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MASTER THESIS

Thermal plasticity of mRNA and miRNA expression in zebrafish (*Danio rerio*) fast muscle

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Abstract

Myogenesis is a complex process that is markedly affected by environmental factors. In particular, the phenotype of myotomal muscle in zebrafish (*Danio rerio*) is known to undergo modifications in response to temperature changes. Despite of their demonstrated importance in numerous biological processes, the role of microRNAs (miRNAs) in regulation of muscle growth and adaptation to environmental changes is still poorly understood in fish. To investigate the thermal plasticity of miRNA expression and their role in myogenesis and thermal acclimation, zebrafish were reared from fertilized eggs to adulthood at three different temperatures (24.3 ± 0.8 ºC, 28.3 ± 0.3 ºC, and 33.4 ± 0.4 ºC). Expression profile analysis revealed differentially expressed miRNAs and mRNAs, which were affected by rearing temperature. Many differentially expressed mRNAs were involved in muscle structure development and contraction, detection of cell damage, energy metabolism and response to oxidative stress. Most miRNAs displaying significant changes in mature transcript levels were not previously reported as miRNAs related to temperature response and muscle development. For example, *miR-218a, miR-200a, miR-100, miR-99, miR-26b, miR-101b* and *let-7e* were shown to be the most significantly differentially expressed miRNAs associated with long-term cold and warm acclimation. Analysis of their putative interaction with differentially expressed mRNAs suggested that these miRNAs may have a role in myogenesis and muscle recovery. This study contributed to our limited understanding of the role of miRNAs in acclimation and muscle development in fish.

Abbreviations

DDR	DNA damage/repair pathway
HMG	High mobility group proteins
HSP	Heat shock proteins
lncRNA	Long non-coding RNA
miRNA	MicroRNA
MPC	Myogenic progenitor cells
MRF	Myogenic regulatory factors
mRNA	Messenger RNA
ncRNA	Non protein-coding RNA
NGS	Next generation sequencing
piRNA	Piwi protein-interacting RNA
RISC	RNA-induced silencing complex
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
UTR	Untranslated region

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1. Introduction

1.1. Zebrafish as a model organism

The zebrafish *(Danio rerio)* is a tropical freshwater fish, belonging to the minnow family (*Cyprinidae*) of order Cypriniformes. It was first proposed as a model organism in the 1970s by George Streisinger at the University of Oregon (Howe et al., 2013). Since then, zebrafish became a useful model organism in genetics and in developmental biology of vertebrates due to their research advantages, which combine the best features of all the other models. First of all, zebrafish are vertebrates, unlike other popular genetics model organisms such as fruit flies (*Drosophila melanogaster*) and nematode worms (*Caenorhabditis elegans*). It is a small organism, which develops relatively fast: at 28 °C, it hatches as a larva at 48-72 hours and becomes sexually mature at about 90 days after fertilization. Zebrafish embryos are transparent, therefore they can be viewed and manipulated at all developmental stages. It also has a short reproduction cycle (3 to 4 months) and produces large numbers of offspring per mating. Each female zebrafish can produce up to 200 eggs per week (Howe et al., 2013; Lawrence, 2007). Moreover, it is convenient to work with zebrafish, because it is cheap and easy to maintain under laboratory conditions. Its genome has been fully sequenced and consists about of 1.75 Gb on 25 pairs of chromosomes, which possesses about 26,000 protein-coding genes. The order of genes along parts of chromosomes (synteny) is often conserved between vertebrates (Broughton et al., 2001). Such features make zebrafish perfect for genetic manipulation. A major advantage of zebrafish as a model species is the possibility of performing gene knockdowns and knockouts to elucidate gene function and for the development of disease models (Harper and Lawrence, 2010). Gene knockdown is a technique, by which the expression of one or more of an organism's genes are reduced, whereas gene knockout is a technique in which one of an organism's genes is made inoperative. The main method of gene knockdown in zebrafish involves the use of morpholinos, which are modified antisense oligonucleotides that block translation of mRNAs or interfere with correct splicing of mRNA. Gene knockouts can be achieved using the recently developed CRISPR/Cas9 system, in which bacterially derived protein Cas9 and a synthetic guide RNA used to introduce a double strand break at a specific location within the genome (Kelly and Hurlstone, 2011).

There are three types of genetic strains of zebrafish: wild-type, mutant and transgenic. Various wild-type zebrafish strains are often used in scientific research (for example AB and TAB strains). Transgenic zebrafish possess DNA from other species that has been inserted

into their genome. Mutant zebrafish carry one or more mutations, which can be spontaneous or chemically induced. There are many transgenic and mutant strains of zebrafish that are used to study different aspects of organism's development, physiology and diseases (Harper and Lawrence, 2010).

1.2. Myogenesis in zebrafish

Zebrafish myogenesis begins in early embryonic life and is modulated by environmental stimuli. The fundamental events in myogenesis are the specification of stem cells to a myogenic lineage (myoblasts), proliferation, cell cycle exit, differentiation, migration and fusion (Johnston, 2006). Embryonic muscle develops in somites, which are formed in pairs along the body axis separated by the notochord and neural tube (Johnston et al., 2011). During zebrafish embryogenesis, the myotome in the somites contains the myogenic progenitor cells (MPCs) that will evolve into the skeletal muscle. The myotome determination is regulated by a complex gene regulatory network that includes a member of T-box family (for example *tbx6*) and is controlled by two pathways: Akt and Notch. The Notch pathway controls cyclic genes involved in the generation of somites, and Wnt and fibroblast growth factor signaling have been shown to control activity of this pathway (Bentzinger et al., 2012). The formation of muscle fibers during embryogenesis is regulated by myogenic regulatory factors (MRFs), namely as *myoD, myf5, mrf4* and *myog*. These transcription factors are highly conserved in teleosts and amniotes (Johnston, 2006). *Myog* works together with other transcription factors to induce and maintain differentiation and the initiation of muscle-specific gene expression (Hinits and Hughes, 2007). The myoblast fuse into multi-nucleated fibers called myotubes and form muscle fibers. The slow muscles in zebrafish are formed from one kind of the MPCs called adaxial cells. The specification of these cells requires signaling from a family of proteins called hedgehog, which is induced by expression of specific transcription factor called *Blimp-1*. The differentiation and morphogenesis of the fast muscle cells are triggered by the migration of adaxial cells. Expression of fast-muscle specific genes also requires several transcription factors such as *Pbx, Six1* and *Six4* (Johnston et al., 2011).

Myotube production and the formation of new fast muscle fibres (hyperplasia) in zebrafish continues until around 40% of the maximum body length and when the final fiber number is established, myotube formation stops. Thereafter, the further growth involves only an increase in fibre length and diameter (hypertrophy) (Johnston et al., 2011). Muscle growth

is regulated by the growth hormone, which acts directly through receptors on the muscle sarcolemma and indirectly by initiating the production and release of insulin-like growth factor (IGF). The IGF system is one of the central pathways regulating protein synthesis in skeletal muscle (Ge et al., 2011; Johnston et al., 2011). The phenotype of myotomal muscle can undergo modifications throughout the life cycle due to the migration, spawning and environmental conditions (Johnston et al., 2011). Muscle regeneration is also part of myogenesis in adults, but unlike embryonic muscle formation, muscle regeneration in adults depends on the injured tissue retaining extracellular matrix scaffolding that serves as a template for the formation of muscle fibers. Tissue regeneration in mature skeletal muscle is provided by MPCs, also called satellite cells in mammals. The specialization of these cells have a higher dependence on extrinsic regulatory factors compared with embryonic stem cells, which rely to a higher degree on intrinsic programming (Bentzinger et al., 2012).

1.3. Influence of temperature on zebrafish development and growth

Zebrafish behaviour, physiology, and development are affected by a number of environmental factors, such as temperature, photoperiod, salinity, oxygen level, food ability, pH, and fish density (Jin et al., 2010; Johnston et al., 2011; Mugoni et al., 2014; Parichy et al., 2009; Sawant et al., 2001; Scott and Johnston, 2012; Sfakianakis et al., 2011; Sfakianakis et al., 2012; Uliano et al., 2010; Ulloa et al., 2013; Villamizar et al., 2012; Villamizar et al., 2014). Among these environmental factors, temperature has the greatest impact and is considered as the ‘‘abiotic master factor’’ regulating physiology of ectothermic animals (Lawrence, 2007). In the wild, zebrafish is indigenous to South Asia and broadly distributed in parts of India, Pakistan, Bangladesh, Nepal, Myanmar, Bhutan and Burma. These areas have a typical monsoon climate with marked seasonal fluctuations. Wide daily (of ∼5 °C during the day) and seasonal fluctuations (from 6 °C in winter to ∼38 °C in summer) of temperatures can be observed in these waters. Data from the laboratory experiments also shown that zebrafish have a maximum thermal tolerance range of 6.7 to 41.7 °C; therefore, they can be classified as eurythermal animals (Lawrence, 2007).

The physiological and molecular mechanisms, which are underlying the tolerance to daily and seasonal temperature variation in ectothermic species, have attracted significant attention in an attempt to understand the potential ecological impacts of global climate change. Temperature has profound effects on the biological systems functions and requires from the organisms the ability to change the physiological phenotype according to the

seasonal temperature conditions (Johnston and Temple, 2002). Phenotypic plasticity is crucial to the ectothermic organisms, because all aspects of their physiology are directly dependent on their thermal environment. The process of phenotype adjustments in an individual or in a tissue, which occurs within the framework of the single genome in response to temperature changes, is called thermal acclimation (Price et al., 2003). Many aspects of cellular biochemistry are involved in this acclimation process. It is based on the producing proteins that are relatively insensitive to temperature and degradation of damaged proteins. Acclimation may also cause changes in the content of membrane lipid and the degree of fatty acid unsaturation, changing in metabolic processes, recruiting different muscle fiber types and synthesizing molecular chaperones in response to changing temperature (Scott and Johnston, 2012).

Several studies shown that temperature is one of the most important environmental factors that modulates zebrafish growth, with major effects on developmental processes at all stages of the life cycle. First of all, temperature has a marked effect on general developmental rate and somitogenesis (Kimmel et al., 1995; Schröter et al., 2008) and larval growth. For instance, Schröter et al. (2008) raised zebrafish between 20 °C and 30 °C and found that somitogenesis frequency depended linearly on temperature and occurred faster at higher temperatures (the temperature coefficient Q_{10}, which describes the fold change in the rate of a process across a 10 °C interval for somitogenesis, was 2.8). These results were also confirmed by Parichy et al. (2009), where zebrafish larvae were raised at three temperatures (24 °C, 28.5 °C and 33 °C) and had significantly different growth rates. It was found that fish from 24 °C group grew slower than 33 °C group fish. A paper by Georga and Koumoundouros (2010) showed that developmental temperature significantly affected the body shape of zebrafish. Sfakianakis et al. (2011) also found that early life temperature had significant effect on muscle ontogeny and body shape, and therefore also had an effect on swimming performance of zebrafish. Moreover, temperature affected zebrafish sex differentiation and hatching (Sfakianakis et al., 2012; Villamizar et al., 2012). Also, water temperature significantly affects zebrafish metabolism, muscle composition and muscle development. Johnston and colleagues (2009) showed that different embryonic temperatures had influence on the recruitment of fast muscle fibers. Total length was larger at 26 °C compared to 22 °C and 31 °C temperature groups. Moreover, fiber number in the 26 °C group was 19% higher than that from the 22 °C and 14% higher than that from the 31 °C treatments. The transcriptional response and changes in energy metabolism of zebrafish in long-term warm and cold acclimation was studied in work of Vergauwen et al. (2010). They performed

thermal acclimation for 4, 14 and 28 days of fish to 34 °C and 18 °C and obtained the up-regulation of heat shock proteins transcripts in warm acclimation, but not in the cold one. They also observed the depletion of energy stores and decrease in condition factor due to warm acclimation at the early stages of zebrafish development, which recovered after 28 days of warm acclimation due to down-regulation of transcripts involved in catabolic processes. Overall, they suggested that warm acclimation was more stressful than the cold one (Vergauwen et al., 2010).

1.4. Regulatory non protein-coding RNA

The term non protein-coding RNA (ncRNA) is commonly employed to functional RNA molecules that are transcribed from DNA but are not translated into proteins (Collins et al., 2011; Fu, 2014; Mattick and Makunin, 2006). Recent evidence from high-throughput transcriptome analyses suggests that the vast majority of the genomes of mammals and other organisms are transcribed as ncRNAs (Fu, 2014; Kaikkonen et al., 2011). ncRNA genes can be located within introns of protein-coding genes or in intergenic regions, and their size can vary from less than 20 nucleotides (nt) to tens of kilobases (Kb) (Collins et al., 2011).

Although ncRNAs are not part of the mRNA transcriptome, they still have essential roles within the cell. There is increasing evidence that ncRNAs mainly fulfill regulatory roles during development, in response to stress and influences of the environment. These RNAs are expressed at substantially lower levels than mRNAs and have a hidden layer of internal signals that control gene expression, chromatin structure, transcription, splicing, RNA editing, translational inhibition, mRNA destruction, or epigenetic memory. Therefore, ncRNAs have essential roles in the physiology and development of organisms (Amaral and Mattick, 2008; Bizuayehu and Babiak, 2014; Collins et al., 2011; Hossain et al., 2012; Huang et al., 2012; Kaikkonen et al., 2011; Zhou et al., 2010). It is known that ncRNAs can interact with genes to up- or down-regulate their expression, to silence translation or even to guide methylation. In particular, some long ncRNAs and miRNAs play essential roles in epigenetic phenomena (Chuang and Jones, 2007; Sato et al., 2011; Tammen et al., 2013; Zhou et al., 2010). There are two broad classes of functional ncRNAs – long ncRNAs and small ncRNAs.

1.4.1. Long ncRNAs

Long ncRNAs (lncRNAs) are non-protein-coding transcripts that are longer than 200 nt. This class of ncRNA is not well studied. Many lncRNAs can be primary transcripts

for the production of short RNAs, which makes the categorization of this group of RNAs very ambiguous (Kaikkonen et al., 2011). However, it is known that lncRNAs have a low level of transcription and, therefore, it is possible that a significant portion of these RNAs performs regulatory functions (Makarova and Kramerov, 2007).

Different mechanisms of transcriptional regulation by lncRNAs have been proposed and the genomic location of these RNAs is an important facet of their regulatory potential. Transcription of lncRNAs regulates the expression of genes in close genomic proximity (*cis*-acting regulation) but can also target distant transcriptional activators or repressors (*trans*-acting) via a variety of mechanisms. Transcription of a lncRNA may promote the accessibility of protein-coding genes to RNA polymerases and lncRNA sequences may themselves convey functions through binding to DNA or proteins (Ponting et al., 2009). Some lncRNAs can regulate transcription indirectly by controlling the sub-cellular localization of transcription factors (Huang et al., 2012; Ponting et al., 2009). lncRNAs also regulate various aspects of post-transcriptional mRNA processing in their splicing. Similarly to small RNAs, lncRNAs can have complementary base pairing with the target mRNA. The formation of duplexes between complementary lncRNAs and mRNA can hide key elements within the mRNA, which are required to bind trans-acting factors that have an effect on a post-transcriptional gene expression (including splicing, translation and degradation of mRNA) (Huang et al., 2012). Many lncRNAs serve as precursors of short ncRNAs, such as siRNA or miRNA.

