Early life exposure to diel thermal variation alters microRNA expression and performance in zebrafish.

by

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Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Freshwater ecosystems are characterized by large thermal variations, especially in the summer months. However, this variability is rarely accounted for in laboratory studies examining physiological processes of fish inhabiting these environments, thereby producing results which lack ecological realism. Understanding how animals respond to and cope with rapid changes in temperature is more crucial than ever as the range of thermal fluctuations in these habitats is expected to increase dramatically due to increases in the frequency and severity of heat waves. Recently, it was posited that epigenetic mechanisms could buffer fish against such thermal fluctuations as they act on a more rapid timescale than genetic adaptation. Thus, the aim of this thesis was to understand how chronic exposure to thermal variability impacts the physiology of freshwater fish, and if these effects are associated with changes in epigenetic modulators called microRNAs (miRNAs) which act by repressing the translation of target genes. This was achieved by subjecting zebrafish (*Danio rerio*) to realistic diel thermal fluctuations (FLUX; 28 ± 5°C) throughout the embryonic and larval stages to evaluate the effects on physiological processes like metabolism and survival, and on the expression of seven thermosensitive miRNAs and three heat shock proteins (HSPs). These fish were compared to those kept at constant control (CTRL; 28°C) and constant elevated (HEAT; 33°C) conditions to allow for comparisons with fish reared under optimal lab conditions and in traditional climate change experiments, respectively. After the developmental stages, the fish from CTRL and FLUX treatments were reared under common control conditions until adulthood to understand if developmental exposure to fluctuations altered miRNA expression profiles in the brain and the upper thermal tolerance (CT_{max}).

This study revealed that while the HEAT conditions reduced survival throughout development, the FLUX thermal regime had no impact on this. Additionally, both FLUX and HEAT conditions significantly altered body weight as well as the miRNA expression profiles during early life stages. Although juveniles from both FLUX and HEAT conditions were able to metabolically compensate to their respective thermal regimes, the degree of compensation was greater in FLUX fish. I found that miR-181a-5p, which regulates pathways associated with mitochondrial biogenesis and respiration, was significantly upregulated in the juveniles from these groups, suggesting that changes in this miRNA could modulate the observed metabolic impacts. Notably, this miRNA remained elevated in the brains of longitudinal adults with FLUX developmental histories, though their CT_{max} was unaffected. Besides its role in modulating mitochondrial function, insights from mammalian models

suggest that this miRNA can also enhance neuronal injury/damage in vertebrate brains. This could indicate that FLUX fish have an altered capacity for recovery following acute thermal stress. Collectively, the findings presented in this thesis demonstrate that developmental plasticity may be regulated by changes in epigenetic processes and underscore the need to incorporate variability into our experiments, as it produces a robust and long-lasting impact on the physiology of fish that is distinct from static temperature exposures. Ultimately, ecologically realistic conditions and the plasticity potential of populations need to be accounted for in investigations to accurately predict how fish will respond to the multiple stressors caused by climate change.

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List of Abbreviations

CT_{max}—Critical thermal maximum

CT_{min}—Critical thermal minimum

CTRL—Constant optimal/control temperature group

DO—Dissolved oxygen

dpf—Days post fertilization

FLUX—Diel thermal fluctuation group

HEAT—Constant elevated temperature group

hpf—Hours post fertilization

HSP—Heat Shock Protein

HSR—Heat Shock Response

KEGG—Kyoto Encyclopedia of Genes and Genomes

miRNA— MicroRNA

MO_2—Oxygen consumption rate

mpf—Months post fertilization

mRNA— Messenger RNA

MS-222—Tricaine methanesulfonate

OCLTT—Oxygen- and capacity-limited thermal tolerance

RT-qPCR—Reverse transcriptase - quantitative polymerase chain reaction

T_{ag}—Agitation temperature

TCA—Tricarboxylic acid cycle

T_{opt}—Thermal optimum

TPC—Thermal performance curve

Chapter 1
General Introduction

1.1 Environmental variability and its impact on ectotherm physiology

In the 6th century BC, renowned Greek philosopher, Heraclitus, famously proclaimed that "change is the only constant" (Robjant, 2014). Although this adage is usually interpreted in a philosophical context, its relevance to freshwater ecosystems (which originally inspired the saying) is undeniable. Specifically, abiotic factors in these environments such as temperature, light, pH, and dissolved oxygen exhibit a great deal of variability over various temporal scales, including annual, seasonal, and diel (Morash et al., 2018). To maximize their performance and fitness under these changing conditions, organisms living in these environments have evolved to possess mechanisms that allow them to alter their physiological processes accordingly (Claireaux & Lefrançois, 2007; Kern et al., 2015; Dillon et al., 2016).

Of these abiotic variables, temperature has the largest impact on the performance and distribution of ectothermic species as it regulates the rates of most biochemical processes (Kern et al., 2015). As such, many studies have explored the impacts of thermal stress on physiological performance indicators like growth (Imsland et al., 1996), development (Schirone & Gross, 1968), metabolism (Sandersfeld et al., 2017) and reproductive output (Pankhurst & Munday, 2011). A majority of these investigations, however, evaluated these parameters under constant elevated thermal conditions as cycling conditions can be technologically or logistically challenging to incorporate into experiments (Morash et al., 2018). While insights from such work have furthered the collective understanding of the mechanisms underlying the response of these animals to changes in body temperature, they have limited applicability to animals under natural conditions as they do not account for the complex thermal patterns they encounter (Calisi & Bentley, 2009; Turko et al., 2023). As an example, models generated using data from static thermal conditions underestimated the developmental rate of striped marsh frogs (*Limnodynastes peronii;* Niehaus et al., 2012) and five *Sepsis* fly species (Khelifa et al., 2019), as well as the growth rate of the tobacco hornworm (*Manduca sexta*; Kingsolver et al., 2015) under naturalistic fluctuating thermal conditions. Thus, understanding how animals respond to environmental changes is crucial, especially because it would help shape management practices for vulnerable ectothermic species (Turko et al., 2023). There is also considerable economic interest in

this as a significant proportion of aquaculture species are reared in open net pens where they are exposed to natural changes in environmental conditions (Brauner & Richards, 2020).

The discrepancy between the rates of physiological processes of organisms in constant and fluctuating conditions arises because ectotherm performance scales non-linearly with temperature through a relationship known as the thermal performance curve (TPC; Fig. 1; Morash et al., 2018). TPCs of most ectothermic species are bell-shaped and negatively skewed such that the thermal optimum (i.e., the optimal temperature for performance; T_{opt}) is closer to the upper critical threshold (CT_{max}) than the lower critical threshold (CT_{min}; Morash et al., 2021). Due to this property, known as Jensen's inequality, organismal performance differs under fluctuating conditions (Colinet et al., 2015; Dowd et al., 2015). For instance, if the mean temperature of an organism's habitat is equal to or greater than the T_{opt} for that species, symmetrical fluctuations around this value would be expected to reduce organismal performance and fitness (Massey & Hutchings, 2020). If the temperature of the environment is close to the CT_{max}, such fluctuations could also be lethal. While the instantaneous performance of the organisms from the two different thermal conditions may not differ at these specific temperatures, the overall performance is altered as a result of the time spent at sub-optimal conditions (Morash et al., 2021). Alternatively, if the mean temperature of the habitat is below the T_{opt}, equivalent fluctuations around the mean would enhance average performance. In line with the predicted impact of fluctuations on the TPCs, both theoretical models and empirical data indicate that rapid temperature changes have a greater impact on ectotherm physiology as compared to gradual changes in average temperature (Ruel & Ayres, 1999; Bozinovic et al., 2011; Estay et al., 2011; Paaijmans et al., 2013; Verheyen & Stoks, 2019).

To understand the specific impact of temperature fluctuations on the physiological processes of a species, factors such as the range of fluctuations experienced, the shape of the curve, and the thermal breadth of the species must be considered (Morash et al., 2018). This is further complicated by evidence which suggests that animals that are chronically exposed to fluctuating temperatures can alter the thermal sensitivity of their physiological processes through acclimation/acclimatization or plasticity, thereby altering the shape of their TPC and buffering the detrimental effects of variability (Hochachka & Somero, 2002; Angilletta, 2009). Some researchers have also theorized that organisms may be able to confine critical physiological processes to short windows of favorable temperatures (i.e., near T_{opt}) throughout the day to maintain their performance (Gilchrist, 1995). For this to be advantageous as a strategy, however, the organism would need to be eurythermal and experience

favorable temperatures fairly frequently or for long durations in its environment (Gilchrist, 1995; Drake et al., 2018; Kefford et al., 2022).

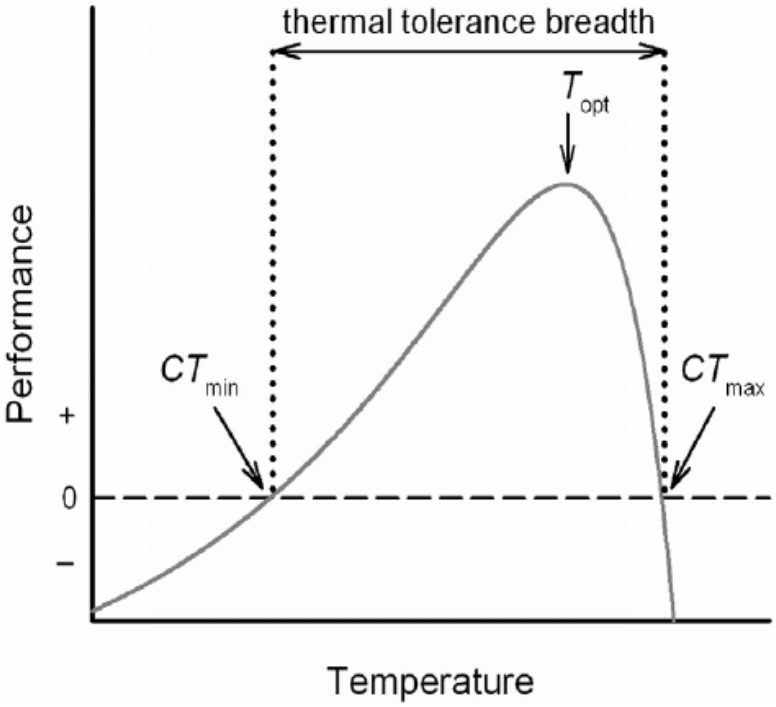


Figure 1.1: Thermal performance curve for ectotherms from Krenek et al. (2012). The thermal optimum (T_{opt}) is the temperature at which performance is optimal, and the upper (CT_{max}) and lower (CT_{min}) critical thresholds indicate the temperature beyond which performance becomes negative.

