal of Inflammation*, *11*(1), 38.

Yang, R. Y., & Liu, F. T. (2003). Galectins in cell growth and apoptosis. *Cellular and molecular life sciences*, *60*(2), 267-276.

Yates, L. A., Norbury, C. J., & Gilbert, R. J. (2013). The long and short of microRNA. *Cell*, *153*(3), 516-519.

Yin C, Salloum FN, Kukreja RC. (2009) A novel role of microRNA in late preconditioning: upregulation of endothelial nitric oxide synthase and heat shock protein 70. *Circ Res*, 104, 572–575.

Zhang, J., Liu, Y., Zhang, X., Pan, J., Nie, Z., Zhang, W., ... & Zhang, Y. (2013). The identification of microRNAs in the whitespotted bamboo shark (Chiloscyllium plagiosum) liver by Illumina sequencing. *Gene*, *527*(1), 259-265.

Zhang, B.C., Zhang, J., Sun, L. (2014). In-depth profiling and analysis of host and viral microRNAs in Japanese flounder (Paralichthys olivaceus) infected with megalocytivirus reveal involvement of microRNAs in host-virus interaction in teleost fish. *BMC Genomics*, 15, 878.

Zhang, X., Tang, N., Hadden, T. J., & Rishi, A. K. (2011). Akt, FoxO and regulation of apoptosis. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1813*(11), 1978-1986.

Zuker, Michael. (2003). Mfold Web Server for Nucleic Acid Folding and Hybridization Prediction. *Nucleic acids research*. 31. 3406-15. 10.1093/nar/gkg595.