During the last few years it has been reported that lncRNAs have essential roles in the mechanism of epigenetic regulation (Ponting et al., 2009). lncRNAs have a role in chromatin remodeling and are involved into the process of gene expression control at a single locus, at multiple chromosomal regions, and throughout entire chromosomes (Andersen and Panning, 2003; Choudhuri et al., 2010; Huang et al., 2012).

1.4.2. Small ncRNAs

Small (or short) ncRNAs are a group of ncRNAs that are include the different types of RNAs such as the small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), miRNAs, and Piwi protein-interacting RNAs (piRNAs) (Huang et al., 2012).

snoRNAs form a class of small RNA molecules that range from 60 to 300 nt in length and are named so, because they are found in the subcompartment of the eukaryotic nucleus called the nucleolus. They have a central role in the chemical modification of other RNAs,

mainly ribosomal RNAs, transfer RNAs and small nuclear RNAs, by directing the enzymes that perform the site-specific modifications of nucleotides in target RNAs via short regions of base-pairing (Tammen et al., 2013). snoRNAs have important roles in rRNA modification during ribosome biogenesis but they are not restricted to this function. It is also evident that they can target other RNAs, including snRNAs and mRNAs (Zhou et al., 2010). Recently, it has been found that at least some snoRNAs exhibit tissue-specific and developmental regulation, and/or imprinting, which indicate a regulatory function of this class of RNAs.

piRNAs are the largest class of small ncRNAs. They are 24–31 nt long and are so named because they can form RNA-protein complexes with Piwi proteins of the Argonaute (Ago) family (Kaikkonen et al., 2011). There are more than 50,000 piRNAs found in mammals, which is significantly larger than the number of other small RNAs of known classes (Makarova and Kramerov, 2007; Zhou et al., 2010). piRNAs are found in clusters throughout the genome and are highly conserved across species. More than 90% of mammalian piRNAs map are uniquely in the genome and cluster to a small number of loci. This class of small ncRNAs is important to maintain the stability of eukaryotic genomes (Costa, 2008). The piRNA complexes are involved in chromatin regulation, epigenetic and post-transcriptional gene silencing, and their primary role is suppression of transposon activity during germ line development (Fu, 2014; Kaikkonen et al., 2011). This kind of RNA has a role in RNA silencing via the formation of an RNA-induced silencing complex (RISC). In zebrafish, piRNAs are implicated in germ cell maintenance and many of them have a role in silencing mechanisms of repetitive elements (Houwing et al., 2007).

siRNAs are a class of double-stranded small RNA molecules. They are formed from dsRNA, which is cleaved by Dicer RNase into siRNA fragments with a two-base overhang on the 3' end and are 20–25 nt long. Dicer facilitates the activation of RISC, which includes one strand of a siRNA that is used as a template for recognizing complementary mRNA (Kaikkonen et al., 2011; Makarova and Kramerov, 2007). This class of small ncRNA performs many functions, but one of the most important is their role in gene regulation. They play a role in RNA interference (RNAi) pathways, where they regulate the expression of specific genes on the transcriptional level using complementary nucleotide sequences (Collins et al., 2011). siRNA also can suppress the transcription of genes, which contain the homologous sequences of siRNA. This phenomenon is called as transcriptional gene silencing. Presently it is known that they are involved in histone modifications and could play a role in control of DNA methylation through Dicer (Hossain et al., 2012). Dicer has an essential role in the epigenetic regulation of heterochromatin function through the induction

of key histone modifiers and induces histone methylation in somatic and embryonic stem cells (Hossain et al., 2012; Tammen et al., 2013).

miRNAs are a class of small, single-stranded RNA molecules, which are approximately 20–24 nt long (Kaikkonen et al., 2011). The genes coding for miRNAs do not always lay near their target genes in the genome. They form clusters throughout the genome and in most cases clustered miRNAs can be transcribed from the single primary transcript. These clustered miRNAs can be related to each other but do not have the same functional relationships. miRNA loci can be found in the introns of protein-coding genes, in the exons of protein-coding genes or can be derived from other classes of ncRNAs (Amaral and Mattick, 2008; Mattick and Makunin, 2006; Rodriguez et al., 2004). It has also been shown that some mammalian miRNAs are derived from processed pseudogenes and from repeats of various transposons, which can play a significant role in developmental processes and epigenetic variation. (Chuang and Jones, 2007). They are synthesized by RNA polymerase II (or polymerase III for some miRNAs) into primary miRNAs (pri-miRNAs), up to thousands ribonucleotides in length (Bizuayehu and Babiak, 2014). The pri-miRNAs are then processed in the nucleus by nuclear RNase III Drosha into shorter (~70 nt) precursor miRNAs (pre-miRNAs). The pre-miRNA is then transported from the nucleus to the cytoplasm and then by cytoplasmic RNase III Dicer processed into the miRNA-5p:miRNA-3p duplex, which includes the final functional mature miRNA and its complementary strand. This duplex is short-lived because the mature miRNA will then be incorporated into the RISC protein complex, whereas the complementary strand is normally degraded (Chuang and Jones, 2007; Kaikkonen et al., 2011). Biogenesis of miRNA is given on Fig.1.

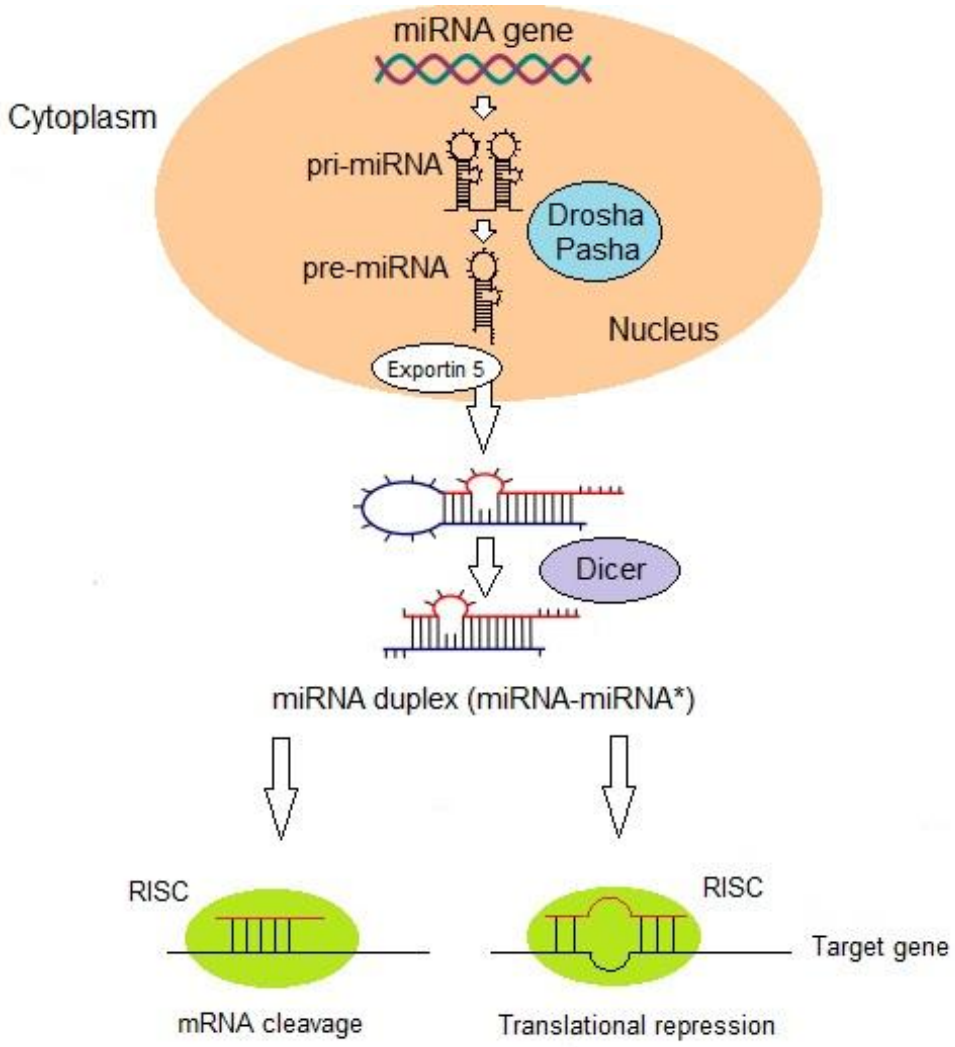

Fig. 1. Biogenesis of miRNA. Pri-miRNAs are produced by Drosha/Pasha complex into the pre-miRNAs in the nucleus, which are then transported into the cytoplasm by Exportin 5. In cytoplasm, pre-miRNAs are processed by Dicer into the miRNA-5p:miRNA-3p duplex, which includes the final functional mature miRNA and its complementary strand. Then, one strand will be included into the RISC (RNA-induced silencing complex), whereas the complementary strand is normally degraded. Then, the RISC complex binds to the target gene and leads to translational repression. Adapted with the publisher's permission from (Brown and Sanseau, 2005), license number 3618770077589).

The main function of miRNA is the inhibition of mRNA translation in cytoplasm (Bizuayehu and Babiak, 2014). miRNAs (as part of RISC) are able to interact with their complementary mRNA, which leads either to degradation of the target mRNA or repression of mRNA translation. mRNA degradation occurs when the miRNA is fully complementary to

the target RNA. Ago proteins as a part of RISC, are cutting the link between mRNA and miRNAs. The resulting products will be destroyed by the systems of mRNA degradation (Hossain et al., 2012; Makarova and Kramerov, 2007). Since the stability of the mRNA can be associated with its translational status, the degradation of target mRNA can be a consequence of the repression of translation. Translational repression of target mRNAs can occur at various stages of translation, either during initiation or at later stages (Makarova and Kramerov, 2007). miRNAs promote deadenylation, which might result in degradation of targeted mRNAs. mRNAs which are repressed by miRNAs, are further stored in processing bodies (P-bodies), which include Ago proteins involved in degradation processes. In some cases (such as certain types of stress), translation can be resumed, but often repression is accompanied by the substantial degradation of target mRNAs (Hossain et al., 2012). miRNAs can also bind to the sites in the 5'UTR and open reading frame of their target mRNA, but 3'UTR target sites are much more frequent. At the 5' end of the miRNA there is a region, "seed", which is located at position 2-7(8) nt. miRNAs targets have the Watson-Crick pairing with this region of miRNAs and most miRNAs have a 7nt match to their targets. It is known that one mRNA can contain multiple sites for the same or different miRNAs. Therefore, each mRNA may be regulated by more than one miRNA. One miRNA can have hundreds of targets and regulate many genes (Saito and Sætrom, 2010).

miRNAs have essential roles in most biological processes. It is known that approximately 50% of all genes are regulated by miRNA. They play central roles in the control of gene expression programs during development and are involved in translational enhancement or post-transcriptional activation of gene expression (Hossain et al., 2012). miRNAs play important roles in many aspects of vertebrate embryogenesis. Dicer and miRNA play an important role during oogenesis and spermatogenesis and are essential for meiotic completion (oocytes lacking Dicer are unable to complete meiosis)(Liu et al., 2010). miRNA-mediated regulation is involved in the functioning of primordial germ cells and in the growth and maturation of oocytes. (Hossain et al., 2012). miRNAs are also involved in adult stem cell pluripotency and differentiation, development of central nervous system, neuronal regeneration, immune cell formation and function, and essential for the metabolic processes (Amaral and Mattick, 2008). miRNAs have a distinct role in regulation of muscle growth and development. For instance in zebrafish, 245 target mRNAs were identified to be post-transcriptionally regulated by muscle miRNAs (Mishima et al., 2009). This class of small ncRNAs is known to be important component of the myogenic regulatory network and has been identified to directly control early myogenic transcriptional regulators (Johnston et al.,

2011). Some of the muscle-specific miRNAs, such as *miR-1, miR-133* and *miR-206* play a major role in embryonic myogenesis (Mishima et al., 2009). For instance, some of the known embryonic Myelin-gene Regulatory Factor (MRF) transcriptional activators like *Pax3* and *Pax7* are reported to be regulated by *miR-206* and in addition they are targeted by *miR-27*, *miR-486* and *miR-1* (Gagan et al., 2012). These muscle-specific miRNAs are present in myogenic cells that express *MyoD* and *Myf5* and play a role in the down-regulation of the Pax factors when the cells differentiate, as well as affecting their proliferation and survival. miRNAs promote muscle regeneration (Liu et al., 2012) and are involved in regulation the muscle phenotype. Some of miRNAs encoded by introns of slow myosin genes are involved in modulation the expression of factors that control slow versus fast fiber type specification. The reciprocal changes in miRNAs and their target mRNAs are able to lead to the cessation of muscle fibre recruitment in adult zebrafish (Johnston et al., 2009). miRNAs are crucial to adaptation to environmental change and response on different kinds of stress, including thermal, oxidative, chemical, physical and osmotic stress (Bizuayehu and Babiak, 2014).

1.5. High-throughput sequencing technologies

In recent years, next-generation sequencing (NGS) technology became the most attractive alternative to traditional methods for global genome and transcriptome studies.

NGS has many advantages comparing to Sanger sequencing (which is considered a first-generation sequencing technology) and microarray-based analysis of the transcriptome. The main disadvantages of microarray-based analysis are its poor sensitivity, low specificity and a limited dynamic range. Moreover, the cross-hybridization, especially when closely related gene family members with highly similar sequence are of particular interest, is an important problem of interpreting microarrays (Fang et al., 2012). These methods are not generally useful for discovering and profiling low-abundance or novel small RNAs. Comparing with microarray-based method, NGS sequencing are able to dissect transcriptome, even for RNAs expressed at extremely low levels (Morozova and Marra, 2008). The other advantages of NGS are the decreasing complexity of experimental procedures and cost relative to other methods (van Dijk et al., 2014; Zhang et al., 2011). NGS is based on the preparation of libraries in a cell free system, which does not require bacterial cloning of DNA fragments. Sequencing reactions are performed in parallel and for detection of the sequencing output is not necessary to use electrophoresis. Moreover, NGS has its ability to produce the

enormous numbers of sequence tags per run by multiplexing the sequencing process and has a high resolution, sequencing depth and throughput (Morozova and Marra, 2008).

Due to their advantages NGS technologies have been widely used in the *de novo* genome sequencing, for the high-throughput transcriptome analysis (such as RNA-seq), for genome-wide mapping of protein–DNA interactions, chromatin immunoprecipitation followed by sequencing (ChIP-seq) (van Dijk et al., 2014) and for ncRNA sequencing.

Nowadays, the most useful and most widely used NGS technologies for transcriptome profiling and small RNAs studies are Illumina and SOLiD sequencing due to their highest throughput and deeper coverage of small RNAs (van Dijk et al., 2014). These technologies are most useful for the research of small RNAs since they allow to find all small miRNA transcripts and quantitatively check precise levels of all miRNAs, discover new miRNAs and even to distinguish between miRNA variants or isomiRs.RNA analysis using NGS technology became the most attractive method for the analysis of eukaryotic transcriptome. Nowadays, it has been applied to investigate the ncRNA transcriptome and small RNA profiling in numerous research studies (Campos et al., 2014; Friedlander et al., 2008; Wang et al., 2009; Wei et al., 2012).