1.2 Diel thermal fluctuations

Much of the existing literature that has explored the effects of thermal variability on ectotherms has focused on the impact of diel thermal fluctuations on the physiology of aquatic organisms like freshwater fish. There are a few reasons for this, with the first being that diel changes in temperature are the most predictable and pervasive thermal patterns in nature as they occur every 24 hours (Kefford et al., 2022). Secondly, because riverine ecosystems experience large diel fluctuations (often exceeding 20°C; Morash et al., 2021), there is a great deal of interest in understanding how organisms living in these environments cope with such large temperature swings over a short period. Such systems often also have a high degree of water mixing, especially in shallow areas, which increases the thermal homogeneity of microhabitats (Kefford et al., 2022). This limits the potential for employing behavioral thermoregulatory strategies like moving into thermal refugia, causing

organisms to rely more heavily on physiological mechanisms to buffer them against the effects of this variability (Kefford et al., 2022).

With climate change, the range of fluctuations experienced by aquatic ecosystems is predicted to increase drastically (Easterling et al., 2000; Rummukainen, 2012; IPCC, 2021). In Canada alone, many reports predict that freshwater bodies will experience an increase of 1.8-6.6°C in mean surface water temperatures along with daily temperature fluctuations exceeding 10°C by the end of the century (Morash et al., 2020; IPCC, 2021). Such effects will likely be driven by an increase in the occurrence and severity of extreme weather events such as droughts and heat waves (Meehl & Tebaldi, 2004), and exacerbated by influences from anthropogenic activities like dam construction (Steel & Lange, 2007), wastewater treatment effluent release (Kinouchi et al., 2007; Łaszewski, 2017), and thermal power generation (Raptis et al., 2016). Vasseur et al. (2014) demonstrated that incorporating diel thermal variation along with the mean temperature in the performance projections of 38 ectotherm species explained 93% of the variance in the species' performance under future climate change conditions. Alternatively, accounting for the mean or diel variation alone explained only a third and half of the variance, respectively. Thus, the precise effects of such rapid temperature changes on ectotherm physiology need to be characterized to predict how the distribution and population dynamics of such species will change in the coming years, and inform conservation strategies towards this (Paaijmans et al., 2013; Vazquez et al., 2017).

Studies that have evaluated the impact of diel fluctuations (with a mean near the T_{opt}) on life history traits like longevity and fecundity in fish have reported mixed effects (Žák & Reichard, 2020; Massey et al., 2022). Alternatively, a general trend towards reduced growth rate (Flodmark et al., 2004; Imholt et al., 2011; Žák & Reichard, 2020), size-at-age (Schaefer & Ryan, 2006), and developmental rate (Dhillon & Fox, 2007) has been observed upon exposure to fluctuations. These changes are often accompanied by an increased tolerance to high temperatures (Feldmeth et al., 1974; Schaefer & Ryan, 2006; Massey, 2023), and in some cases, to other co-occurring stressors such as hypoxia as well (Massey, 2023). As suggested by Morash et al. (2021), these physiological effects may be mediated by alterations in feeding behavior, growth efficiency, and/or metabolism under fluctuating conditions. An increase in the energetic demand, resulting from activation of energetically expensive cellular protective mechanisms (like the induction of the heat shock response; HSR), depletion of glycogen reserves, and maintenance of performance across a wide range of temperatures, is proposed to be the main driver of these effects. However, empirical support for this is scarce, as some studies indicate

that chronic exposure to fluctuations lowers whole-animal metabolic rate, while others suggest the opposite. Additionally, only a small proportion of these investigations have explored the molecular/cellular mechanisms underlying these changes (Podrabsky & Somero, 2004; Lim & Bernier, 2022; Caklec, 2023), presenting an avenue for further research.

1.3 Phenotypic plasticity driven by epigenetic mechanisms

Fish can buffer their performance from rapid environmental changes through phenotypic plasticity, a process by which a genotype can produce various phenotypic outcomes depending on the environmental conditions (O'Dea et al., 2016). The alteration in phenotype can include changes in various molecular, morphological, physiological, and behavioral traits, and can confer adaptive benefits to the organism in the context of its environment (Beldade et al., 2011). Naturally, not all traits are created equal. Some may only be capable of plasticity during critical periods, while others may exhibit 'robustness' throughout the organism's lifespan and remain unchanged despite a high degree of environmental variation (Beldade et al., 2011). As an example, while nutritional status during development can significantly alter the size of the eyestalks (an ornament for sexual selection) in male fruit flies (*Drosophila melanogaster*), it has a negligible impact on the development of the nervous system and gonadal size (David et al., 1998; Cheng et al., 2011; Tang et al., 2011). Although there has been a longstanding debate on whether phenotypic plasticity can impact the evolutionary trajectory of a species, the consensus seems to be that it favors adaptive evolution through multiple processes, including the accumulation of cryptic changes (genetic variation which does not manifest in phenotypic alterations; reviewed by Pfennig et al., 2010; Beldade et al., 2011). Results from theoretical models also suggest that such plasticity is often critical in buffering population dynamics from environmental variability, especially when abiotic factors follow relatively predictable temporal patterns and provide consistent cues (Reed et al., 2010; Seebacher et al., 2014).

Developmental plasticity, which occurs in response to environmental changes during the early life stages, has come to the forefront of climate change discussions for its potential to shield populations against the effects of high temperature and thermal variability (Burggren, 2018; Massey & Hutchings, 2021; Pottier et al., 2022). The reason for this is three-fold. First, ectotherms are usually the most sensitive to temperature extremes when they are immature due to their undeveloped physiological systems and their highly limited ability for dispersal as embryos and larvae (Coulter et al., 2015; Burggren, 2018; Dahlke et al., 2020). Secondly, most cells are in the process of differentiating into

specialized cell types during the embryonic and larval stages (West-Eberhard, 2003; Martin et al., 2011). So, any deviations in this developmental program could permanently alter the fate of these cells, thereby influencing the phenotype of the adult, and consequently, its fitness (West-Eberhard, 2005; Bateson et al., 2014; Vagner et al., 2019). Finally, as Burggren (2018) emphasized, the ability of an adult fish to exhibit plasticity in response to thermal challenges is inconsequential for the population if the developmental stages cannot alter their response to these changes and fail to reach maturity. Despite the results of numerous studies detailing the plastic response of fish to diel thermal fluctuations during development, as well as their impacts on physiological processes during adulthood (Schaefer & Ryan, 2006; Coulter et al., 2015; Eme et al., 2018; de Alba et al., 2021; Massey et al., 2022), the proximate mechanisms underlying these long-term alterations have yet to be identified.

Epigenetic mechanisms are an important source of phenotypic plasticity in fish as they can alter gene expression without modifying the original DNA sequence of cells (O'Dea et al., 2016; Eirin-Lopez & Putnam, 2019; Venney et al., 2022). These marks can be reliably replicated and transferred to daughter cells during cell proliferation, resulting in the maintenance of epigenetic changes throughout the organism's lifespan (Skinner, 2011). Additionally, these epigenetic patterns can also be transmitted to subsequent generations via germ cells, thus allowing populations to potentially adapt to rapid environmental changes in a shorter time frame than through genetic adaptation (Kristensen et al., 2018; Eirin-Lopez & Putnam, 2019; Major et al., 2020).

There are three main processes through which epigenetic changes can occur, namely, methylation of DNA, modification of histones, and regulation of gene expression via small, non-coding RNAs (Champroux et al., 2018). While all three marks can be altered in response to environmental stressors, there is little evidence that alterations in DNA methylation and histone patterns are reliably preserved across generations (de Waal et al., 2012; Jiang et al. 2013; Radford et al., 2014; Iqbal et al., 2015). This is likely the result of the epigenetic reprogramming that occurs at the mid-blastula transition in most vertebrate embryos, where such changes are erased (at least, in part) and de novo marking occurs (Mhanni & McGowan, 2004; Lindeman et al., 2011; Wu et al., 2011). The ability of non-coding RNAs to bypass this reprogramming, influence other epigenetic marks, and their ease of measurement through sequencing and qPCR, have made them the subject of a large body of research that investigates the role of molecules in developmental and transgenerational plasticity (Bartel, 2009; Jodar et al., 2013; Wang & Liu, 2022; Heinrichs-Caldas et al., 2023).