1.6. Aims of this study

Muscle growth in fish is a complex process and the role of miRNAs in regulation of muscle growth and adaptation to environmental change is still poorly understood. My hypothesis is that the mRNA and miRNA transcriptomes display thermal plasticity, which may be associated with different growth phenotypes. The overall aim of this thesis was to investigate differences in the transcriptional response in fast muscle of zebrafish reared at three different temperatures (24.3 ± 0.8 ºC, 28.3 ± 0.3 ºC, and 33.4 ± 0.4 ºC) using next-generation sequencing (Illumina).

Specific objectives were as follows:

1. To prepare mRNA TruSeq libraries for Illumina (MiSeq) sequencing.
3. To construct and sequence (Illumina) small RNA TruSeq libraries.
4. To determine alterations in mRNA and miRNA expression levels with temperature.
5. To integrate mRNA and miRNA data with the muscle phenotype.

2. Materials and methods

2.1. Fish husbandry, design of the temperature experiment and sampling

The first generation of AB strain zebrafish was maintained at 28 ºC in a recirculation system at the zebrafish rearing facility of the University of Nordland, Bodø, Norway. Males and females were kept separately and were mixed together on a night before breeding. Breeding of zebrafish was performed in recirculation system, in 10 l tanks. There were 6 breeding group, each consisted of 3 females and 2 males. Breeding boxes (1 l plastic tank with glass marbles) were placed into each of 10 l tanks with fish at the 8.30 AM, before switching light on. In total, approximately 2100 eggs were obtained. Then, eggs were divided to three groups (700 eggs per group) and placed in 3 l tanks, which were then placed in 30 l tanks with 3 experimental temperatures: 24.3 ± 0.8 ºC, 28.3 ± 0.3 ºC, and 33.4 ± 0.4 ºC (Table 1). Temperature of water was checked every 5 min by Ebro temperature loggers EBI 310 (Xylem Inc., Germany). Hatching occurred after 120 dd (day·degree) in the 24 ºC, 84 dd in the 28 ºC and 66 dd in the 33 ºC group. On 184 dd in the 24 ºC, 140 dd in the 28 ºC and 132 dd in the 33 ºC group, larvae started free swimming and they were released from the 3 l tanks in the nets, which were also placed in the 30 l holding tanks. When fish became bigger, they were released from the nets to 30 l tanks and were kept there until sampling. For each temperature group, there were 3 replicate tanks. The average density of fish was 7 fish per liter.

Table 1. Experimental group abbreviations that will be used throughout the work. Sample abbreviations include group abbreviation, fish number and number of tank from which fish were taken.

Experimental group abbreviation	Temperature (ºC)	Sample abbreviation
A	24.3 ± 0.8	1A2, 2A2, 4A2, 7A2, 8A2, 2A3
B	28.3 ± 0.3	6B1, 7B1, 8B1, 1B2, 6B3, 7B3
C	33.4 ± 0.4	1C2, 3C2, 9C2, 10C2, 11C2, 4C3

Water-renewal and filter cleaning were performed weekly. Water for renewal was taken from two 100 l tanks at 24 ± 1 ºC and 33 ± 1 ºC (water at 28 ± 1 ºC was obtained by mixing water from these two tanks). To check the water quality, Sera Aqua-Test Set was used (Water Quality Test Kit for Aquariums). The pH (optimal pH 6-8), nitrites (optimal level <1 mg/l) and nitrates (optimal level <50 mg/l) were checked once a week in each tank. For this experiment, 12L:12D (lights on from 9 AM to 9 PM) photoperiod was used. Fish after 184 dd in 24 ºC, 140 dd in 28 ºC and 132 dd in 33 ºC were fed twice a day at 11 AM and 3 PM with SDS-200 (Special Diets Services, UK).

After 4 months, 10 adult fish from each temperature group were sampled. These fish were euthanized by incubation in buffered solution of sodium bicarbonate with tricaine methanesulfonate (MS-222, 300 mg/l). Then total length (TL, mm), fork length (FL, mm) and weight (mg) of fish were measured immediately. Pieces of white muscle from the tail (Fig. 2) were carefully dissected, placed in 1.4 mm Lysing matrix tubes with ceramic beads (FastPrep), snap frozen in liquid nitrogen and kept at -80 ºC prior to analysis.

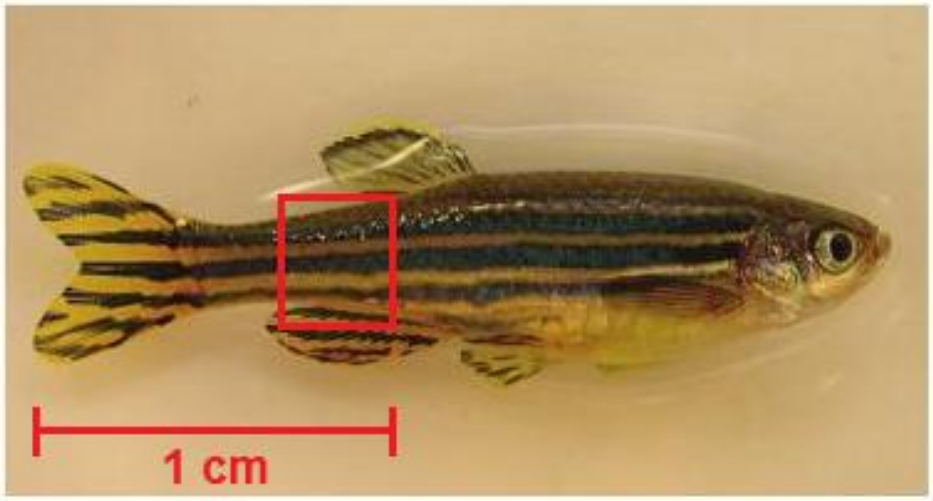

Fig. 2. Adult male zebrafish. Fast muscle samples were collected from the myotomal region posterior to the dorsal fin (red frame).

The remaining fish were anesthetized in a buffered solution of sodium bicarbonate with MS-222 (100 mg/l) and their total length, fork length and weight was also measured. All procedures used in this study were carried out in accordance with the guidelines set by the National Animal Research Authority (Forsøksdyrutvalget, Norway).

2.2. RNA extraction

RNA extraction was performed using AllPrep DNA/RNA Mini Kit (Qiagen, Germany) from fast muscle of 10 fish per temperature group (3 fish per each of 2 tanks and 4 fish from the third tank, from each temperature group). The amount of starting material for each extraction was 30 mg. In each of the tubes 750 µl of lysing RLT plus buffer with 7.5 µl of β-mercaptoethanol (Sigma-Aldrich®, Norway) was added to a frozen tissue and then it was homogenized on a Precellys®24 (Bertin Technologies, France) for 2×20 s at 4,000×g. The homogenate was centrifuged at 12,000×g for 30 s to remove cell debris and then the lysate was transferred to the AllPrep DNA Mini spin column (Qiagen, Germany) placed in 2 ml collection tube and centrifuged at 20,000×g for 30 s. The flow-through homogenate was placed in 2 ml microcentrifuge tube. To separate nucleic acids from proteins, 150 µl of chloroform was added to the homogenate, mixed well and centrifuged (at 4 ºC, 20,000×g) for 3 min. The upper layer of the supernatant was placed in a new 2 ml microcentrifuge tube. Afterwards, 80 µl of Proteinase K was added to this aqueous phase to remove the proteins and release nucleic acids by the inactivation of RNases. Then, two steps of ethanol filter washing were performed and mix was transferred to an RNeasy Mini spin column placed in a collection tube and centrifuged for 15 s (20,000×g). Then, 500 µl of RPE buffer was added to the RNeasy mini spin column and centrifuged for 15 s at 20,000×g. After that, 80 µl of incubation mix (mix of DNase I and RDD buffer) was placed directly onto RNeasy mini spin column membrane and incubated for 15 min at room temperature. Then, 500 µl of FRN buffer was added to the spin column and centrifuged for 15 s (20,000×g). After this step, the flow-through was saved and placed again onto the spin column in new collection tube and washed with 500 µl of RPE buffer and then 500 µl of 96-100% ethanol. The RNeasy Mini spin column was placed in a new 1.5 ml collection tube and 45 µl of RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at 8,000×g to elute the RNA. The eluate was placed again onto the column membrane and centrifuged for 1 min at 8,000×g to achieve a higher concentration of RNA. The quality and quantity of RNA was assessed by Agilent Technologies TapeStation. The isolated RNA was stored at -80 °C.

2.3. Library preparation and sequencing

For the library construction and sequencing, RNA samples from 6 fish in each temperature group were used. Samples were pooled in 6 pools (each pool contained 1 fish from each of 3 temperature groups).

2.3.1. RNA-seq libraries

mRNA libraries were prepared according to the TruSeq® RNA Sample Preparation v2 LS Protocol from Illumina, as illustrated in Fig. 3).

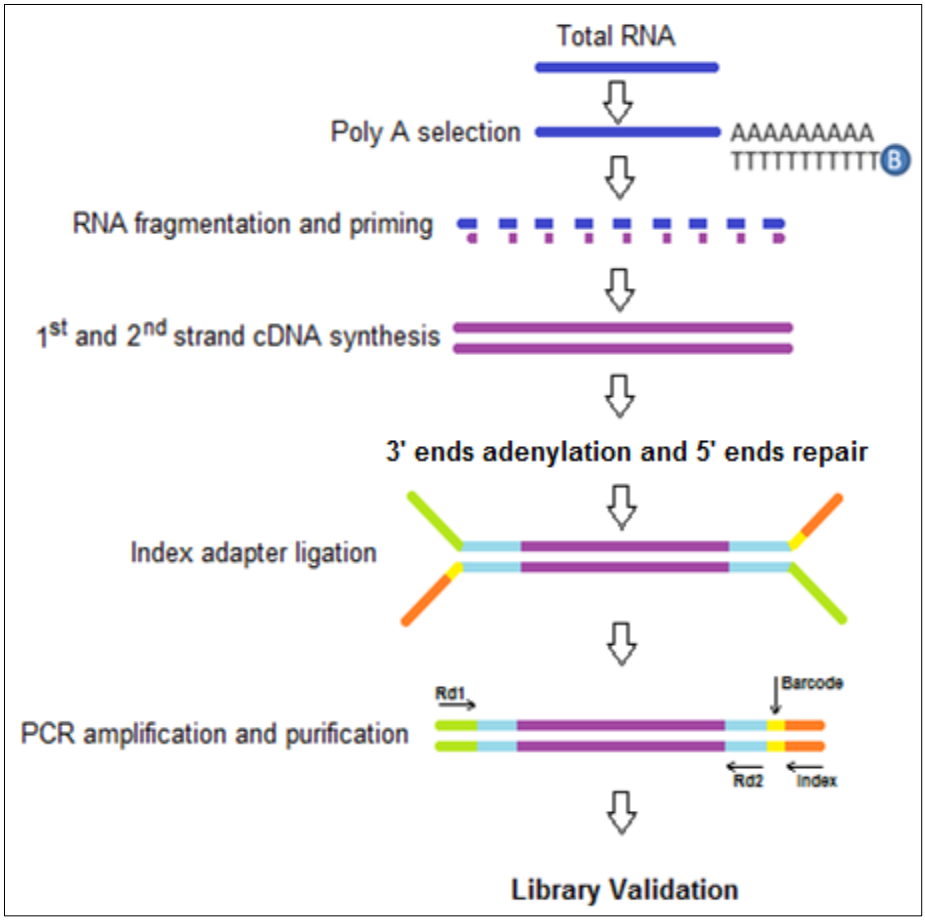

Fig. 3. Illumina RNA library preparation workflow; adapted from (Labome, 2013).

mRNA was purified using oligo-dT attached magnetic beads using two rounds of purification. Incubation in a GeneAmp® PCR System 9700 (Life Technologies, USA) was performed to denature the RNA and bind polyA RNA to the oligo-dT beads. PolyA-bound beads were separated from the solution using magnetic stand and washed twice to remove the all unbound RNA. Then the mRNA from the beads was eluted. Afterwards, beads were washed, incubated in thermocycler and eluted again to fragment and prime the RNA. After the second elution, RNA was fragmented and primed for cDNA synthesis. Then, first strand cDNA synthesis was performed. Supernatant with the fragmented and primed RNA was added First Strand Master Mix (FSM) and this mix was incubated in the thermal cycler. Immediately after incubation, Second strand Master Mix was added to the mix with the first

strand, incubated in a thermal cycler, washed twice with ethanol and mixed with Resuspension Buffer to obtain double stranded cDNA (ds cDNA). After ds cDNA synthesis, End repair was performed. End Repair Mix was added to the plate with ds cDNA and the plate was incubated. After incubation, there were two steps of ethanol washing. Then, Resuspension Buffer was added to the plate. Blunt-ended fragments were received. Before adaptor ligation, adenylation of 3' ends was performed. For that, A-tailing Mix was added to experimental plate and the plate was incubated in a thermal cycler. After, to this plate was added Ligation Mix and RNA Adapter Index (from TruSeq RNA Sample Prep Kit v2 Set A Indexed Adapter Sequences) and plate was incubated. Stop Ligation Buffer was then added to plate to inactivate the ligation and then the AMPure XP beads were added. Afterwards, 2 steps of ethanol washing were performed and Resuspension Buffer was added.

2.3.2. Small RNA libraries

For the small RNA library preparation, TruSeq® Small RNA sample preparation protocol was used, as shown in Fig. 4.

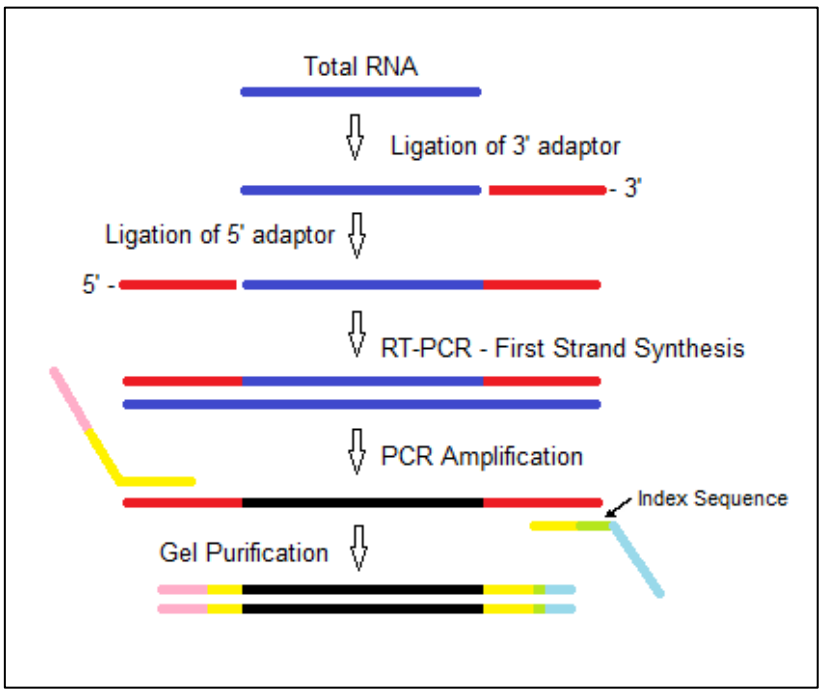

Fig. 4. TruSeq Small RNA sample preparation kit workflow; adapted from the (Illumina, 2010).

RNA 3' adapter was ligated to the total RNA. RNA 3' Adapter and Total RNA in nuclease-free water were mixed and incubated in the thermal cycler. Then, Ligation Buffer, RNase Inhibitor and T4 RNA Ligase 2 were mixed together and added to the reaction tube with RNA. This mix was incubated, and then Stop Solution (STP) was added to the reaction tube and mix was incubated again. RNA 5′ Adapter (RA5) was aliquoted, mixed with T4 RNA Ligase and added to the reaction tube, which contained RNA with ligated 3' Adapter. The reaction tube with this mix was placed to the thermal cycler. After adapters were ligated to each ends of RNA molecules, the reverse transcription was performed to create single-stranded cDNA. For that, adapter-ligated RNA was mixed with RNA RT Primer, and then incubated. The mix of 5X First Strand Buffer, 12.5 mM dNTP mix, 100mM DTT, RNase Inhibitor and SuperScript II Reverse Transcriptase was added to the reaction tube and then, the tube was placed to thermal cycler. The cDNA was then amplified. For that, the master mix of Ultra Pure Water, PCR Mix (PML), RNA PCR Primer (RP1) and RNA PCR Primer Index (RPIX) was prepared. RNA PCR Primer Indexes from TruSeq Small RNA Indices A Box (Index Sequences 1-12) was used. This master mix was added to the reaction tube, and then this tube was placed to thermal cycler with following PCR cycling conditions: 98 ˚C for 30 s, 11 cycles of 98 ˚C for 10 s, 11 cycles of 60 ˚C for 30 s, 11 cycles of 72 ˚C for 15 s and 1 cycle of 72 ˚C for 10 min. Then, each sample was run on Agilent Technologies 2100 Bioanalyser using high sensitive (HS) Agilent DNA 1000 chip. The amplified cDNA constructs were mixed with DNA Loading Dye and loaded on the 6% PAGE Gel and run for 60 min at 145 V. Then it was stained with Ethidium Bromide and viewed with UV-transilluminator. The bands with miRNAs, which were identified by UV-transilluminator, were cut out from the gel and placed into the Gel Breaker tubes, which were then centrifuged. Ultra pure water was added to these tubes, and they were then shaking at room temperature for 3 hours to elute the DNA. The eluate and gel debris were transferred to the top of 5 μm filter and centrifuged. The filter was then removed and mix of Glycogen, 3M NaOAc, and 0.1 X Pellet Paint and pre-chilled (-25 ºC to -15 ºC) 100% ethanol was added to the eluate. This mix was centrifuged and the supernatant was removed leaving the pellet intact. The pellets were then washed with 70% ethanol and resuspended in 10 mM Tris-HCI, pH 8.5. This ethanol precipitation was performed to obtain a more concentrated library. The library was validated on Agilent Technologies 2100 Bioanalyser using high sensitive (HS) Agilent DNA 1000 chip.

2.3.3. High-throughput sequencing on the MiSeq

RNA reads were obtained from six Illumina libraries prepared from pools of triplicate samples of adult zebrafish muscle tissue from 24 °C, 28 °C and 33 °C temperature groups. The RNA-seq libraries were quantified using qPCR. For the qPCR, the KAPA library quantification kit for Illumina sequencing (Kapa Biosystems, USA) using Light Cycler 480® qPCR instrument (Roche Applied Science). All processes were performed according to the kit manual, with reaction volume 10 µl. Quality control was performed on Agilent Technologies 2100 Bioanalyser using high sensitive (HS) Agilent DNA 1000 chip. The MiSeq Reagent Kit v3 (150 cycles) for the paired-end read sequencing of the RNA-seq libraries (76 cycles in each direction).

Small RNA reads were obtained from six Illumina libraries prepared from pools of triplicate samples of adult zebrafish muscle tissue from 24 °C, 28 °C and 33 °C temperature groups. The average length of the amplified cDNA template was assessed in Agilent 2100 Bioanalyzer with the high sensitivity DNA chip kit (Agilent Technologies). Each small RNA library template was sequenced on the Illumina platform using the MiSeq Reagent Kit v3 (150 cycles), single-end read sequencing and 51 cycles.

2.4. Bioinformatics analysis

The Cutadapt version 1.8 was used to find and remove adapter sequences from the 3' ends of sequencing reads. Then, fastx_collapser from the FASTX toolkit was used to collapse, identify and count identical reads. After trimming, the collapsed reads were aligned against the reference zebrafish genome (Danio_rerio, version Zv9) by Bowtie2 version 2.2.5 (Langmead and Salzberg, 2012). Bowtie2 uses a computational strategy known as ‘indexing’ to speed up its mapping algorithms and the Burrows-Wheeler transform to store a memory-efficient representation of the reference genome. For each read, Bowtie 2 proceeds in four steps: extraction of 'seed' substrings from the read and its reverse complement, alignment of substrings to the reference in an ungapped fashion assisted by the full-text minute index, seed alignments and calculation of their positions in the reference genome from the index, seeds extention into full alignments. Bowtie 2 was run with default options and performed end-to-end read alignment.

The htseq-count (Anders et al., 2014) script (with parameters: -s yes -i Name -t miRNA) was used to identify and count the overlap of reads with known zebrafish mature miRNAs, which were obtained from miRBase v. 21 Sequence database

(http://www.mirbase.org/search.shtml). Reads without perfect alignment were removed. The Limma package (Ritchie et al., 2015) was used for statistical analysis of smallRNA data. Limma is part of the Bioconductor project (http://www.bioconductor.org) and in current study was used standard pipeline and parameters. miRNA target prediction was performed with MicroCosm v. 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), which used the genomic sequence from the Ensembl (http://www.ensembl.org/index.html). MicroCosm Targets uses the miRanda computational prediction algorithm to identify the potential miRNA target sites. Every potential target site which was detected for a given miRNA, was checked to see whether the site is conserved in orthologous transcripts from other species and this program make an assumptions that each target is conserved in at least two species.

For RNA-seq data Bowtie2 version 2.2.5 was used to align each paired-end library to the reference zebrafish genome (with parameters -k5 and paired reads). Then, the featureCounts Rsubread package (with parameters: -p -t exon -S -T 16 -M -g) was used to count all reads that overlapped with annotated transcripts from the Zebrafish RefSeq database (downloaded Feb. 9, 2015). The Limma package (with standard pipeline and parameters) was used to identify genes differentially expressed between samples from the three different groups of treatment, with a significance level of $P<0.05$. These differentially expressed transcripts were fed into DAVID v. 6.7 for functional analysis (Huang da et al., 2009; Huang da et al., 2009). This tool provides batch annotation and gene-GO term enrichment analysis to highlight the most relevant GO terms associated with a given gene list and moreover, uses the KEGG pathway enrichment analysis.

3. Results

3.1. Temperature effects on fish growth and mortality

The highest mortality occurred in cold temperature group (24 °C). In the warm group (33 °C) mortality was lower than in the cold group, but still higher than at the reference temperature (28 °C). The hatching period was also affected by temperature. In the reference temperature group, hatching occurred three days after fertilization, while in cold and warm groups it is occurs on fifth and second day. The total length and weight of fish were affected by the temperature (Fig. 5).

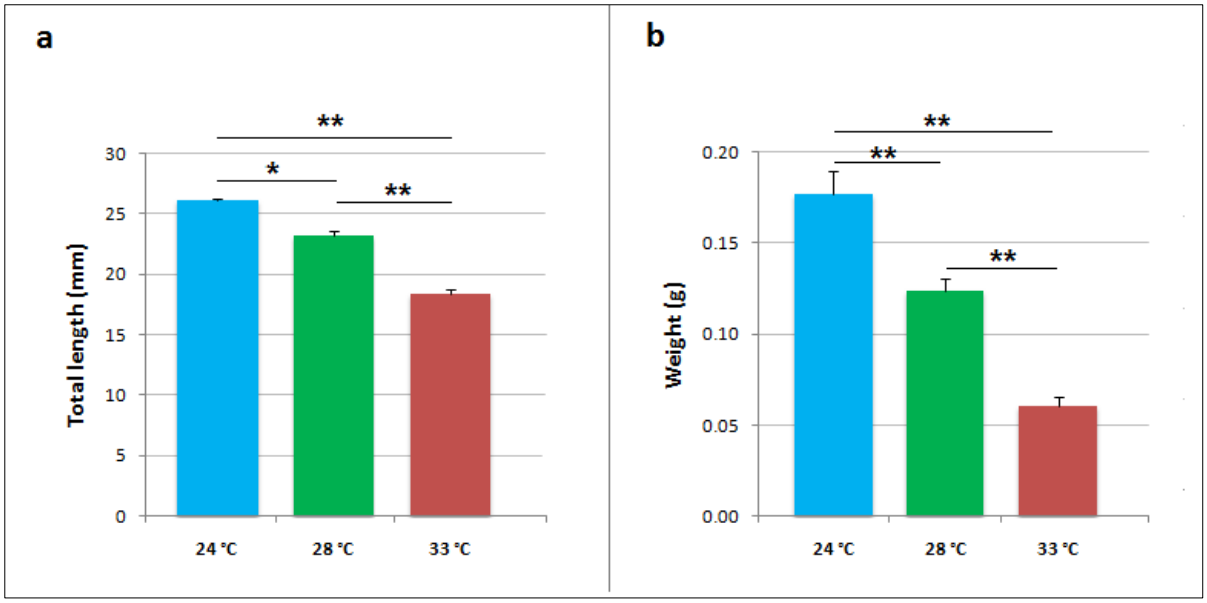

Fig. 5. a. Comparison of 4-month old adult zebrafish a. total length (mm) and b. weight (g) among the temperature groups. Asterisks indicate significant differences (*P<0.05, **P<0.001).

It was found that adult fish from the 24 °C group after 4 month were larger (TL 26.1±0.06 mm, mean±SE) than fish, which have been reared at the 33 °C (TL 18.4±0.04 mm, mean±SE) and bigger than fish from reference, 28 °C temperature group (TL 20.4±0.16 mm, mean±SE). The same trend was identified for fish weight – fish from the 24 °C (weight 0.18±0.01 g, mean±SE) were heavier than fish from reference (weight 0.12±0.01 g, mean±SE) and 33 °C (weight 0.06±0.01 g, mean±SE) groups.

3.2. Analysis of small-RNA sequencing data

3.2.1. Characterization of small RNA libraries

The Illumina sequencing platform has a quality filter pipeline that provides per-nucleotide Phred quality scores representing the probability that a given base call is erroneous. This filter uses the standard threshold of chastity >= 0.6 and the reads datasets from Illumina platform contained only sequences that had passed through it. In all experimental libraries, a yield of reads that passed Illumina quality and vector filtering was between 22,144,752 and 26,464,932. Prior to the analysis of small RNA, adapter sequences from the 3' ends of sequencing reads were removed. Identical reads were identified, collapsed and counted before trimming them (Table 2).

Table 2. Sequencing statistics of Illumina reads from zebrafish muscle small RNA libraries; average number of reads across 6 pooled libraries (each pool includes three libraries - one from each of the three temperature groups, where A – 24 ˚C group, B – 28 ˚C group, C – 33 ˚C group). Table include the total number of reads obtained after sequencing, number of reads passed through Illumina quality filter (PF), quality score 30 (Q30 means that error probability is 0.001 or 1 in 1000), number of trimmed reads and their average size (bp).

Pools	Sample ID	Total Reads	PF Reads	> Q30 (%)	Trimmed reads	Average read size (bp)
1	1A2-1B2-1C2	33,324,422	26,464,932	93.9	25,238,894	21
2	2A2-6B1-3C2	30,318,836	25,220,138	93.5	23,305,321	21
3	4A2-7B1-4C3	29,798,688	24,941,004	94.6	23,481,192	21
4	7A2-8B1-9C2	26,264,176	22,144,752	94.8	20,805,440	22
5	8A2-6B3-10C2	27,185,084	23,232,164	94.3	21,524,689	22
6	2A3-7B3-11C2	25,483,272	23,024,334	96.2	21,772,001	22

After trimming, the collapsed reads were mapped to Zv9 zebrafish genome using Bowtie2 program. Reads mapped to known zebrafish mature miRNAs (obtained from miRBase v. 21) were identified and counted (Fig. 6).

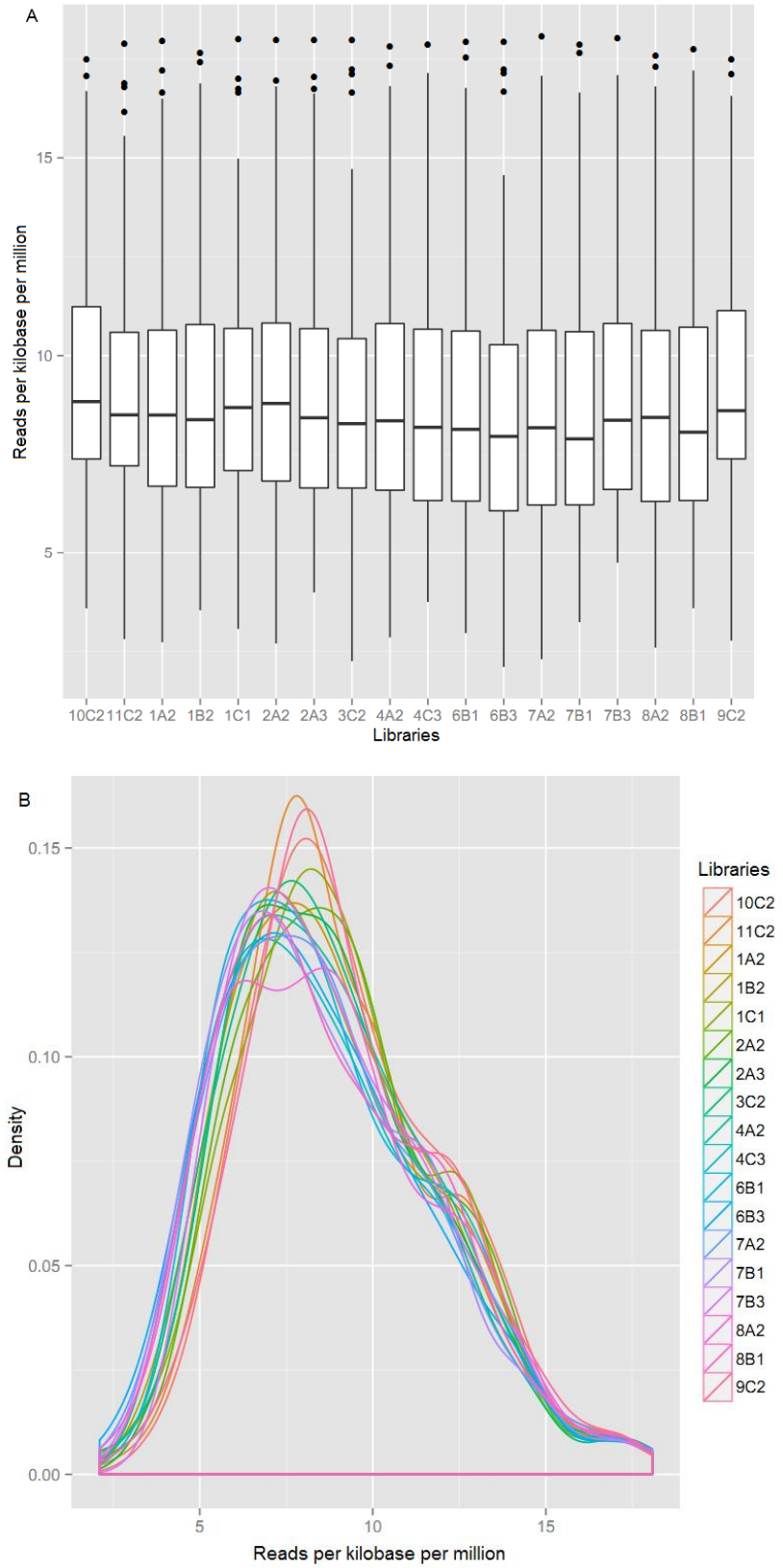

Fig. 6. A. The number of counts for each library after normalization and removing rare counts; B. Counts density distribution in each sample. Library abbreviations include fish number, group abbreviation (where A – 24 ˚C group, B – 28 ˚C group, C – 33 ˚C group) and number of tank from which fish were taken.