1.4 Role of microRNAs in response to environmental changes

MicroRNAs (miRNAs) are a class of short (~22 nucleotides long) non-coding RNAs that serve as epigenetic modulators by either degrading or preventing the translation of one or more mRNAs (Cai et al., 2009). They were first discovered three decades ago as modulators of development in the roundworm (*Caenorhabditis elegans*) and have since been studied extensively across multiple taxa for their prominent role in regulating mRNA expression (Lee et al., 1993; Wightman et al., 1993). Studies estimate that nearly six out of every 10 mRNAs in mammals contain regions for miRNAs to bind to, highlighting their widespread importance for physiological processes (Friedman et al., 2009; Guo et al., 2010). Although no such approximation exists for teleost fish, a similar proportion of mRNAs are predicted to be targeted by miRNAs given how conserved gene regulatory networks are across vertebrates (Bizuayehu & Babiak, 2014).

MiRNAs undergo processing through a complex multi-step pathway before they can exert their regulatory effects on their target mRNAs. In the most common biogenesis pathway, these molecules are initially derived by transcription of the DNA by either RNA polymerase II or III in the nucleus, which results in the formation of transcripts with one or more hairpin loops called primary miRNAs (pri-miRNAs; Georgakilas et al., 2014). Drosha, an RNAse III enzyme that is part of the microprocessor protein complex, excises the loops to generate shorter transcripts known as precursor miRNA (pre-miRNA; Lee et al., 2003; Denli et al., 2004). The pre-miRNAs are then transported into the cytosol via the exportin-5 complex, where another member of the RNAse type III family called Dicer cleaves the terminal loops to produce a miRNA duplex (Yi et al., 2003). One strand from the duplex, referred to as the guide strand, is loaded onto the Argonaute2 (Ago2) protein to form the RNA-induced silencing complex (RISC; Lee et al., 2002; Lee et al., 2003; McGeary et al., 2019). The miRNA in the RISC contains a seed region on its 5’ end comprising of a few nucleotides that can bind to the 3’ untranslated region of multiple mRNAs (Bartel, 2009; McGeary et al., 2019). The degree of complementarity between these regions is a crucial determinant of the regulatory impact of the miRNA, with complete complementarity resulting in the degradation of the mRNA and incomplete pairing (which is more common in biological systems) repressing its expression (Guo et al., 2010; Bizuayehu and Babiak, 2014). MiRNAs produced in one tissue are also capable of targeting mRNAs in other tissues as they can be released into circulation where they remain highly stable because of their association with protein complexes (Arroyo et al., 2011; Turchinovich et al., 2016).

As such, a singular miRNA is able to down-regulate the production of hundreds of proteins involved in various biochemical pathways, thus fine-tuning multiple physiological processes.

Over the last two decades, miRNAs have garnered significant attention as their expression levels have been shown to be altered across various fish tissues and developmental stages by changes in water temperature (Johnston et al., 2009; Campos et al., 2014; Bizuayehu et al., 2015; Ikert et al., 2020), dissolved oxygen (Lau et al., 2014; Heinrichs-Caldas et al., 2023), salinity (Wang et al., 2015), health status (Cadonic et al., 2023) as well as exposure to xenobiotics (Craig et al., 2014; Qiang et al., 2017; Wang et al., 2019). Such alterations in miRNA expression arise through modifications in biogenesis and mode of action of these molecules and can modulate several key signaling and cytoprotective pathways (Yin et al., 2009; Leung & Sharp, 2010; Ha & Kim, 2014; Cao et al., 2023). For instance, many miRNAs can regulate the expression of heat shock proteins (HSPs) involved in the HSR (Yan et al., 2012; Liu et al., 2022a; Raza et al., 2022). Under various stressful conditions, inducible HSPs such as HSP47 (also known as SERPINH1), HSP70, and HSP90α act as molecular chaperones that protect proteins from damage and facilitate the refolding or denaturation of damaged proteins to maintain cellular function (Lindquist & Craig, 1988; Fangue et al., 2006; Stefanovic et al., 2016; Jeyachandran et al., 2023). As highlighted by Li et al. (2009), regulation of gene expression by miRNAs rather than transcription factors could allow organisms to respond to rapid environmental changes quicker because miRNAs are synthesized faster than proteins and exert their effects by directly targeting mRNAs instead of influencing transcriptional processes. For this reason, and because miRNAs are subjected to constant turnover, miRNAs are increasingly being used as reliable biomarkers of disease states in mammals and humans (Weiland et al., 2012; Iannaccone, et al., 2018). While Ikert et al. (2021) demonstrated that miRNAs in the mucus, plasma, and even the surrounding environment could serve as biomarkers reflecting the condition of fish, this technique is in its early stages and requires a better characterization of the miRNA profiles in this group before it can be used reliably. For instance, a distinct miRNA expression pattern must be associated with a specific stressor, regardless of developmental stage, sex, and population, so it can be utilized to infer the health/stress level of populations and guide focused remediation efforts.

1.5 Research objectives and hypotheses

The overarching aim of this thesis was to identify changes in miRNA and HSP mRNA expression in fish reared under diel thermal fluctuations throughout development, and investigate whether these changes underlie long-term plastic responses in physiological processes. The objectives were to:

1. Delineate how diel thermal fluctuations affected the expression of seven thermally sensitive miRNAs and three HSPs, as well as whole-animal performance indicators like survival and metabolism during early life stages.
2. Determine how this ontogenetic exposure influenced adult thermal tolerance and morphology, and if any differences in miRNA and HSP abundance seen during development persisted until this stage. In adults, gene expression was measured in the brain as this tissue relies greatly on aerobic metabolism and has been suggested to be the factor that limits the upper thermal tolerance of an organism (Andreassen et al., 2022; Ern et al., 2023).

I thus hypothesized that exposure to thermal fluctuations during development would differentially regulate fish performance via alterations in miRNA expression, and that these effects would be irreversible. Given that the expression of HSPs can be regulated on multiple levels upon exposure to thermal stress, including via miRNAs, the expression of these genes was also expected to be differentially expressed. Specifically, these fish were compared with those kept at constant optimal temperatures as well as at constant elevated temperatures. This comparison aimed to reveal how they differed from fish reared under highly controlled laboratory conditions and in traditional climate change experiments, respectively.

1.6 Study species

For this project, I will be using zebrafish (*Danio rerio*), a fish that is typically found along the shallow shorelines of the Ganges and Brahmaputra rivers in India, Bangladesh and Nepal (Spence et al., 2008). The rapid development, small size, high fecundity, and ease of rearing, make this fish a popular model for research into developmental biology, genetics, and physiology (Spence et al., 2008; Dai et al., 2014). Despite the fact that these fish encounter wide diel and seasonal thermal fluctuations in their natural habitats, they are maintained under constant thermal conditions in the laboratory to optimize their performance (Spence et al., 2008; Massey, 2023). Thus, to ensure that studies using zebrafish yield results with environmental and translational significance, it is important to evaluate their physiological and molecular processes under variable conditions. Finally, since the

zebrafish genome has been sequenced, and more than 1500 miRNA sequences have been identified in this species, it is an ideal model for epigenetic studies (Thatcher et al., 2008; Cao et al., 2023).

Chapter 2
Methods and Results

2.1 Methods

2.1.1 Overview of experimental design

Fertilized zebrafish (*Danio rerio*) embryos were exposed to one of three developmental thermal regimes: constant control (CTRL; 28°C), constant heat (HEAT; 33°C), or diel fluctuations (FLUX, 28 ± 5°C; Fig. 2.1). A subset of fish was sampled at the proposed end of the embryonic period when the larvae are free swimming and possess a protruding mouth (5 days post fertilization; dpf; Kimmel et al., 1995) to quantify the relative abundance of miRNAs and mRNAs of interest. At the presumed start of the juvenile phase (30 dpf; Kimmel et al., 1995), whole-body oxygen consumption rate (MO_2) was measured in fish from another subset, which were then sampled for the same molecular analysis. All remaining fish were raised under common CTRL conditions until adulthood. At 6 months post fertilization (mpf), the fish were subjected to critical thermal maximum (CT_{max}) tests and the brains were sampled to assess for differences in molecular responses.

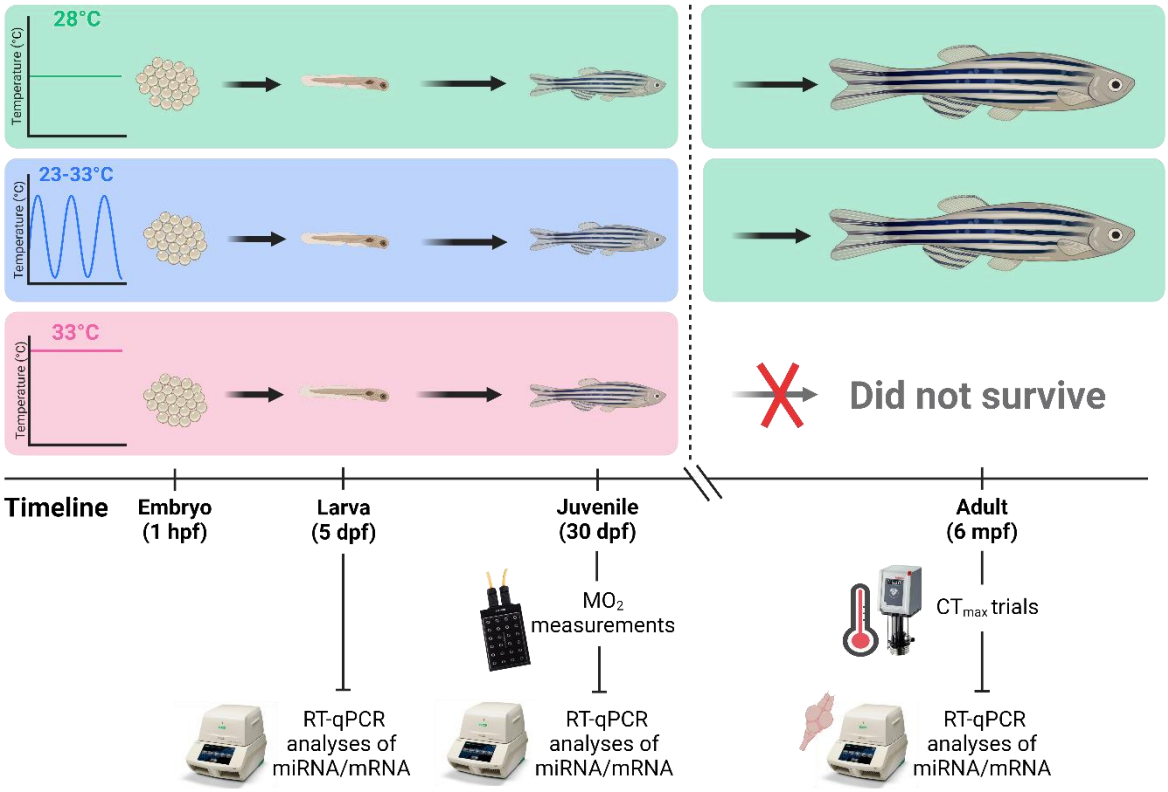


Figure 2.1: Overall experimental design. Fish were exposed to different developmental temperature profiles, and were sampled at various stages during development to quantify the effect of these temperatures on miRNA and mRNA expression profiles. Created with BioRender.com