In total, 206 known miRNAs were identified, which could be grouped into 101 distinct families (Fig. 7).

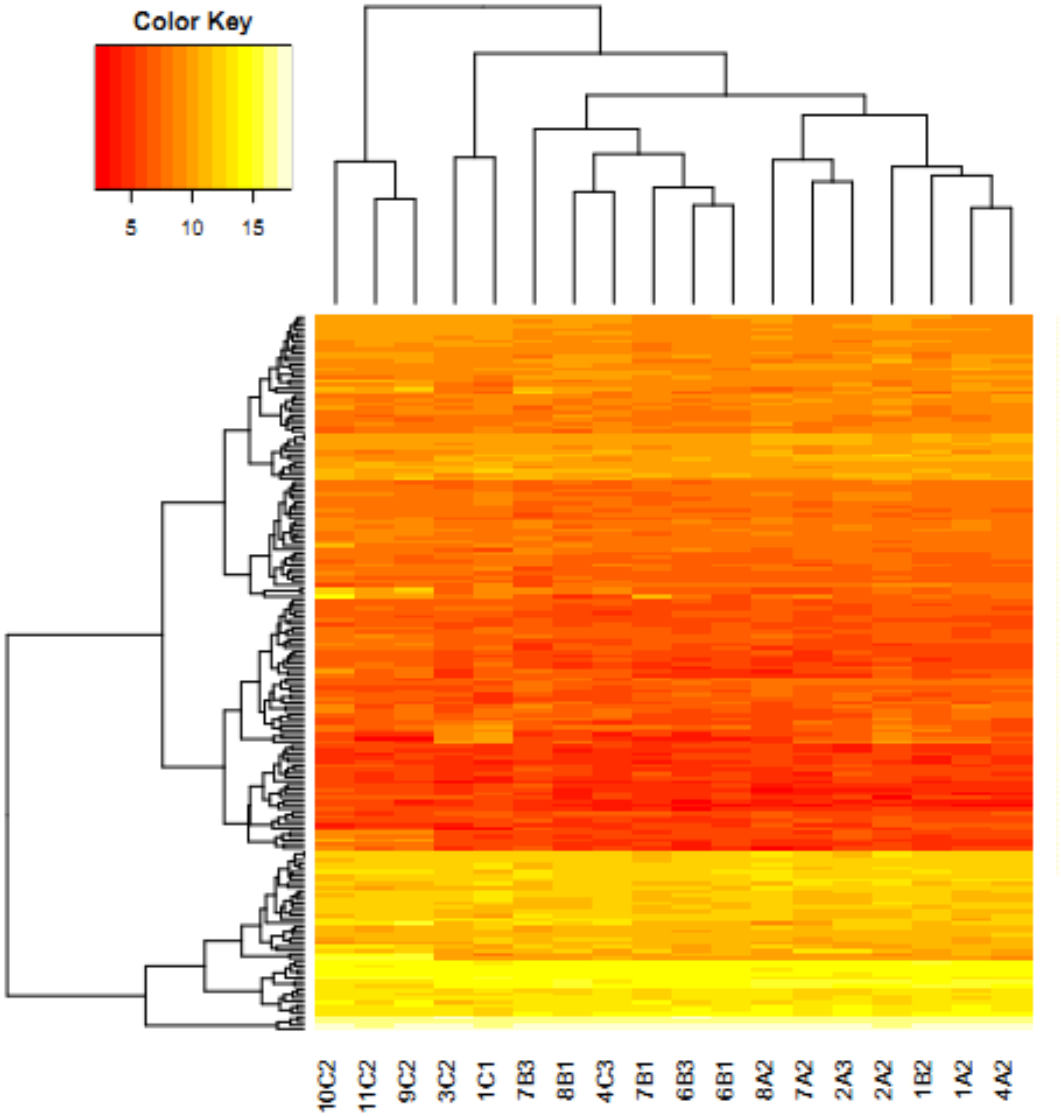

Fig. 7. Heatmap generated using R shows an overview of miRNA expression across all experimental conditions. Red and yellow indicate low and high miRNA expression, respectively.

3.2.2. Analysis of differential expression

Differentially expressed miRNAs were identified and tested for statistical significance. The differentially expressed miRNAs in zebrafish fast muscle between 24 ˚C and reference group and between 33 ˚C and reference group are shown in Tables 3 and 4. The most significant differences were observed between warm (33 ˚C) and cold (24 ˚C) temperature groups (Table 5).

Table 3. Differentially expressed miRNAs in zebrafish fast muscle between 24 ˚C and 28 ˚C temperature groups. Positive and negative fold-changes indicate up- and down-regulation in the 24 ˚C compared to 28 ˚C group, respectively.

miRNA	Fold change	P-value	miRNA	Fold change	P-value
miR-125b	3.6	0.02	*miR-145*	1.7	0.04
miR-181c	3.1	0.02	*miR-222a*	1.6	0.02
miR-107a	3.0	0.03	*miR-21*	1.6	0.02
miR-99	2.3	0.001	*miR-133a*	1.5	0.03
miR-27a	2.0	0.02	*let-7g*	1.5	0.04
miR-1388	1.9	0.01	*miR-130c*	-0.6	0.02
let-7e	1.8	<0.001	*miR-27e*	-0.6	0.01
miR-196b	1.8	0.03	*miR-101b*	-0.5	<0.001
miR-181a	1.8	0.02	*miR-26b*	-0.5	<0.001
let-7c	1.7	0.01	*miR-735*	-0.5	0.02
miR-125c	1.7	0.02	*miR-10b*	-0.4	0.02

Table 4. Differentially expressed miRNAs in zebrafish fast muscle between 33 ˚C and 28 ˚C temperature groups. Positive and negative fold-changes indicate up- and down-regulation in the 33 ˚C compared to 28 ˚C group, respectively.

miRNA	Fold change	P-value	miRNA	Fold change	P-value
miR-200a	6.2	0.04	*miR-133c*	1.9	0.04
miR-141	6.1	0.04	*miR-130a*	1.8	0.04
miR-138	3.9	0.04	*miR-26b*	1.7	0.01
miR-301	3.9	0.03	*miR-455*	1.7	0.04
miR-190a	2.9	0.04	*miR-130c*	1.6	0.04
miR-218a	2.7	0.03	*miR-27c*	-0.5	0.03
miR-460	2.5	0.03	*miR-100*	-0.5	0.01
miR-101b	2.3	0.04	*miR-125b*	-0.5	0.04

Table 5. Differentially expressed miRNAs in zebrafish fast muscle between 24 ˚C and 33 ˚C temperature groups. Positive and negative fold-changes indicate up- and down-regulation in the 24 ˚C compared to 33 ˚C group, respectively.

miRNA	Fold change	P-value	miRNA	Fold change	P-value
miR-200a	-5.6	0.03	*miR-196b*	2.1	0.01
miR-141	-4.9	0.04	*let-7e*	2.1	<0.001
miR-733	4.3	0.01	*miR-125c*	2.0	0.004
miR-10b	-4.1	0.003	*miR-1388*	2.0	0.008
miR-181c	3.5	0.02	*miR-92b*	2.0	0.02
miR-190a	-3.5	0.01	*miR-27a*	1.9	0.02
miR-153c	-3.4	0.02	*miR-181a*	1.9	0.008
miR-454a	-3.3	0.01	*miR-130c*	-1.9	0.001
miR-101b	-3.2	0.003	*miR-27e*	-1.9	0.002
miR-22b	3.2	0.04	*miR-19c*	-1.9	0.02
miR-10a	-3.1	0.02	*miR-27c*	1.9	0.004
miR-99	3.0	<0.001	*miR-130a*	-1.9	0.01
miR-100	2.9	<0.001	*miR-101a*	-1.8	0.02
miR-216b	-2.9	0.04	*let-7c*	1.8	0.008
miR-26b	-2.8	<0.001	*miR-21*	1.8	0.008
miR-735	-2.6	0.004	*miR-210*	-1.7	0.03
miR-125b	2.5	0.004	*miR-133a*	1.6	0.01
miR-218a	-2.4	0.01	*miR-130b*	-1.6	0.04
miR-2188	2.4	0.04	*miR-338*	-1.6	0.03
miR-218b	-2.2	0.03	*miR-27c*	-1.6	0.03
miR-29a	-2.2	0.04	*let-7g*	1.5	0.01

After group-comparison, the most highly differently expressed miRNAs that were up- or down-regulated among temperature groups, and which had the highest number of reads per million (RPKM) and lowest false discovery rate (FDR) were identified. The most abundant miRNAs in the fast myotomal muscle of adult zebrafish were *miR-26b*, *miR-99*, *miR-100*,

miR-101b, *miR-125b*, *miR-125c*, *miR-141*, *miR-153*, *miR-200a*, *miR-218a*, *miR-735* and *let-7e* (Fig. 8).

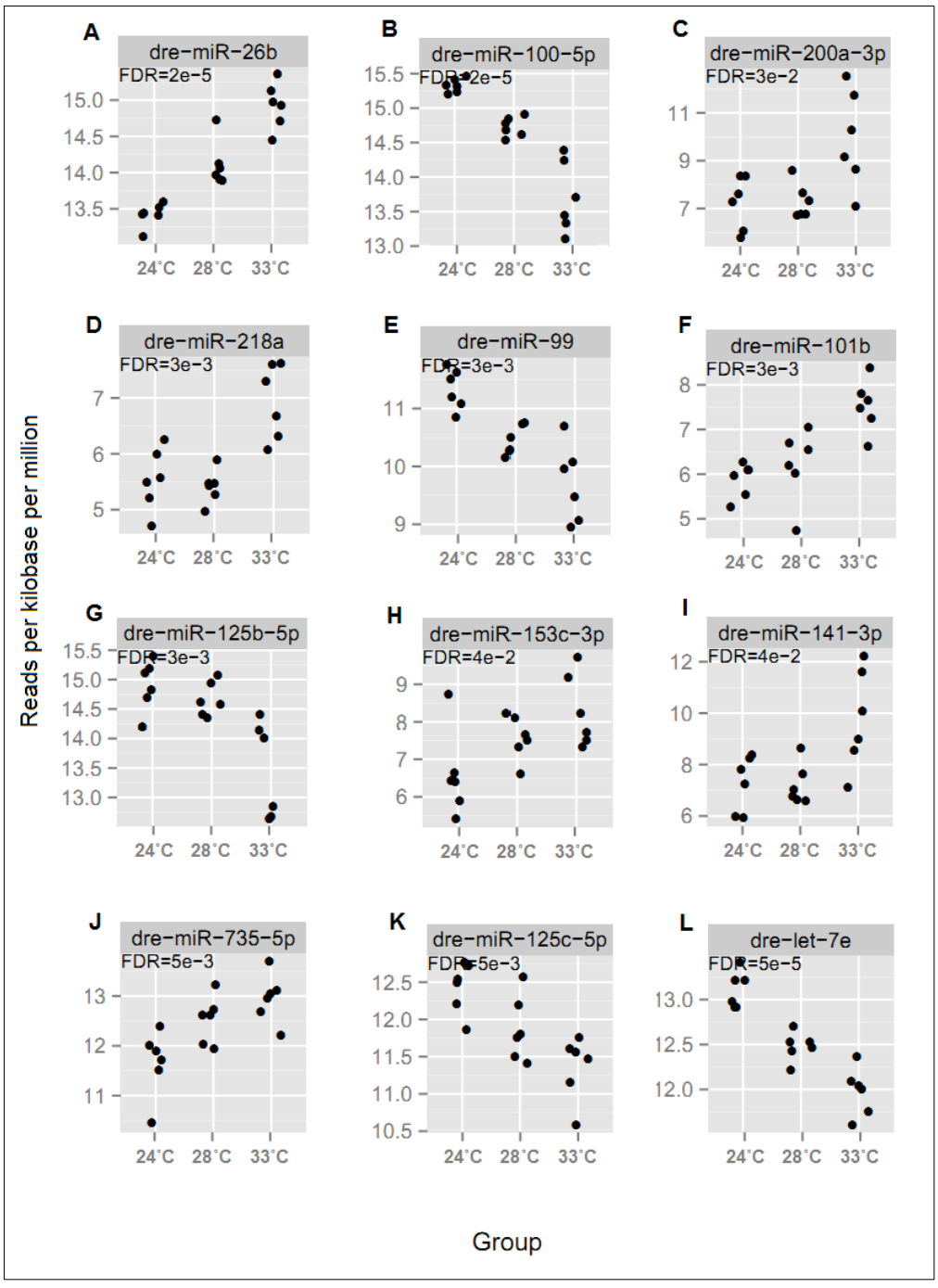

Fig. 8. The most significant differentially expressed miRNAs with the highest RPKM and FDR <0.05. Each dot represents the individual sample from the temperature group.

The up-regulated miRNAs in the 33 ˚C group comparing to the 28 ˚C group, were *miR-200a* (6.3 fold change), *miR-141* (6.0 fold change), *miR-218a* (2.7 fold change), *miR-101b* (2.3 fold change) and *miR-26b* (1.7 fold change), while the *miR-100* (-1.9 fold change) and *miR-125b* (-2.2 fold change) were down-regulated.

Comparison of 24 ˚C and 33 ˚C groups showed that *miR-99* (3 fold change), *miR-100* (2.9 fold change), *miR-125b* (2.6 fold change) and *let-7e* (2.1 fold change) were up-regulated in 24˚ C, while *miR-200a* (-5.6 fold change), *miR-141* (-4.9 fold change), *miR-153* (-3.4 fold change), *miR-101b* (3.2 fold change), *miR-26b* (-2.8 fold change) and *miR-218a* (-2.5 fold change) were down-regulated in this group.

3.2.3. miRNA target prediction

Computationally predicted targets for the significantly differentially expressed miRNAs were determined using MicroCosm Targets v.5. Putative targets for the most differentially expressed miRNAs were identified (Table 6). The genes that were predicted targets for these miRNAs are involved in different metabolic processes (in particular in energy metabolism, lipid and carbohydrate metabolism), immune response, muscles contraction, attachment and regeneration, and response to the different environmental stress (including oxidative and temperature stress).