2.1.2 Broodstock care

Adult zebrafish were obtained from Big Al's Aquarium Services (Kitchener, ON, Canada) and were housed in a freshwater recirculating system (Integrated Aqua System, CA, USA) in the WATER facility at the University of Waterloo. Fish were maintained at 27°C in system water (dechlorinated city water, pH ~7.6) on a 14 h:10 h light:dark cycle and stocked at a density of 3.33 fish/L in 30 L plastic aquaria. The fish were fed twice daily, with ground Gemma Micro 300 pellets (Skretting, Westbrook, Maine, USA) in the morning and live hatched brine shrimp in the afternoon. Animal care and experimental procedures outlined in this study followed the stipulations set by the University of Waterloo Animal Care Committee (AUP #45327).

2.1.3 Developmental thermal regimes

Two months after arrival to the facility, 36 breeding pairs were established based on secondary sexual characteristics and each pair was induced to spawn by placing into a 1 L crossing tank with a grated insert (Aquaneering, San Diego, CA, USA) overnight. A clutch of approximately 50 fertilized embryos (hereafter, each clutch is referred to as a family) was collected from each crossing tank using a fine mesh strainer within an hour of the lights being turned on at 6:00 am. Each family was immediately transferred into a 125 mL glass beaker containing 100 mL of embryo medium (5mM NaCl, 0.17 mM KCl, 0.33 mM MgSO4, 0.33 mM CaCl2, 0.0004% Methylene Blue), and was randomly assigned to one of three treatment groups: CTRL (28°C), FLUX (28 ± 5°C), or HEAT (33°C; n=12 families per treatment). These specific temperatures were selected for the CTRL and FLUX treatments because the mean in both conditions would be the same (28°C). Moreover, while 26–28°C is considered to be the optimal temperature range for rearing zebrafish (Spence et al., 2008; Scott & Johnston, 2012), 23–33°C represents the range of fluctuations that zebrafish commonly encounter in the wild as well as the thermal limits beyond which performance and survival reduce markedly (Schnurr et al., 2014; Feugere et al., 2021; Massey, 2023).

Each treatment consisted of three 30 L water bath with the same thermal regime and the families were split across the water baths evenly, resulting in 4 families per bath. The location of all the water baths was randomized along the bench top (Fig. 2.2). Each water bath was fitted with a 120 V Eheim Universal 600 Water Pump (Deizisau, Germany) to promote water circulation and a waterproofed Thermocron iButton data logger to record the water temperature every 10 min. Clear plastic dip was used to waterproof the iButtons as a study by Roznik and Alford (2012) showed that doing so reduced the chances of equipment malfunction and had little impact on temperature readings.

For the CTRL and HEAT water baths, heating wands were used to maintain the water temperature. For the FLUX water baths, Qubit Biology Heat Pump systems (Kingston, ON, Canada) were used to create the fluctuating thermal regime. Each system was comprised of a BK Precision DC Regulated power supply and a Peltier heat pump system (1686A) located above the water bath, as well as a stainless-steel heat exchanging coil and A447 thermometer placed in the water (Caklec, 2023). To begin the heating ramp from 23°C to 33°C, the voltage on the BK power supply would be set to 7.0 V every morning at 8:00 AM, which caused the water to be drawn up from the heat exchanging coil and over an evaporator followed by a condenser within the heat pump system. Alternatively, the cooling back to 23°C was initiated at 8:00 PM when the voltage was reduced to 2.0 V. The water temperature

was lowered by reversing the order in which it passed over the evaporator and condenser. The thermometer provided real-time feedback to the heat pump system regarding temperature so it could adjust the power and water flow to reach the temperature set point. With this system, however, the specific ramp rate could not be controlled. As the experiment was conducted in the summer (July 2023), the water in the tanks heated faster than it cooled. Specifically, the maximum temperature was reached around 4:30 PM, and maintained until 8:00 PM. Alternatively, the ramp back down to 23°C was more uniform, with the lowest temperature being attained around 8:00 AM. The fish were reared under a 12 h:12 h light:dark regime and the specific timings for the light changes corresponded to the voltage changes.

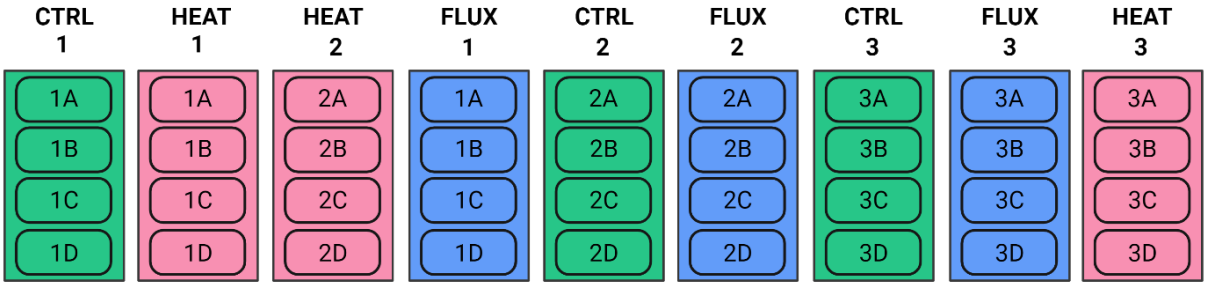


Figure 2.2: Arrangement of water baths from different treatment groups during the study. Each thermal regime consisted of three water baths, each containing four families. The location of the baths was randomized along the experimental area. Created with BioRender.com

2.1.4 Larval maintenance and sampling

From the embryonic stage at 1 hour post fertilization (hpf) to the free-swimming larval stage at 5 dpf, the fish were maintained in their original 125 mL glass beakers within the water baths. Every day, the eggs were cleaned and survival was recorded at 9:00 AM, followed by an 80% medium change. The replacement media was heated at 28°C, 24°C, and 33°C for the CTRL, FLUX, and HEAT treatments, respectively, to minimize the temperature change the embryos experienced. The temperature for the FLUX reservoir tank was chosen based on the approximate temperature of the FLUX water baths at this time. At approximately 9:00 AM on the day the fish reached 5 dpf, 10 individuals from each family were collected using a transfer pipette and euthanized by an overdose of tricaine mesylate (MS-222, 400 mg/L, Millipore-Sigma-Aldrich, Oakville, ON, Canada). Individuals from a family were dried gently with a Kimwipe and placed into a pre-weighed 2 mL microcentrifuge tube. The tubes were weighed again using an analytical balance (MX104, Mettler Toledo,

Mississauga, ON, Canada) with a readability of 0.1 mg, and average larval weight of a family was obtained by dividing the total family weight by 10. Each tube was then frozen by placing on dry ice, followed by storage in a -80 ºC freezer for further RNA extraction and analysis.

The remaining larvae from each family were moved from their beakers into 1 L crossing tanks filled with 50% system water and 50% embryo medium positioned inside the water baths. From 6 dpf to 30 dpf, a 70% water change was conducted daily at 9:30 AM using system water to reduce the accumulation of nitrogenous waste in the tanks. As with the embryo medium, water for the changes was drawn from reservoir tanks set to temperatures corresponding to those in the experimental treatment tanks. One hour prior to the water changes, larvae were fed ad libitum with Gemma 75 (Skretting, Westbrook, ME, USA). Specifically, the CTRL, FLUX, and HEAT groups were fed at 28°C, 23°C, and 33°C, respectively. During this time, survival was assessed and dissolved oxygen (DO) concentration in each tank was measured using a DO sensing probe and meter system (CellOx 325 probe and Oxi 3210 handheld meter; WTW, Weilheim, Germany).

2.1.5 Juvenile respirometry and sampling

At 30 dpf, MO_2 of each family was quantified using a 24-well microplate respirometry system (Loligo Systems, Viborg, Denmark) equipped with oxygen sensor spots. A fluorescence reader was positioned beneath the microplate to convert the response of the sensor in each well to a value representing the partial pressure of oxygen (P_{O_2}) within the chamber. The microplate system was connected to a flow through water bath for temperature regulation and was placed on an oscillating shaker to ensure even mixing of oxygen in the wells.

For each trial, 20 out of the 24 wells in the plate contained juveniles, while the other four served as blanks to measure background oxygen consumption. Six families were chosen at random to be used in a trial and three to four measurements were made for each family, with one measurement being made using two juveniles in a well. The location of the measurements for each family were randomized on the plate as well. Due to the large size of the fish from the HEAT treatment compared to the other groups, one measurement for some families within this group consisted of a singular juvenile in a well. Additionally, three families from this group were excluded from the experiment at this point because two of these families had no survivors, and the other only had one.