Table 6. Putative mRNA targets from zebrafish muscles for the most significantly expressed miRNAs.

miRNA	Name of putative miRNA target	Ensembl gene ID	Function	Score	ΔG_{duplex} (kcal mol^{-1})
miR-26b	*uqcr1* (ubiquinol-cytochrome c reductase core protein I)	ENSDART00000074128	Energy metabolism Cardiac muscle contraction	20.0	-20.84
	klhl40a (kelch-like family member 40a)	ENSDARG00000039052	Muscle structure development Skeletal muscle fiber development Negative regulation of protein ubiquitination	18.8	-18.77
	myl7 (myosin, light chain 7, regulatory)	ENSDART00000040013	Cardiac muscle cell proliferation and tissue development Focal adhesion Regulation of actin cytoskeleton Tight junction	18.5	-12.65
	ufd1l (ubiquitin fusion degradation 1-like)	ENSDART00000034968	Ubiquitin-dependent protein catabolic process	18.7	-20.40
miR-200a	*dmd* (dystrophin)	ENSDART00000019287	Muscle attachment Actin binding	19.8	-26.42
miR-218a	*idh3b* (isocitrate dehydrogenase 3 (NAD+) beta)	ENSDART00000065777	Carbohydrate and lipid metabolism Carbohydrate metabolism Energy metabolism	19.8	-22.41
	wnt10a (wingless-type MMTV integration site family, member 10a)	ENSDART00000007308	Hedgehog signaling pathway Wnt signaling pathway Cell fate commitment	18.8	-21.90
	six4a (SIX homeobox 4a)	ENSDART00000048110	Regulation of transcription DNA binding	18.3	-18.20

miR-153	*adipoqb* (adiponectin)	ENSDART00000078901	PPAR signaling pathway Adipocytokine signaling pathway Lipid metabolism in skeletal muscle	19.4	-11.54
	ankrd28 (Ankyrin repeat domain-containing protein 28)	ENSDART00000104920	Repair and regeneration of muscle tissue following damage due to stress	18.4	-21.50
miR-101b	*pkmp* (pyruvate kinase, muscle, b)	ENSDART00000024013	Energy metabolism Carbohydrate and lipid metabolism Carbohydrate metabolism Nucleotide metabolism	19.3	-21.86
	tnnt1 (troponin T1, skeletal, slow)	ENSDART00000044154	Muscle contraction	17.6	-18.20
miR-100	*hsp90ab1* (Heat shock protein HSP 90-beta)	ENSDART00000020084	Generating immune responses Protein processing in endoplasmic reticulum Progesterone-mediated oocyte maturation	18.8	-29.59
	hspb1 (heat shock protein 1)	ENSDART00000097378	Regulation of myofibril size Response to heat MAPK signaling pathway VEGF signaling pathway	14.9	-18.33
	ube2n (ubiquitin-conjugating enzyme E2N)	ENSDART00000099932	DNA damage response Detection of DNA damage Postreplication repair	17.6	-31
miR-141	*rac1* (ras-related C3 botulinum toxin substrate 1a)	ENSDART00000047769	Myoblast fusion Skeletal muscle regeneration GTP binding	18.6	-16.20
	rmi1 (RMI1, RecQ mediated genome instability 1)	ENSDART00000060657	Fanconi anemia pathway Repair of damaged DNA	18.9	-17.30
	egf (epidermal growth factor)	ENSDART00000074639	Gap junction ErbB signaling pathway Focal adhesion	18.6	-15.40

			Regulation of actin cytoskeleton FoxO signaling pathway MAPK signaling pathway		
miR-99	*hsp90ab1* (Heat shock protein HSP 90-beta)	ENSDART00000020084	Generating immune responses Protein processing in endoplasmic reticulum Progesterone-mediated oocyte maturation	17.9	-27.71
	smarca5 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5)	ENSDART00000025529	ATP-dependent chromatin remodeling Chromatin remodeling DNA damage response	17.3	-19.50
	*usp5 (*ubiquitin specific protease 5)	ENSDART00000029121	The Ubiquitin-Proteasome Pathway	17.0	-22.20
	ube2n (ubiquitin-conjugating enzyme E2N)	ENSDART00000099932	DNA damage response Detection of DNA damage Postreplication repair	16.7	-27.90
let-7e	*tpi1a* (triosephosphate isomerase 1a)	ENSDART00000037007	Carbohydrate and lipid metabolism Carbohydrate metabolism Carbon metabolism	18.0	-18.80
	hspa8 (Heat shock 70 kDa protein 8)	ENSDART00000099994	Environmental Information Processing Transport and catabolism Transcription Protein processing in endoplasmic reticulum	17.7	-13.98
	*hspd1 (*heat shock 60 protein 1)	ENSDART00000078595	Protein refolding Tissue regeneration RNA degradation ATP binding	17.6	-16.90

3.3. Analysis of RNA-sequencing (RNA-seq) data

3.3.1. Library characterization

All experimental libraries yielded large numbers of reads that passed Illumina quality and vector filtering (Table 7). Each of paired-end RNA-seq read was aligned to the Zv9 reference zebrafish genome.

Table 7. Sequencing statistics of Illumina reads from zebrafish muscle total RNA libraries; average number of reads across 6 pooled libraries (each pool includes three libraries - one from each of the three temperature groups, where A – 24 ˚C group, B – 28 ˚C group, C – 33 ˚C group). Table includes the total number of reads obtained after sequencing, number of reads passed through Illumina quality filter (PF), quality score 30 (Q30 means that error probability is 0.001 or 1 in 1000) and number of trimmed reads.

Pools	Samples ID	Total Reads	PF Reads	> Q30 (%)	Trimmed reads
1	1A2-1B2-1C2	33,603,044	29,271,780	94.8	28,846,704
2	2A2-6B1-3C2	27,617,008	25,109,806	96.4	24,482,831
3	4A2-7B1-4C3	24,483,200	22,774,860	96.9	22,366,469
4	7A2-8B1-9C2	38,714,548	30,347,292	92.3	29,985,635
5	8A2-6B3-10C2	33,833,956	29,517,720	94.2	28,900,606
6	2A3-7B3-11C2	31,588,672	27,955,660	95.1	26,333,360

All reads, which overlapped with annotated transcripts from the Zebrafish RefSeq database (downloaded February 9th, 2015) were counted (Fig. 9).

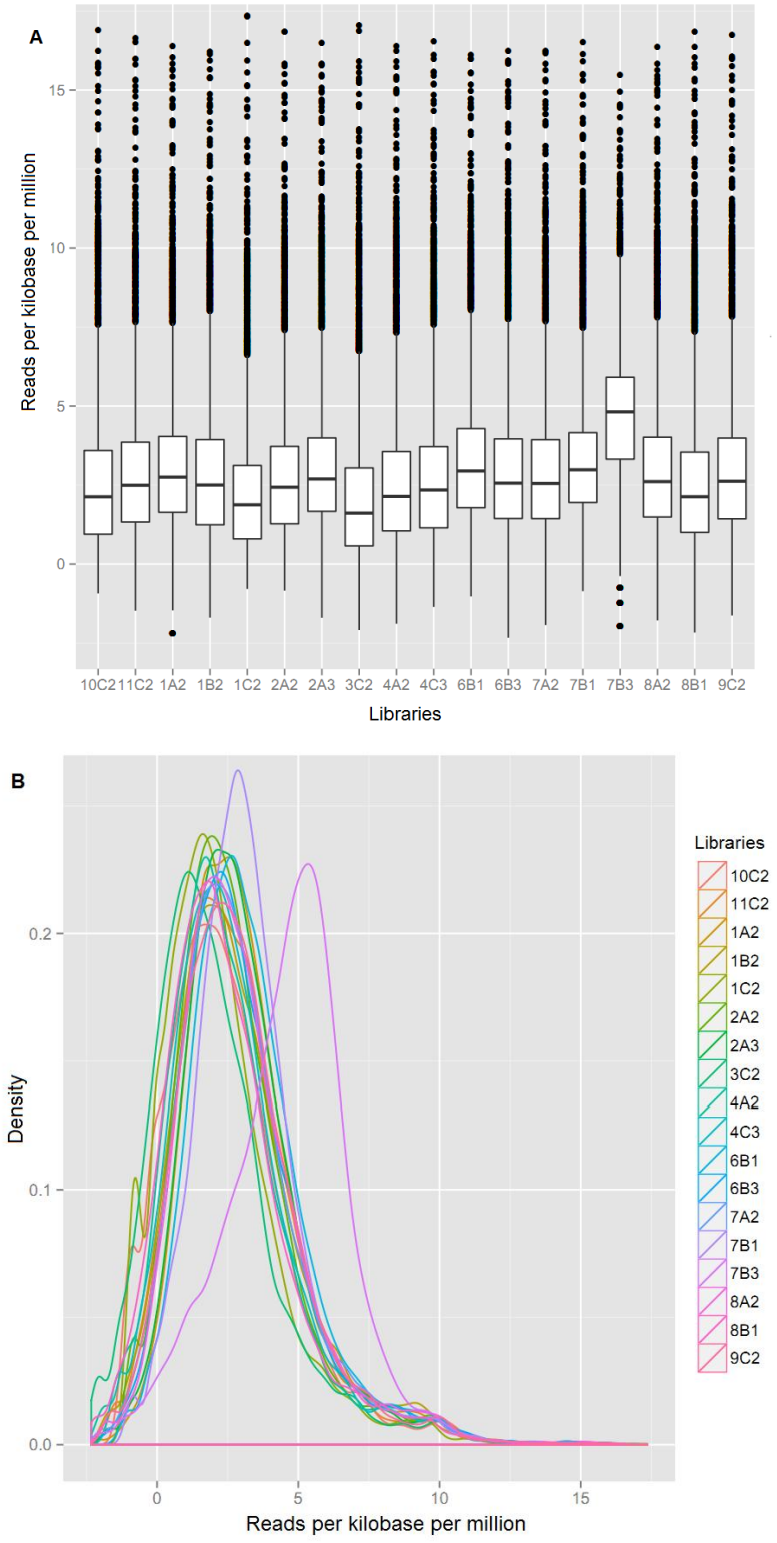

Fig. 9. A. The number of counts for each library after normalization and removing of rare counts; B. Counts density distribution in each sample. Library abbreviations include fish number, group abbreviation (where A – 24 ˚C group, B – 28 ˚C group, C – 33 ˚C group) and number of the tank from which each fish was taken.

3.3.2. Analysis of differential expression

The Limma package for R was used to perform to test the differentially expressed genes for statistical significance using the Benjamini-Hochberg correction. There were 17 up-regulated (fold change from 2.5 to 42.8) genes and 11 down-regulated (fold change from -2.6 to -13.9) genes in 24 ˚C group comparing to 28 ˚C group, 15 up-regulated (fold change from 2.0 to 7.4) genes and 1 down-regulated (fold change -0.4) genes in normal 28 ˚C group comparing to cold and warm groups, 30 up-regulated (fold change from 3.4 to 49.1) genes and 306 down-regulated (fold change from -2.0 to -86.2) genes in the 33 ˚C group compared to the 28 ˚C group. The most significantly up- and down-regulated transcripts in all three experimental temperature groups are given in Tables 8-12.

Among the significant up-regulated transcripts in the 24 ˚C group we observed genes involved in chromatin binding and remodeling, such as *hmgb3a* and *six4a*; different solute carrier family transporter molecules, such as *slco2a1, slc16a4* and *slc22a16*; genes involved in energy metabolism and response to oxidative stress (like *arl11* and *gpx1a*); members of signaling cascades – *rcan3, thbs4b* and *hfe2*. Some genes (such as *tnn1a1, mdka, pcolce (2 of 2)* and *thbs4b*) are involved in tissue morphogenesis, collagen binding, muscle contraction and growth factor activity were also up-regulated in the 24 ˚C group. Genes involved in ATP binding and phosphorylation (*mark1*), skeletal system development (*bglap*) and different heat shock proteins (for example *hspb1, hsp90aa1.1, hsp90aa1.2*) were down-regulated in the 24 ˚C group. A number of transcripts up-regulated in the 24 ˚C group showed the opposite trend in the 33 ˚C group. A similar situation was observed and for some down-regulated transcripts.

Among the up-regulated transcripts identified in the 33 ˚C group, heat shock proteins - *hspa1b, hspa8,* and including *hspb1, hsp90aa1.1, hsp90aa1.2*. This group showed up-regulatation of genes related to the muscle structure development and muscle contraction (such as *homer1b* and *zgc:86709*), genes responsible to the ATP binding and metal ion binding (for example *mylpfb, zgc:195170, mat2ab, cib2*), and genes responsible for RNA binding (*zgc:194878*), DNA binding and regulation of transcription (for example *helt, h2afx (9 of 17), tbx6* and *hoxb6b*). Down-regulated genes in the 33 ˚C group included genes involved in immune response (*mhc1uka*), lipid metabolism, energy metabolism and metal ion binding (*nt5c2b, hxd1, sftpbb, zgc:64065, bmp2k* and *epdr1*). Moreover, different solute carrier family transporter molecules (such as *slc29a4, slc22a3, slc2a1*) and transcripts

responsible for DNA binding such as *apobec (2 of 2)* and *hmgb3a* were down-regulated in this group, while in the 24 ˚C group they were found among the up-regulated transcripts).

The most significantly up-regulated transcript in the 24 ˚C group was *hmgb3a* (42.8 fold change), which was also the most significantly down-regulated transcript in 33 ˚C group (-86.2 fold change). Among the most significantly down-regulated transcripts in the 24 ˚C group were *hsp90aa1.1* (-13.9 fold change), *hsp90aa1.2* (-9.3 fold change) and *hspb1* (-5.2 fold change). In the 33 ˚ C group, *hspa1* (49.1 fold change), *hspa8* (14.6 fold change), *hspb1* (12.9 fold change), *pklr* (11.3 fold change) and *serpinh1b* (10.4 fold change) were the most significantly up-regulated, while among down-regulated transcripts it were the *zgc:92880* (-27.1 fold change), *zgc:112242* (-25.3 fold change) and *tmem120a* (-12.2 fold change).

Table 8. Top 15 up-regulated genes in the 24 ˚C temperature group. Positive fold-changes indicate up-regulation in the 24 ˚C compared to reference group.

Gene	NCBI gene ID	Gene name	Gene Ontology Function	Fold change	P-value
hmgb3a	NM_001122836	high-mobility group box 3a	chromatin remodeling, chromatin binding	42.8	<0.001
slco2a1	NM_001089582	solute carrier organic anion transporter family, member 2A1	organic anion transporter polypeptide (OATP)	7.3	0.01
tnni1al	NM_001020668	troponin I, skeletal, slow like	muscle contraction	6.7	0.002
slc16a4	NM_001080599	solute carrier family 16	major Facilitator Superfamily (MFS) transmembrane transporter	5.9	0.01
prf1.5	NM_001123246	perforin 1.5	Membrane attack complex component/natural killer-cell-mediated cytolysis	5.9	0.04
arl11	NM_199693	ADP-ribosylation factor-like 11	small GTP-binding protein, ADP-ribosylation factor	4.4	0.03
slc22A16	NM_001025488	solute carrier family 22	transmembrane transporter	3.7	0.02
mdka	NM_131070	midkine-related growth factor	tissue morphogenesis, neural tube formation, growth factor activity	3.6	0.02
pcolce (2 of 2)	NM_001030181	procollagen C-endopeptidase enhancer	collagen binding, heparin binding, peptidase activator activity	3.5	0.03
rcan3	NM_001005392	regulator of calcineurin family member 3	calcium-mediated signaling, regulation of calcineurin-NFAT signaling cascade	3.2	0.03
six4a	NM_131718	sine oculis homeobox homolog 4a	DNA binding, regulation of transcription	2.9	0.006
gpx1a	NM_001007281	glutathione peroxidase 1a	oxidoreductase activity, peroxidase activity, response to oxidative stress	2.9	0.02
thbs4b	NM_173226	thrombospondin 4b	calcium ion binding, growth factor activity, cell adhesion, TGF-beta signaling pathway	2.7	0.02
cahz	NM_131110	carbonic anhydrase	response to osmotic stress, carbonate dehydratase activity, metal ion binding, carbon dioxide transport	2.6	0.03
hfe2	NM_001045281	hemochromatosis type 2	bone morphogenetic protein (BMP) signalling pathway	2.5	0.02

Table 9. Top 9 down-regulated genes in group 24 ˚C temperature group. Negative fold-changes indicate down-regulation in the 24 ˚C compared to reference group.