The trials were initiated by placing the juveniles in the wells filled with system water at 28°C and connecting the microplate system to a water bath at the same temperature. The wells were covered

with an adhesive PCR plate film, silicone pad, and plastic brick to create a seal. The fish were allowed to acclimate for 30 min, after which the P_{O_2} of the wells was measured for 30 min. The water bath was then replaced with another one held at 33°C so the metabolic response of juveniles to an acute temperature increase could be quantified. To minimize the temperature differential between the wells and the water bath, the plate was unsealed and ~70% of the water in the wells was carefully replaced with 33°C system water using a transfer pipette before the trial was resumed following the same protocol as the previous temperature.

Following each trial, juveniles were euthanized with an MS-222 overdose, blotted with a Kimwipe, and weighed individually using an analytical balance. Individuals from the same family were pooled into a 2 mL microcentrifuge tube, flash frozen using liquid nitrogen, and stored at -80°C for further RNA extraction and analysis.

MO_2 of each well was calculated using the formula outlined in Zimmer et al. (2019),

$$MO_2 = (\Delta P_{O_2} \times \alpha_{O_2} \times V)/m \quad (1)$$

where ΔP_{O_2} is the difference in partial pressure of oxygen over 0.5 hr (mmHg/h), α_{O_2} is the oxygen solubility constant in freshwater (pmol/L/mmHg; 1.5906 for 28°C and 1.4770 for 33°C; Boutilier et al., 1984), V is the volume of the well (0.0017 L), and m is the wet weight of the juveniles in the well (g). When there were two juveniles in the well, the sum of their mass was used. In all trials, background ΔP_{O_2} was averaged across blank wells and subtracted from measurements of juveniles. The MO_2 for each family was determined by averaging all the measurements made from that family. The average weight of juveniles from each family was also calculated for further comparisons.

2.1.6 Adult CT_{max} trials and sampling

The juveniles from the CTRL and FLUX conditions remaining after sampling at 30 dpf were transferred into 30 L tanks in the same recirculating system as the parental generation, separated by family. All families were maintained at 28°C, following the same light cycle as their earlier developmental stages until 6 months post fertilization (mpf). Since there were no juveniles left in the HEAT group, it was not included in the adult study. Only families with 10 or more individuals remaining were selected for subsequent analyses, which resulted in five families per treatment. The remaining juveniles were fed everyday until satiety with Gemma Micro 150 (Skretting, Westbrook,

Maine, USA) in the morning and live brine shrimp in the afternoon until the expected onset of adulthood at 3 mpf (Kimmel et al., 1995), after which their diet followed that of their parents.

At 6 mpf, the thermal tolerance of the longitudinal adults was assessed through CT_{max} trials and sampled for analysis for miRNA and mRNA abundance. For each trial, one family was used. One pair of fish (a male and female) each was sampled at three timepoints to assess if the treatment groups differed in their molecular response to the thermal challenge, and if so, pinpoint when these differences occurred. The timepoints used were as follows: baseline (where fish were haphazardly sampled from their holding tanks at 28°C without exposure to any thermal challenge), CT_{max} (where fish were sampled upon losing equilibrium, which occurred approximately 45 min into the trial at 39-42°C), and recovery (where fish were allowed to recover at 28°C in a tank for 2 hr after reaching CT_{max}). The duration of the recovery period was selected based on work by Morgan et al. (2018), which showed that zebrafish adults return to normal swimming and feeding patterns within 15 min after a CT_{max} test. Sampling involved documentation of length (in cm), weight (in g), and sex, followed by dissection of brain tissue, snap freezing, and storage at -80°C. Morphometric endpoints like Liver Somatic Index (LSI = [liver weight/body weight] × 100) and Fulton's condition factor (K = [body weight/ (length3) *100)] were calculated to assess the status of the fish.

CT_{max} trials were conducted by placing one pair each into one of two mesh boxes positioned at the top of a 30 L tank filled with system water at 28°C. In each trial, one box was randomly assigned to contain the pair that would be euthanized at CT_{max}, while the other contained the pair that would be transferred to the recovery tank. The CT_{max} tank was fitted with a Julabo portable immersion circulator (Corio CD; Julabo, Seelback, Germany) to control the rate of change of water temperature and circulation, as well as an air stone to oxygenate the water (Weber et al., 2023). After a 15 min acclimation, the water temperature was increased at the standard CT_{max} rate of 0.3°C/min (Becker and Genoway, 1979).

Both agitation (T_{ag}) and CT_{max} points were recorded, where T_{ag} corresponded to the temperature at which the fish began to display erratic swimming patterns and CT_{max} represented the temperature at which the fish lost equilibrium and was unable to return to an upright position within five seconds. Fish from the mesh box assigned to the CT_{max} timepoint were netted and euthanized with an overdose of MS-222 upon losing equilibrium, while the fish from the recovery box were moved into the recovery tank, which was a 30 L tank with a heating wand to maintain the temperature, an air stone for aeration, and a pump for mixing water. After 2 hr in this tank, these fish were also euthanized. The

trials spanned three days, with the sequence being determined by randomly selecting both treatment and family.

2.1.7 RNA extraction, cDNA synthesis, and transcript abundance quantification

For the larval and juvenile samples, eight families were used for all treatments, with the same families being used at both timepoints. Total RNA was extracted from frozen larval, juvenile, and adult brain samples using the miRNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for animal tissues without any deviation. Samples were eluted using nuclease free water and stored at -80°C before further processing. RNA concentration was then determined using a Qubit 3.0 Fluorometer with the Qubit RNA Broad Range assay kit (ThermoFisher Scientific, Waltham, MA, USA). Following this, cDNA was synthesized using 1000 ng of RNA for the larval and juvenile samples and 900 ng for the adult brain samples according to the manufacturer's protocol for the miRNA 1st-Strand cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA). For each tissue type, one pooled cDNA sample was created to validate the efficiency of primers using standard curves. All samples were stored at -20°C until subsequent analysis.

Relative abundance of three heat shock proteins (HSPs) and seven miRNAs was quantified in all samples using RT-qPCR (Table 2.1). The miRNAs were selected based on previous findings demonstrating that their expression is sensitive to temperature changes in fish (Bizuayehu et al., 2015; Qiang et al., 2017; Ikert and Craig, 2020; van Gelderen et al., 2022). A seven-point standard curve ranging from a dilution of 12x to 768x was constructed for all targets using the pooled samples to ensure that the primers had an efficiency between 90 to 110% and a R^2 value greater than 0.98 for all tissues. Each qPCR mixture consisted of 3 µL of sample cDNA, 1 µL of 3.125 µM forward primer, 1 µL of 3.125 µM reverse primer, and 5 µL SYBR® Green Supermix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Qiagen Universal Primer (Hilden, Germany) was used as the reverse primer for all miRNAs evaluated.

A CFX96 Thermal Cycler (Bio-Rad Laboratories, CA, USA) was used to perform the amplification for each plate, on which samples were run in duplicate. The amplification profile followed involved an incubation at 95°C for 30s, followed by denaturation at the same temperature for 10 s, and annealing at 60°C for 20 s repeated over 40 cycles. Melt curves of each run were analyzed to ensure that only a singular product had formed. From this, it was determined that miR-214 and miR-499-5p

had to be excluded from quantification for the adult brain samples due to non-specific amplification, despite troubleshooting attempts including changing the concentration of primers and annealing temperature (Table 2.1). Relative expression of mRNA and miRNA targets was normalized using the geometric means of two reference genes, namely, 18s and rps18, as they were found to be stably expressed across all samples in the three tissue types. For the larval and juvenile samples, the normalized relative expression values of the FLUX and HEAT groups were presented relative to that of the CTRL group to facilitate visual identification of differences. Similarly, the CTRL group at baseline served as the reference for comparing transcript levels for the brain samples.

Table 2.1: Primer sequences for mRNA and miRNA measured using RT-qPCR. The forward (F) and reverse (R) primers for each of the targets quantified are detailed below, along with their efficiencies in the three tissue types. As the sequence of the Universal Primer is proprietary information, amplicon lengths are not listed for the miRNAs measured.

Target	Accession number	Primer sequence (5' – 3')	Efficiency (%)	Amplicon length (bp)	M score
18s	FJ915075.1	**F:** ATGGCCGTTCTTAGTTGGTG **R:** GAACGCCACTTGTCCCTCTA	**5 dpf:** 101.2 **30 dpf:** 109.0 **6 mpf brain:** 108.9	130	**5 dpf:** 0.356 **30 dpf:** 0.815 **6 mpf brain:** 0.854
rps18	NM_173234	**F:** GAGGTTGAGAGGGTGGTGAC **R:** AAGGACCTGGCTGTATTTCCC	**5 dpf:** 98.6 **30 dpf:** 94.3 **6 mpf brain:** 96.1	111	**5 dpf:** 0.356 **30 dpf:** 0.815 **6 mpf brain:** 0.854
hsp47	NM_131204.2	**F:** GTCAGCCACGACCTTCAGAA **R:** TGCCGGAAATGTTGGACAGA	**5 dpf:** 102.3 **30 dpf:** 106.0 **6 mpf brain:** 109.2	91	N/A
hsp70	AF210640.1	**F:** AAAGCACTGAGGGACGCTAA **R:** TGTTCAGTTCTCTGCCGTTG	**5 dpf:** 105.2 **30 dpf:** 109.7 **6 mpf brain:** 108.1	127	N/A
hsp90	BC134081	**F:** CACGATCATGGCGATAAGTG **R:** ACAGCGGTTTGGTTTTGTTC	**5 dpf:** 90.7 **30 dpf:** 92.6 **6 mpf brain:** 108.6	100	N/A
let-7d-5p	MIMAT0001762	**F:** TGAGGTAGTTGGTTGTATGGTT **R:** Universal Primer	**5 dpf:** 109.2 **30 dpf:** 109.4 **6 mpf brain:** 99.8	N/A	N/A
miR-103-3p	MIMAT0001816	**F:** AGCAGCATTGTACAGGGCTATGA **R:** Universal Primer	**5 dpf:** 99.5 **30 dpf:** 106.7 **6 mpf brain:** 109.7	N/A	N/A
miR-122	MI0001965	**F:** AGAGATGGAGTGTGACAATGG **R:** Universal Primer	**5 dpf:** 108.4 **30 dpf:** 109.9 **6 mpf brain:** 103.7	N/A	N/A
miR-181a-5p	MIMAT0001623	**F:** AACATTCAACGCTGTCGGTGAGT **R:** Universal Primer	**5 dpf:** 92.9 **30 dpf:** 108.9 **6 mpf brain:** 100.0	N/A	N/A
miR-214	MIMAT0001283	**F:** ACAGCAGGCACAGACAGGCAG **R:** Universal Primer	**5 dpf:** 101.5 **30 dpf:** 108.1 **6 mpf brain:** N/A	N/A	N/A
miR-301a-3p	MIMAT0001870	**F:** CAGTGCAATAGTATTGTCAAAG **R:** Universal Primer	**5 dpf:** 109.3 **30 dpf:** 102.3 **6 mpf brain:** 109.5	N/A	N/A
miR-499-5p	MIMAT0003749	**F:** CCAGCGTGTTAAGACTTGCAG **R:** Universal Primer	**5 dpf:** 108.8 **30 dpf:** 97.3 **6 mpf brain:** N/A	N/A	N/A

2.1.8 Bioinformatic analysis

Potential mRNA targets of the miRNA measured in this study were predicted using the bioinformatic tool miRanda (v3.3), which accounts for multiple parameters including the complementarity between the miRNA's seed region and the 3' UTR of the mRNA and the free

energy associated with duplex formation (Enright et al., 2003). Highly conservative criteria were used to prune the gene lists obtained as any interactions with a pairing score less than 140 and/or a binding energy value higher than -20 were excluded (Cadonic et al., 2020; Ikert et al., 2021). The remaining mRNA targets were entered into the Database for Annotation, Visualization and Integrated Discovery (DAVID) to determine the significantly enriched (Benjamini–Hochberg corrected $p < 0.05$) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with these genes.

2.1.9 Statistical analysis

Thermal profile data from the iButtons was imported into a datasheet using the OneWireViewer software and a time-series graph was constructed by averaging the values of the water baths in each treatment. Originally, the DO levels (averaged over the 30-day experimental period) in the tanks were compared using a two-way ANOVA that evaluated the effects of thermal regime, water bath, and their interaction. Since the effects of the water bath and the interaction were not significant, all families in a treatment were pooled and only the effect of treatment was evaluated using a one-way ANOVA.

Statistical analysis for the larval and juvenile stages of this experiment was conducted using Graphpad Prism version 8. Kaplan-Meier survival curves were computed for the three treatments compared using a Mantel-Cox (log-rank) test to determine whether treatment affected this parameter over the 30-day experimental period. As outlined in Martin et al. (1987), five individuals were removed from all curves at 5 dpf to account for the sampling. As with the DO analysis, the larval and juvenile weights from different treatments were compared using separate one-way ANOVA tests as no significant effect of water bath was detected through the two-way ANOVA analysis.

Separate one-way ANOVA tests were used to evaluate the differences in the normalized relative expression of transcripts measured between treatments at both developmental stages. Post hoc comparisons were made using Tukey's HSD tests when the ANOVA was deemed to be significant. Assumptions like normality and homoscedasticity of data were confirmed using Shapiro-Wilk's test and Levene's test, respectively. If the assumptions were not satisfied, the data was subjected to a log or square root transformation. Outliers in each group were identified using the ROUT method (Q= 1%) and removed when necessary. Finally, a two-way repeated measures ANOVA was carried out to determine how the MO_2 of families from different thermal regimes varied between the two test temperatures, 28°C and 33°C. The normality assumption was validated using Shapiro-Wilk's test,

while the sphericity assumption was considered to be satisfied as there were only two timepoints for the repeated measure. Holm-Sidak multiple comparison tests were used for post hoc analysis.

All results for the longitudinal adult experiment were computed using R version 4.3.2. Linear mixed effects models (LME) were fitted using the 'lme4 package to investigate the effect of developmental thermal regime on the adult parameters measured. Separate models were constructed with CT_{max}, T_{ag}, LSI, K, and body weight as the response variables using the 'lmer' function. In all these models, treatment served as a fixed effect and family was used as a random effect to account for the nested structure of the data. To compare the relative abundance of transcripts measured between treatment groups, similar models were generated for each gene measured with treatment and timepoint as the fixed predictor variables, the interaction between the two, and family as a random factor. Residuals from the models were plotted against treatment and timepoint to examine linearity for both factors and assessed for normality using the Shapiro-Wilk test. Levene's test was conducted to evaluate homogeneity of the variance of the residuals.

When the assumptions were not met, data was log transformed and reevaluated as necessary. If the assumptions were still violated (particularly, in the case of non-linearity), a generalized additive mixed model (GAMM) was constructed using the 'gamm' function in the 'mgcv' package. These models accounted for treatment as a fixed factor, timepoint as a smooth function with three knots, and the interaction between the two factors as a factor-smooth interaction. As with the LME models, family was included as a random effect. For all significant models, post-hoc analysis involved comparisons of estimated marginal means using the 'emmeans' function from the package of the same name (Lenth et al., 2018). The Kenward–Rogers method of estimating degrees of freedom and the Tukey method of adjusting p values were employed. If the interaction between fixed factors was significant, contrasts involved comparisons between treatment groups within each timepoint, as well as between timepoints within a treatment group. Statistical significance was indicated by a p value less than 0.05 for all analyses.

2.2 Results

2.2.1 Temperature profiles and DO concentration

Temperature profiles of each treatment group obtained from the iButtons are depicted over the span of 48 hours in Fig. 2.3A, as well as the entire experimental period in Fig. 2.3B. The average

temperature over the 30 days, the temperature during sampling at 5 dpf, and the minimum and maximum temperatures attained in each water bath are listed in Table A.1. Throughout the experiment, the DO concentration in the tanks exceeded 5.0 mg/L, and did not differ significantly between treatments (One-way ANOVA, $p = F_{2,33} = 2.223$, $p = 0.123$; Table A.2).

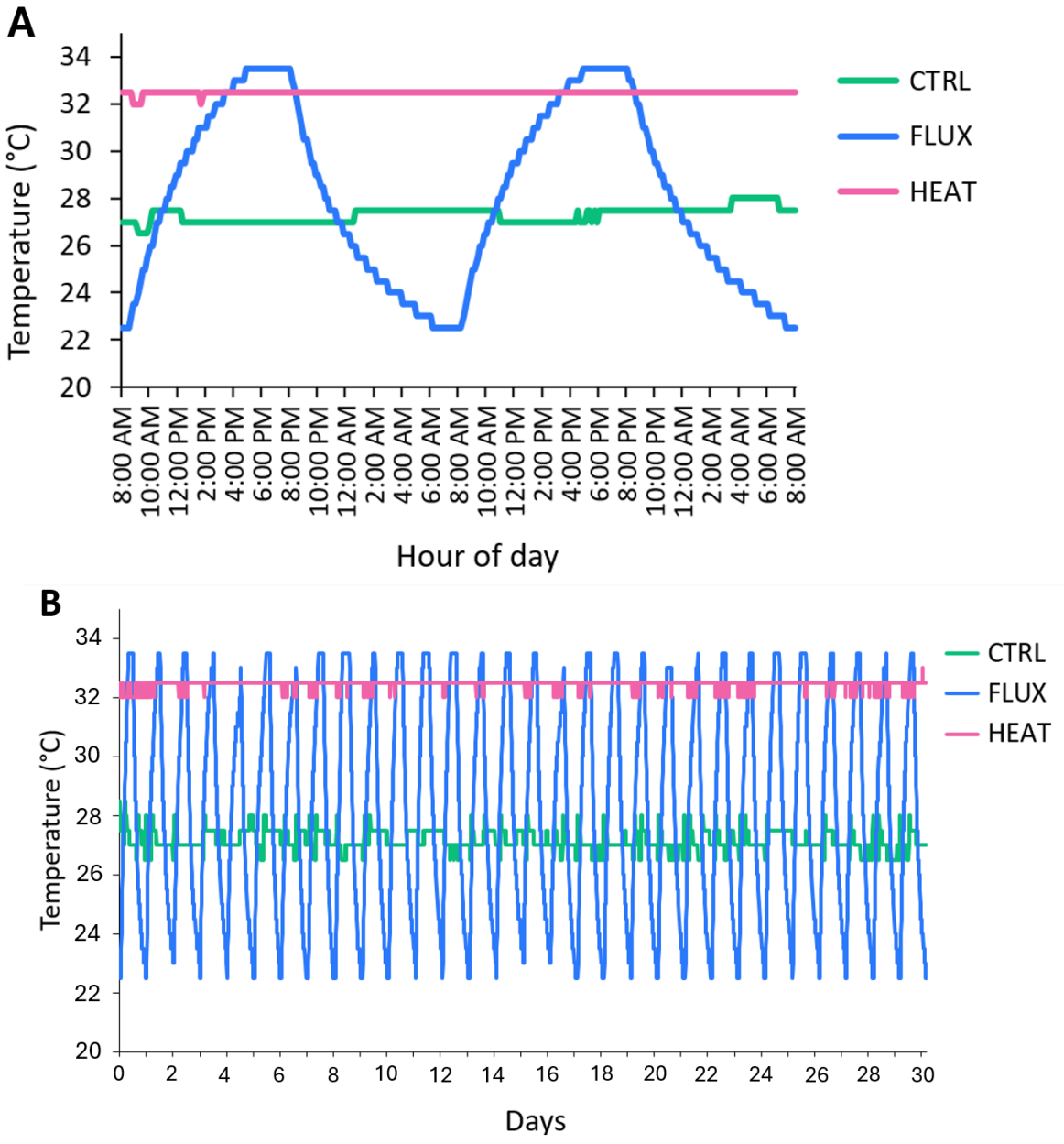


Figure 2.3: Trace of the water temperatures in the different treatment groups over A) 48 hours and B) the 30-day experimental period as measured by iButton loggers.