Gene	NCBI gene ID	Gene name	Gene Ontology Function	Fold change	P-value
hsp90aa1.1	NM_131328	heat shock protein 90, alpha 1 (cytosolic)	stabilizes proteins against heat stress, skeletal myofibril assembly, muscle cell development, NOD-like receptor signaling pathway	-13.9	0.01
hsp90aa1.2	NM_001045073	heat shock protein 90, alpha 2 (cytosolic)	nucleotide binding, ATP binding, NOD-like receptor signaling pathway, Progesterone-mediated oocyte maturation, stress response,	-9.3	0.02
hspb1	NM_001008615	heat shock protein, alpha-crystallin-related, 1	regulation of myofibril size, response to temperature stimulus, response to heat. thermotolerance in vivo and support of cell survival under stress conditions. MAPK signaling pathway, VEGF signaling pathway	-5.2	0.004
bglap	NM_001083857	osteocalcin; bone gamma-carboxyglutamate (gla) protein	skeletal system development, ossification, bone mineralization, bone development, calcium ion binding, metal ion binding	-4.4	0.04
mark1	NM_001114476_2	MAP/microtubule affinity-regulating kinase 1	ATP binding, phosphorylation	-4.0	0.04
rsrp1	NM_001045308	arginine/serine-rich protein 1	protein binding	-3.9	0.004
myoz1b	NM_001002447	myozenin 1b	Calcineurin-binding	-3.4	0.04
c1h11orf73	NM_001017577	chromosome 11 open reading frame 73	cellular response to heat, Hsp70 protein binding, protein import into nucleus	-2.7	0.04
smco4	NM_001291899	single-pass membrane protein with coiled-coil domains 4	integral component of membrane	-2.6	0.03

Table 10. Top 10 up-regulated genes in the 28 ˚C temperature group. Positive fold-changes indicate up -regulation in the reference group compared to 24 ˚C and 33 ˚C groups.

Gene	NCBI gene ID	Gene name	Gene Ontology Function	Fold change	P-value
hspb6	NM_001100958	heat shock protein, alpha-crystallin-related, b6;	response to temperature stimulus, response to heat, response to abiotic stimulus	7.4	0.005
pfkfb4l	NM_198816	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4, like	ATP binding, Fructose and mannose metabolism	5.7	0.01
gpt2	NM_001098757	glutamic pyruvate transaminase (alanine aminotransferase) 2	Alanine, aspartate and glutamate metabolism	4.2	0.02
lpin1	NM_001044353	lipin 1	fatty acid catabolic process	4.1	0.02
mxi1	NM_131237	max interacting protein	transcription regulator activity	4.0	0.03
nudt4a	NM_200110	nudix (nucleoside diphosphate linked moiety X)-type motif 4	hydrolase activity, oxidation reduction, transition metal ion binding	3.5	0.03
sesn1	NM_001002660	sestrin 1	determination of left/right symmetry, Mediating Nodal Signaling, response to reactive oxygen species	3.2	0.03
rxrga	NM_131217	retinoid x receptor, gamma a	DNA binding, metal ion binding, steroid hormone mediated signaling pathway, PPAR signaling pathway	3.1	0.03
coq10b	NM_001017747	coenzyme Q10 homolog B (S. cerevisiae)	coenzyme	3.1	0.03
kiaa	NM_001045179	KIAA	RNA binding	2.8	0.04
acadm	NM_213010	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	lipid metabolism, Fatty acid metabolism, PPAR signaling pathway, oxidoreductase activity	2.7	0.04
gapdh	NM_001115114	glyceraldehyde-3-phosphate dehydrogenase	NAD binding, NADP binding, oxidoreductase activity, apoptotic process, Glycolysis / Gluconeogenesis	2.1	0.04
ptp4a3	NM_213181	protein tyrosine phosphatase type IVA, member 3;	phosphatase activity, protein dephosphorylation	2.1	0.04
aldoaa	NM_194377	aldolase a, fructose-bisphosphate, a	Glycolysis/Gluconeogenesis, Pentose phosphate pathway, Fructose and mannose metabolism	2.0	0.04

Table 11. Top 20 up-regulated genes in the 33 ˚C temperature group. Positive fold-changes indicate up-regulation in the 33 ˚C compared to reference group.

Gene	NCBI gene ID	Gene name	Gene Ontology Function	Fold change	P-value
hspa1B	NM_001100062	heat shock 70kDa protein 1B (HSPA1B)	nucleotide binding, ATP binding, MAPK signaling pathway, Endocytosis, stress response	49.1	0.004
hspa8 (1 of 2)	NM_001200012	Heat shock 70kDa protein 8	signal transduction, apoptosis, protein homeostasis	14.6	0.01
zgc:194878	NM_001136256	reverse transcriptase	RNA binding, transition metal ion binding,	13.1	0.004
hspb1	NM_001008615	heat shock protein, alpha-crystallin-related, 1	regulation of myofibril size, response to temperature stimulus, response to heat. thermotolerance in vivo and support of cell survival under stress conditions. MAPK signaling pathway, VEGF signaling pathway	12.9	<0.001
pklr	NM_201289	pyruvate kinase, liver and RBC	Glycolysis / Gluconeogenesis, Purine metabolism, Pyruvate metabolism.	11.3	0.01
serpinh1b	NM_131204	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1b	stress response, collagen fibril organization, maturation of collagen molecules, enzyme inhibitor activity	10.4	<0.001
mylpfb	NM_001004668	Myosin light chain, phosphorylatable, fast skeletal muscle b	increase in myofibrillar ATPase activity, calcium ion binding, metal ion binding	6.6	0.02
hsp90aa1.1	NM_131328	heat shock protein 90-alpha 1	stabilizes proteins against heat stress, skeletal myofibril assembly, muscle cell development, NOD-like receptor signaling pathway	5.6	0.01
zgc:195170	NM_001130638		cation transport, ion transmembrane transport, response to ATP, extracellular ATP-gated cation channel activity	5.4	0.03
mat2ab	NM_001014296	Methionine adenosyltransferase II, alpha	ATP binding, alkali metal ion binding. Cysteine	5.1	0.03

		b	and methionine metabolism, Selenoamino acid metabolism		
helt	NM_207065	hey-like transcription factor	DNA binding, regulation of transcription	4.9	0.03
h2afx (9 of 17)	NM_001103164	H2A histone family, member X	DNA binding, response to DNA damage stimulus, DNA recombination and repair	4.9	0.04
cib2	NM_200706	calcium and integrin binding family member 2	calcium ion binding, integrin-mediated signaling pathway	4.8	0.01
hsp90aa1.2	NM_001045073	heat shock protein 90-alpha 2	nucleotide binding, ATP binding, NOD-like receptor signaling pathway, Progesterone-mediated oocyte maturation, stress response	4.7	0.02
tbx6	NM_153666	T-box 24	DNA binding, transcription factor activity, hydrolase activity, transcription regulator activity	4.6	0.04
hoxb6b	NM_131538	homeo box B6b	DNA binding, transcription factor activity, transcription regulator activity	4.5	0.04
chrna6	NM_001042684	cholinergic receptor, nicotinic, alpha 6	ion channel activity, cation channel activity, channel activity, passive transmembrane transporter activity	4.3	0.03
mtnr1c	NM_001161484	melatonin receptor 1C	melatonin receptor activity, signal transducer activity, G-protein coupled receptor signaling pathway/	3.9	0.03
zgc:86709	NM_001002066	actin, alpha, cardiac muscle 1a	ATP binding, Cardiac muscle contraction	3.7	0.03
homer1b	NM_001002496	homer homolog 1	muscle structure development	3.4	0.03

Table 12. Top 20 down-regulated genes in the 33 ˚C temperature group. Negative fold-changes indicate down-regulation in the 33 ˚C compared to reference group.

Gene	NCBI gene ID	Gene name	Gene Ontology Function	Fold change	P-value
hmgb3a	NM_001122836	high-mobility group box 3a	DNA binding, chromatin binding, chromatin remodeling	-86.2	<0.001
zgc:92880	NM_001003431	zgc:92880	heme binding, oxygen transporter activity, metal ion binding	-27.1	0.04
zgc:112242	NM_001020668	pyruvate dehydrogenase kinase, isozyme 3b	part of troponin complex, involved in muscle contraction	-25.3	<0.001
tmem120A (2 of 2)	NM_001005946	zgc:103681	membrane component	-12.2	0.001
mhc1uka	NM_001045460	major histocompatibility complex class I UCA gene;	antigen processing and presentation, immune response	-9.8	0.02
ankrd29	NM_001020760	ankyrin repeat domain 29	repair and regeneration of muscle tissue following damage due to stress	-9.7	0.006
slco2a1	NM_001089582	solute carrier organic anion transporter family, member 2A1	Transporter	-7.5	0.01
epdr1	NM_001002416	ependymin related protein 1	calcium binding, metal ion binding, regulation of cell adhesion	-6.9	0.01
zgc:65811	NM_200552	zgc:65811	membrane component	-6.9	0.02
arhgap4a	NM_200444	Rho GTPase activating protein 4a	negative regulation of cell migration, timing of cell division, signal transduction, spine morphogenesis	-6.9	0.02
bmp2k	NM_001008644	BMP2 inducible kinase	ATP binding, protein phosphorylation	-6.7	0.03
klc3	NM_001077178	Kinesin light chain 3	microtubule motor activity	-6.4	0.005
zgc:64065	NM_201334	Phosphatidylinositol-specific	phosphoric diester hydrolase activity, lipid	-6.2	0.04

		phospholipase C X domain-containing protein 1 (PI-PLC X domain-containing protein 1)	metabolic process		
sftpbb	NM_001171066	Surfactant protein Bb	metal ion binding, lipid metabolic process	-6.0	0.01
apobec2 (2 of 2)	NM_001168463	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 2	skeletal muscle organ development, sensory epithelium regeneration, heart looping, positive regulation of DNA binding	-5.8	0.03
kxd1	NM_001003469	KxDL motif containing 1	ATP binding, metal ion binding	-5.6	0.04
twf1b	NM_001002304	twinfilin, actin-binding protein, homolog 1b	Actin-binding protein, negative regulation of actin filament polymerization	-5.5	0.04
nt5c2b	NM_001079966	5,-nucleotidase, cytosolic Iib	inosine monophosphate (IMP) metabolism	-5.1	0.01
slc22a3	NM_001114460	Solute carrier family 22 (organic cation transporter), member 3	Transporter	-5.1	0.02
slc29a4	NM_001080572	Solute carrier family 29 (nucleoside transporters), member 4	Transporter	-4.8	0.02

3.3.3. Pathway analysis

The canonical pathways which were observed for these differentially expressed transcripts are given in Table 13-14. Some of the important pathways among the up-regulated transcripts in the 24 ºC group were extracellular matrix (ECM)-receptor interaction, focal adhesion pathway, GnRH signaling and metabolic pathways (metabolism of amino acids, lipid metabolism and energy metabolism). Several down-regulated transcripts in the 24 ºC group were involved in protein processing in endoplasmic reticulum, progesterone-mediated oocyte maturation and NOD-like receptor signaling pathways. Most of up-regulated transcripts in the 28 ºC group were involved in metabolic pathways (such as energy metabolism, metabolism of amino acids, carbohydrate metabolism, lipid metabolism, biosynthesis of antibiotics and amino acids), PPAR signaling pathway, pentose phosphate pathway (part of carbohydrate metabolism), adipocytokine signaling pathway and p53 signaling pathway. Some of up-regulated transcripts in the 33 ºC group were involved in metabolic pathways (for example carbohydrate metabolism, nucleotide metabolism, energy metabolism, metabolism of amino acids, biosynthesis of antibiotics and amino acids), focal adhesion, tight junction, regulation of actin cytoskeleton, neuroactive ligand-receptor interaction, MAPK (mitogen-activated protein kinase) signaling pathway, VEGF (vascular endothelial growth factor) signaling pathway, protein processing in endoplasmic reticulum, progesterone-mediated oocyte maturation and NOD-like receptor signaling pathway. The down-regulated transcripts in the 33 ºC group were involved in some metabolic pathways (such as energy metabolism, metabolism of amino acids, nucleotide metabolism and metabolism of cofactors and vitamins).

Table 13. The canonical pathways for down-regulated transcripts in 24 ˚C and 33 ˚C temperature groups compared to reference group. Obtained from KEGG Pathway Analysis based on differentially regulated transcripts.

Down-regulated transcripts in 24 ˚C group	Down-regulated transcripts in 33 ˚C group
dre04141 Protein processing in endoplasmic reticulum dre04914 Progesterone-mediated oocyte maturation dre04621 NOD-like receptor signaling pathway	dre01230 Biosynthesis of amino acids dre01210 2-Oxocarboxylic acid metabolism dre00250 Alanine, aspartate and glutamate metabolism dre01200 Carbon metabolism dre00230 Purin metabolism dre00240 Pyrimidine metabolism dre00760 Nicotinate and nicotinamide metabolism dre00480 Glutathione metabolism dre00590 Arachidonic acid metabolism

Table 14. The canonical pathways for up-regulated transcripts in 24 ˚C and 33 ˚C temperature groups compared to reference group, obtained from KEGG Pathway Analysis based on differentially regulated transcripts.

Up-regulated transcripts in 24 ˚C group	Up-regulated transcripts in 33 ˚C group
dre00480 Glutathione metabolism dre00590 Arachidonic acid metabolism dre00910 Nitrogen metabolism dre04512 ECM-receptor interaction dre04145 Phagosome dre04510 Focal adhesion dre04912 GnRH signaling pathway	dre00620 Pyruvate metabolism dre00230 Purine metabolism dre01200 Carbon metabolism dre00270 Cysteine and methionine metabolism dre00010 Glycolysis/Gluconeogenesis dre04510 Focal adhesion dre04530 Tight junction signaling dre04810 Regulation of actin cytoskeleton dre04080 Neuroactive ligand-receptor interaction dre01230 Biosynthesis of amino acids dre01130 Biosynthesis of antibiotics dre04010 MAPK signaling pathway dre04141 Protein processing in endoplasmic reticulum dre04914 Progesterone-mediated oocyte maturation dre04621 NOD-like receptor signaling pathway dre04370 VEGF signaling pathway

4. Discussion

In the current study, we used high-throughput sequencing to examine the long-term temperature effect on thermal plasticity of ncRNA expression and gene expression in zebrafish reared at 24 °C (cold group), 28 °C (reference temperature) or 33 °C (warm group) from fertilized egg to adulthood.