2.2.2 Survival analysis

A Kaplan-Meier curve analysis was conducted to assess the effect of developmental thermal regime on fish survival until 30 dpf. This analysis revealed that survival differed significantly between the three groups over the duration of the experiment (Mantel-Cox, $\chi^2 = 258.6$, df = 2, p <0.0001; Fig. 2.4). Overall, pairwise comparisons showed that this result was mediated by the low survival rate seen in the HEAT group (7.78%) relative to that of the CTRL (60.22%, p<0.0001) and FLUX (58.61%, p<0.0001) groups. While all three curves appear to be similar until the first sampling point at 5 dpf, there is a marked reduction in survival of HEAT fish around 8 dpf. Finally, there was no significant difference (p = 0.13) between the survival of the CTRL and FLUX groups over the entire experimental period.

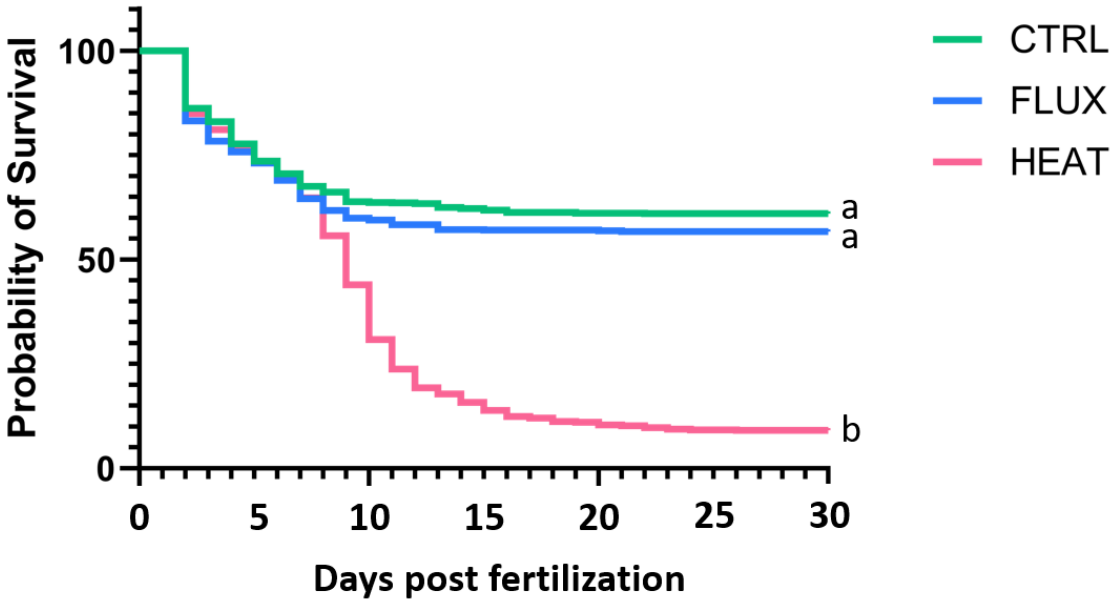


Figure 2.4: Survival of zebrafish reared under different developmental temperature profiles until 30 days post fertilization (dpf). Curves were calculated for each treatment by pooling individuals across families (n = 694 - 705 individuals per treatment), and accounted for fish sampled at 5 dpf. Comparison of curves using a Mantel-Cox test showed that treatment had a significant impact on survival ($\chi^2 = 258.6$, df = 2, p <0.0001). Curves with different letters are significantly (p>0.05) different from each other.

2.2.3 Larval mRNA and miRNA expression

Of three HSPs measured, *hsp47* was the only one whose expression was not significantly affected by developmental thermal regime (One-way ANOVA, $F_{2,20} = 1.19$, p = 0.325; Fig. 2.5A).

Alternatively, the relative abundance of *hsp70* (One-way ANOVA, $F_{2,20} = 3.684$, $p = 0.043$; Fig. 2.5B) was significantly altered by the rearing temperatures as the levels were 4-fold higher in the HEAT group relative to the CTRL (Tukey post-hoc test, $p = 0.040$). Notably though, the expression of *hsp70* in FLUX larvae did not differ significantly from those in the CTRL (Tukey post hoc test, $p = 0.675$) or HEAT groups (Tukey post hoc test, $p = 0.18$). A similar pattern was seen for *hsp90*, as the transcript was found to be approximately twice as abundant in the HEAT larvae compared to the CTRL and FLUX groups (One-way ANOVA, $F_{2,20} = 5.97$, $p = 0.0093$; Tukey post hoc test, $p = 0.011$ [HEAT vs. CTRL], $p = 0.044$ [HEAT vs. FLUX]; Fig. 2.5C).

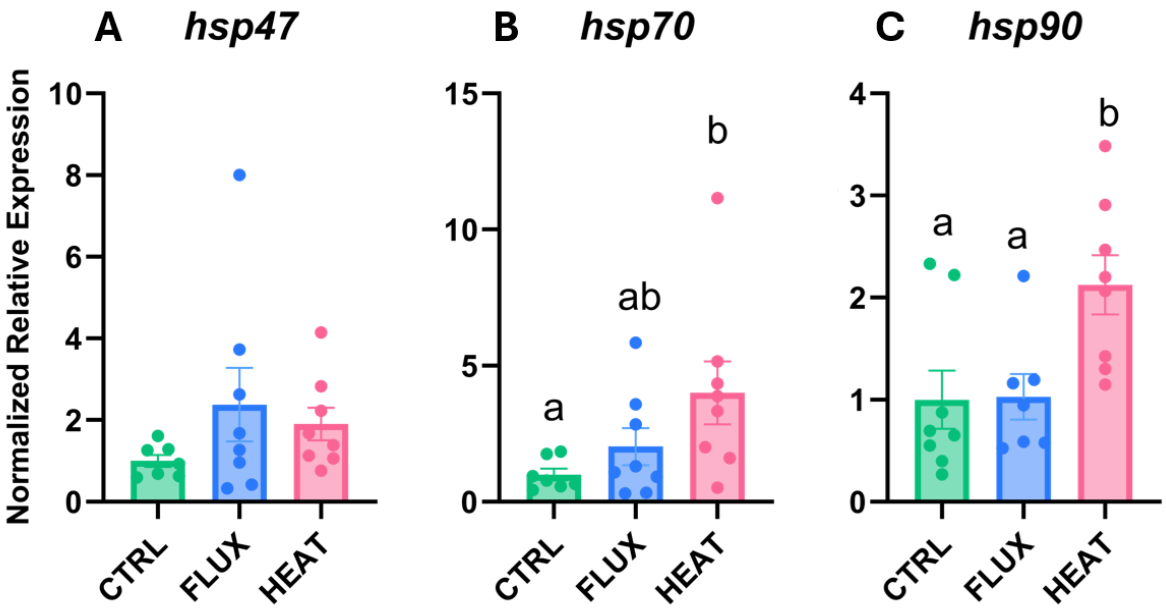


Figure 2.5: Normalized relative expression of the three HSPs measured in zebrafish larvae at 5 days post fertilization (dpf). Fish were either exposed to CTRL, FLUX, or HEAT temperature profiles from 1 hpf until they were sampled. At 5 dpf, the effect of developmental thermal regime of (A) *hsp47*, (B) *hsp70*, and (C) *hsp90* expression was assessed using one-way ANOVAs. Bars represent the mean ± SEM of the treatment, and those having different letters are significantly different from others as per Tukey's post hoc comparisons ($p < 0.05$; n = 7-8 families/treatment).

Notably, the treatment groups did not differ significantly with respect to the expression of four out of the seven of the miRNAs measured, i.e., miR-103-3p, miR-122, miR-214, and miR-499-5p (One-way ANOVAs, $p > 0.05$; Table A.3; Fig. 2.6B,C,E,G). While treatment was found to have a significant effect on the expression of let-7d-5p (One-way ANOVA, $F_{2,20} = 6.19$, $p = 0.0077$; Fig. 2.6A), pairwise comparisons showed that neither the FLUX group (Tukey post hoc-test, $p = 0.395$)

nor the HEAT group (Tukey post hoc-test, p = 0.103) was significantly different when compared to the CTRL. Thus, the treatment effect was primarily driven by the lowered abundance of the transcript in the HEAT group relative to the FLUX one (Tukey post-hoc test, p = 0.006). This was also the case for miR-181a-5p (One-way ANOVA, $F_{2,20}$ = 3.956, p = 0.0348; Fig. 2.6D), where the FLUX and HEAT groups differed significantly from each other (Tukey post-hoc test, p = 0.0368), but not from the CTRL (Tukey post-hoc test, p = 0.113 and p = 0.842, respectively). In contrast, however, the expression of miR-301a-3p was significantly downregulated in the HEAT group (One-way ANOVA, $F_{2,20}$ = 9.209, p = 0.0013; Fig. 2.6F) compared to the CTRL (Tukey post-hoc test, p=0.0034) and FLUX (Tukey post-hoc test, p=0.0036) groups.