We observed the influence of rearing temperature on the general development of zebrafish. The hatching period was affected by temperature. The present results are consistent to previous studies (Schaefer and Ryan, 2006; Schmidt and Starck, 2010; Sfakianakis et al., 2012; Villamizar et al., 2012). In several research studies there was reported that temperature significantly affects on the zebrafish weight and body length. For instance Parichy et al. (2009) found that temperature has certain influence on zebrafish larvae growth rates. They observed a faster growth under warm treatment comparing to reference conditions and cold treatment. In other research studies, fish from the group under cold treatment showed similar results in comparison with ones from group under warm treatment, but they were smaller and grew slower than fish, which have been reared in reference conditions. For instance Johnston et al. (2009) found that zebrafish, which have been reared at 22 °C were bigger (TL 27.8 mm) than those, which have been reared at 31 °C (TL 23 mm), but still smaller than fish reared at reference conditions (TL 29.8 mm). Other results were obtained by Sfakianakis et al. (2011). In their research, zebrafish had been reared at 22, 28 and 31 °C, and it was found that fish under 31 °C treatment (TL 33.1 mm) were bigger than fish, which had been reared at reference temperature (TL 32.7 mm) and under cold treatment (TL 32.6 mm). Similar trend in their study was observed for fish weight: fish under 31 °C treatment (0.36 g) were bigger than fish, which had been reared at reference temperature (0.35 g) and under cold treatment (0.35 g). In current study zebrafish, which have been reared at 24 °C (TL 26.1 mm, weight 0.18 g), were bigger and heavier than fish, which have been reared at reference temperature (TL 20.4 mm, weight 0.12 g) and 33 °C (TL 18.4 mm, weight 0.06 g). This result in current study might be associated with density of fish in tank during the rearing. Parichay et al. (2009) in their work also showed that at high density zebrafish grew slowly than fish reared at low density. In present study, due to the high mortality, the density of fish per tank in cold temperature group was much smaller compared to the other groups, while the density per tank in normal temperature group was the highest.

The influence of rearing temperature on zebrafish muscle phenotype and gene expression was observed in present study. Overall, 206 miRNAs were identified in fast

muscle of zebrafish with a wide range of relative abundances. The most abundant and significantly differentially expressed were *miR-100, miR-26b, let-7e, miR-99, miR-101b, miR-141, miR-153c, miR-200a* and *miR-218a*. The up- and down-regulation of these miRNAs in response to cold and warm acclimation was observed and the putative targets for these miRNAs were identified. In zebrafish muscle was also identified 376 differentially expressed transcripts, for which the up- and down-regulation in response to different types of rearing temperature was observed. Moreover, some of these transcripts were identified as putative targets for differentially expressed miRNAs.

The wide range of thermal tolerance in zebrafish is achieved by their ability to change their physiological phenotype. Several research studies reported that the phenotype of zebrafish myotomal muscle can undergo modifications in response to heat and cold temperature stress and long-term temperature acclimation. In zebrafish, these changes include myogenesis and expression of related genes (Du et al., 2008; Johnston et al., 2009; Johnston and Temple, 2002; Long et al., 2012; Malek et al., 2004; Murtha and Keller, 2003; Schaefer and Ryan, 2006; Schmidt and Starck, 2010; Schnurr et al., 2014; Schröter et al., 2008; Scott and Johnston, 2012; Vergauwen et al., 2010; Wang et al., 2014).

Zebrafish myogenesis is a tightly regulated process that is critically modulated by Wnt signaling. Wnt pathways regulate the differentiation of muscle stem cells and mediate the growth and recovery of muscle fibers in adult zebrafish (Bentzinger et al., 2012; von Maltzahn et al., 2012). One of the members of the high mobility group (HMG) family, *hmgb3* has been shown to be involved in modulation of Wnt-signalling. *Hmgb3* is a chromatin-associated nuclear protein that interacts with transcription factors, by enhancing their binding to DNA and their transcriptional activity (Nemeth et al., 2003). In a study with mice, which was performed by Maciotta et al. (2012), it was reported that *hmgb3a* are involved during muscle regeneration. In particular, they observed a strong down-regulation of this gene upon chronic and acute muscle injury. In zebrafish *hmgb3a* was reported as a regulator of dorsoventral mesoderm genes and dorsoventral patterning (Cao et al., 2012), but the involvement of *hmgb3a* in zebrafish muscle regeneration has not been previously reported. In the present study, *hmgb3a* was the most significantly up- and down-regulated transcript under cold and warm temperature, respectively, when compared to 28 °C. We observed a strong up-regulation of this gene (42.8 fold change) in the cold group and down-regulation (-86.2 fold change) in the warm group compared to the reference group. Based on the present result and on Maciotta's work, I suggest that in zebrafish *hmgb3a* might be also part of the pathways controlling muscle regeneration.

T-box transcription factors as well as Wnt signaling are essential regulators of formation and differentiation of muscle stem cells. One of these factors is a *tbx6*. The myotome determination is regulated by a gene regulatory network that includes this gene. *tbx6* is a necessary component of a segmentally restricted myogenic inhibitor, which is necessary for the development of the skeletal muscle (Windner et al., 2012). In zebrafish the complete loss of this gene leads to the disruption of segmentation and loss of anterior somite compartment identity. Tbx6 is known to interact with Hsp70 and heat shock is known to induce the ubiquitous expression of *tbx6* (Windner et al., 2012). We observed the up-regulation of *tbx6* in the warm temperature group (comparing to reference and cold groups). Another transcription factor, which also plays an important upstream role in myogenesis in zebrafish, is sine oculis–related homeobox (Six). The *six1* and *six4* genes are part of regulatory cascade that directs skeletal muscle progenitors toward the myogenic lineage. Six proteins act as cofactors to activate the MRFs (such as *pax3, myoD, mrf4*, and *myog*) expression in the myotome (Bentzinger et al., 2012; Bismuth and Relaix, 2010). In the present research, *six4a* transcript was observed among the significantly up-regulated transcripts in the cold temperature group (compared to reference and warm groups) and predicted as a putative target for *miR-218a*, which had an opposite expression and was up-regulated in warm group comparing to reference and cold groups. Previously the *miR-218a* was not reported as a participant of skeletal muscle development, but now I suggest that it might be involved in control of this process, since among the putative targets for this miRNA there can be obtained genes involved in Hedgehog and Wnt signaling pathways. These pathways are known to have a key role during zebrafish embryonic muscle development via controlling the expression of MRFs and play essential role in the maintaining of skeletal muscle homeostasis in adult fish (von Maltzahn et al., 2012). Overall, the data from this thesis suggest that the up-regulation of *tbx6* in the warm group can be involved in muscle recovery and renewal process, while the *si4a* (in cold group) can be involved in muscle growth and maintaining of muscle homeostasis.

The maintenance of muscle cellular homeostasis is essential to the fish. Temperature can causes the damage of nucleic acids and proteins on the cellular level and one way to recover the cellular homeostasis is by producing heat shock proteins (HSP) (Feder and Hofman, 1999). They tend to perform chaperone function by stabilizing new proteins to ensure correct folding and involved in preventing protein denaturation or processing denatured proteins (helping to refold proteins that were damaged by the cell stress), therefore appear to play a role in protecting cells against different environmental influences and

stresses. This group of proteins is known to be involved in tolerance of hyperthermia, tolerance of ischemia/hypoxia, recovery from translational and transcriptional inhibition following heat shock, regulation of heat-shock response (Feder and Hofman, 1999; Murtha and Keller, 2003). Moreover, recent research studies have shown the roles of HSPs during embryogenesis, myogenesis, growth and development of organism. For instance, the member of the molecular chaperone family Hsp90 (consisting of Hsp90α and Hsp90β) is known to be ubiquitously expressed in all eukaryotic cells and have a distinct role in cell proliferation, differentiation, stress management, and tissue development in a variety of species, including zebrafish (Du et al., 2008). For instance Sass *et al.* in their research in 1996 and 1999 found that *hsp90α* expressed in a spatial and temporal manner in zebrafish and have a role in the differentiation of slow and fast muscle lineages during development (Sass et al., 1999). Srikakulam and Winkelmann in their work (2004) also found that Hsp90 forms a complex with newly synthesized myosin proteins and is involved in myosin folding and assembly (Srikakulam and Winkelmann, 2004). A crucial role of *hsp90α1* in myofibril assembly during muscle development of zebrafish was reported by Du et al. (2008). They identified that knockdown of *hsp90α1* expression disrupted myofibril organization in skeletal muscles of zebrafish embryos, which led to paralyzed fish embryos without skeletal muscle contraction. Other classes of HSPs such as Hsp70 are also able to form a complex with nascent myosin filaments and involved in muscle recovery after damage. Hsp70 is crucial for protein homeostasis due to its ability to mediate repair and degradation of altered or denatured proteins (Du et al., 2008). Several studies investigated the alteration expression of HSPs in response to temperature upshift or stress. Murtha and Keller (2003) found that expression of *hsp70* was up-regulated in zebrafish brain, liver and muscles in response to heat shock. They also found the up-regulation of *hsp47*, but only in zebrafish brain and despite that they observed the expression of *hsp90α* and *hsp90β* in brain, muscles and liver, they didn't find the up-regulation of these HSPs in response to heat stress. The up-regulation of *hsp70* in response to heat shock has been also reported in work of Airaksinen et al. (2003). The down-regulation of *hsp70* and *hsp90* in zebrafish skeletal muscle in response to the low temperature treatment was shown in work of Malek et al. (2004). Vergauwen and colleagues in their work in 2010 found that *hsp70* level was up-regulated due to long-term warm acclimation, but not due to cold. In present study, the up-regulation of *hsp47*, *hsp70* (such as *hspa1b* and *hspa8*), *hsp90 (*such as *hsp90α1.1* and *hsp90α1.2)* and *hspb1* (hsp, alpha-crystallin-related) was observed in warm temperature group (33˚ C) comparing to cold and reference groups and probably this trend can be related with muscle damage and their recovery processes in this group. A down-

regulation of *hsp90α1.1, hsp90α1.2* and *hspb1* (hsp, alpha-crystallin-related) was found in the cold group (comparing to reference and warm groups). Some of these HSPs were predicted as putative targets for the significantly differentially expressed miRNAs which were identified in the current study. For instance, *hspb1* is one of putative targets of *miR-100*, *hspa8* and *hspd1* are targets of *let-7e*, and *hsp90ab* is putative target for *miR-99* and *miR-100*. Previously, was not reported about the involving of these miRNAs in thermal response in zebrafish and the question of their role in it requires further investigation.

Other gene family which is known to be related to protein folding and repair and involved in muscle recovery after damage is the Ankyrin gene family (Miller et al., 2003). The ankyrin repeats are one of the most common protein–protein interaction platforms in nature, which have been found in numerous proteins with functions like cell signaling, cytoskeleton integrity, transcription and cell–cycle regulation, inflammatory response, development, cell-fate decisions during development and interaction with ubiquitin. Several ankyrin repeats were found in members of the TRP cation channel family, which include heat and cold sensitive receptors. Some of the ankyrin repeats, which is called muscle ankyrin repeat proteins (MARPs) are known to be involved in regulation of muscles stress response pathways (Miller et al., 2003; Mosavi et al., 2004). In the present study, members of this gene family were found among the putative target for the *miR-153* (for example *ankrd28*), which was up-regulated in warm group and the *ankrd29* was found among the significantly down-regulated transcripts in warm group comparing to cold and normal temperature groups. This gene still not well described and previously was not reported as a type of MARPs, but based on result of this study it is possible to suggest that it can be associated with muscle recovery and stress response. This new finding warrants further investigation.

The detection, repair and degradation of damaged proteins play an essential role in the stress response and maintaining of muscle cellular homeostasis. It is known that temperature activates the pathways associated with DNA damage signaling and repair. The ability of normal cells to detect DNA damage and activate specific signaling networks to enable the DNA repair (including initiation of apoptosis when occur excess damage, which is too severe and not retrievable) is the crucial for maintaining genomic stability under the stresses condition (Mueller et al., 2013). The DNA damage repair (DDR) response is a signal transduction pathway, which activated upon DNA damage. Many genes involved in this pathways and it is known that miRNAs also involved in regulatory networks affecting the DNA damage/repair process (Tessitore et al., 2014). The recognition of damaged proteins there is one of the important parts of these DDR pathways. One of the first cellular responses

to the DNA damage is the phosphorylation of *h2ax* (Burma et al., 2001; Paull et al., 2000). Phosphorylation of *h2ax* appears to play a critical role in the damage-signaling pathways and known to increases linearly with the severity of the damage (Burma et al., 2001). In the present study the *h2afx* gene were found to be significantly up-regulated in the 33 ˚C group comparing to other groups and apparently indicates serious muscle damage, which occurs in this group.

Other important part of the DDR pathways is the rapid degradation of damaged proteins. One of the major protein degradation pathways in the eukaryotes is the ubiquitin-proteasome pathway. Ubiquitin is a polypeptide that attached to the proteins, which were marked for degradation. Ubiquitination is a multistep process, which include the ubiquitin activation by ubiquitin-activating enzyme, transfer of activated ubiquitin to ubiquitin-conjugating enzymes, then to ubiquitin ligase and then to a specific target protein. These polyubiquinated proteins are then recognized and degraded by a proteasome (Cooper, 2000). In current study, the genes involved to the protein ubiquitination were identified as the putative targets for some of the significantly differentially expressed miRNAs in zebrafish muscles. For instance the *ube2n* (ubiquitin-conjugating enzyme E2n) was predicted to be a putative target for both, *miR-100* and *miR-99* and the *usp5* (ubiquitin specific peptidase 5), which was identified among the putative target of *miR-99*. *ube2n* and *usp5* are known to be responsible to the detection and response to the DNA damage and to the post-replication repair and protein degradation. The *ufd1l* (ubiquitin fusion degradation 1-like) is a putative target of *miR-26b* (which in current study was up-regulated in the 33 ˚C group comparing to the 28 ˚C group, and down-regulated in 24 ˚C group). Other target of *miR-26b* - *klhl40a* (kelch-like family member 40a) are also known to be involved in the negative regulation of the protein ubiquitination. Talking about the members of Kelch-like protein family, through their role in protein ubiquitination and degradation, some of them are also involved in negative regulation of Wnt signaling and linked to skeletal myogenesis (Abou-Elhamd et al., 2009). Thus, based on this we can suggest, that *miR-26b* with their target *klhl40a* can also be involved in skeletal muscle myogenesis in zebrafish.

Of vital importance to the integrity of the genome is the cell's ability repair its DNA. One of the DNA repair complexes is ATP-dependent chromatin remodeling complex, which include several different protein families that able to remodel the way DNA is packaged (Mueller et al., 2013; Tang et al., 2010). One of these protein families is SWI/SNF (SWItch/Sucrose NonFermentable). The SWI/SNF chromatin remodeling factor

snf2h/smarca5 is required for efficient recruitment of DDR proteins and involved to the DNA damage detection and repair, and known to be target for the *miR-99* (Mueller et al., 2013).

5. Conclusions

A total of 376 transcripts were differentially expressed in fast muscle of zebrafish between the different temperature groups. These transcripts are involved in regulation of muscle recovery, renewal and growth, different metabolic processes and response to environmental stressors, including thermal and oxidative stress. Some of them were predicted as putative targets for several miRNAs, which were also significantly differentially expressed in response to different rearing temperatures. A number of differential expressed miRNAs had not been previously associated with skeletal muscle development in zebrafish, namely as *miR-26b*, *miR-99*, *miR-100* and *miR-218a*. These miRNAs were shown to target heat shock proteins, genes involved in processes of protein and DNA damage, degradation and repair, transcription factors and genes involved in regulation of myogenesis. The potential involvement of these miRNAs in zebrafish acclimation and myogenesis warrants further investigation. Taken together, the results of this thesis indicate that rearing temperature is an essential factor in zebrafish growth and has a strong influence on thermal plasticity of mRNA and miRNA expression in fast muscle.

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