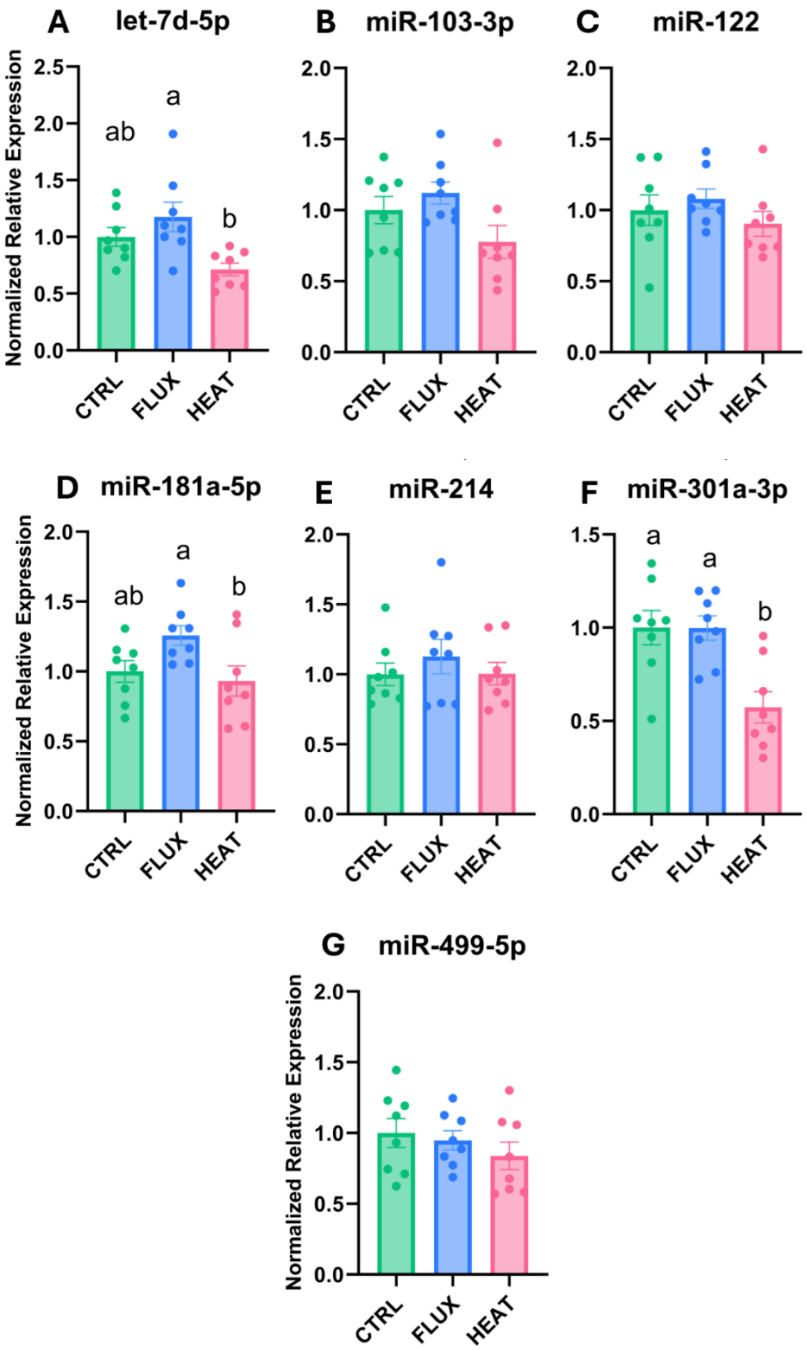


Figure 2.6: Normalized relative expression of the seven miRNAs measured in zebrafish larvae at 5 days post fertilization (dpf). Fish were either exposed to CTRL, FLUX, or HEAT temperature profiles from 1 hpf until they were sampled. At this point, the expression of (A) let-7d-5p, (B) miR-103-3p, (C) miR-122, (D) miR-181a-5p, (E) miR-214, (F) miR-301a-3p, and (G) miR-499-5p was quantified. Bars represent the mean ± SEM of the treatment, and those having different letters are

significantly different from each other (One-way ANOVA, Tukey's post hoc comparisons, $p < 0.05$; n = 7-8 families/treatment).

2.2.4 Juvenile respirometry

A two-way repeated measures ANOVA was conducted to assess the effect of developmental thermal regime and temperature on the MO_2 of the 30 dpf juveniles. The analysis revealed a significant effect of treatment ($F_{2,30} = 12.90$, $p < 0.0001$), temperature ($F_{1,30} = 26.99$, $p < 0.0001$), as well their interaction ($F_{2,30} = 7.396$, $p = 0.0024$) on the MO_2 (Fig. 2.7). At 28°C, families reared in the HEAT condition had a lower MO_2 relative to those in the CTRL ($p = 0.0047$) and the FLUX conditions ($p = 0.029$), as per Holm-Sidak post hoc comparisons. Additionally, there was no statistical difference between the MO_2 of the CTRL and FLUX groups (Holm-Sidak test, $p = 0.389$).

Upon increasing the water temperature to 33°C, there was a significant increase in the MO_2 (nearly 36%) of the CTRL group relative to its MO_2 at 28°C (Holm-Sidak test, $p < 0.0001$; Fig. 2.7). While a similar but smaller increase was detected for the HEAT group (Holm-Sidak test, $p = 0.011$), the MO_2 of the FLUX group was not significantly altered (Holm-Sidak test, $p = 0.647$). As such comparing the treatment groups at 33°C revealed that the MO_2 of the CTRL group was nearly 1.4-times higher than that of the FLUX (Holm-Sidak test, $p < 0.0001$) and HEAT (Holm-Sidak test, $p < 0.0001$) groups, respectively. At this testing temperature, the FLUX and the HEAT families did not differ significantly with respect to their MO_2 (Holm-Sidak test, $p = 0.469$).

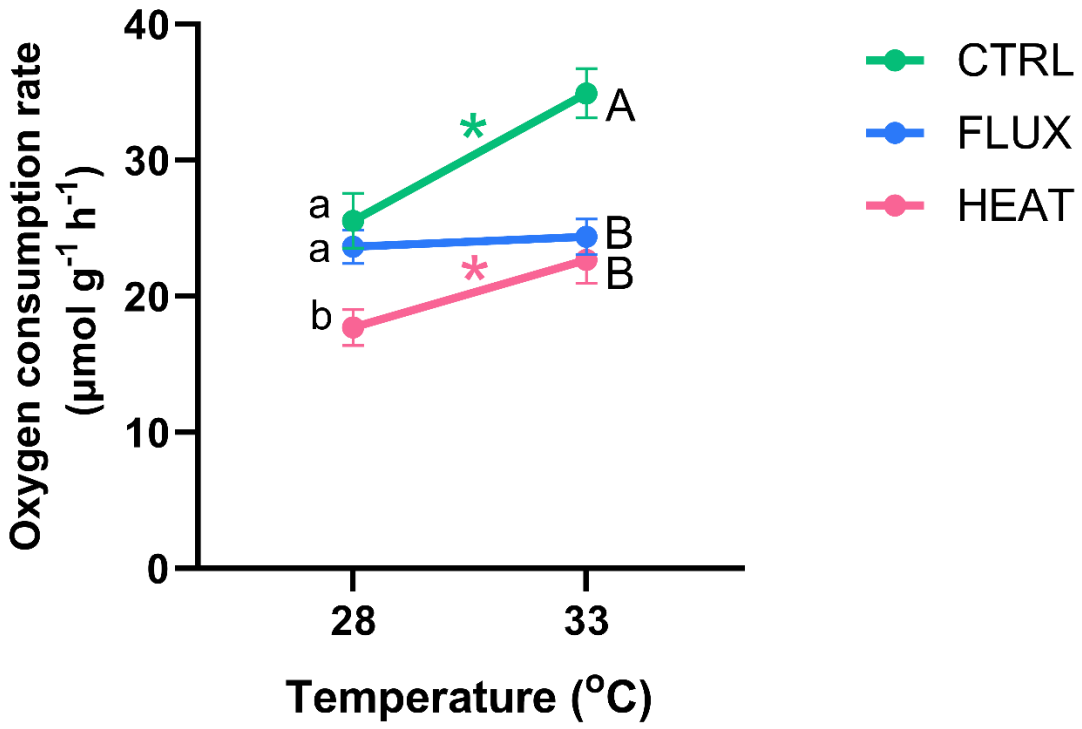


Figure 2.7: Whole body oxygen consumption (MO_2) of juvenile zebrafish from different thermal treatments at 30 days post fertilization (dpf). The MO_2 of families was initially quantified at 28°C, followed by measurement at 33°C. Differences in this parameter across temperature and treatment were determined using a two-way repeated-measures ANOVA. Different lowercase letters indicate significant differences between treatments at 28°C, while different uppercase letters indicate significant differences between treatments at 33°C as per Holm-Sidak post hoc comparisons (n = 9-12 families/treatment). Asterisks indicate significant differences within a treatment group across temperatures (Holm-Sidak post hoc test). Values are presented as mean ± SEM.

2.2.5 Juvenile mRNA and miRNA expression

The expression of all the HSPs measured in the 30 dpf juveniles was not significantly different between treatment groups (One-way ANOVA, Table A.4, $p > 0.05$; Fig. 2.8A-C).

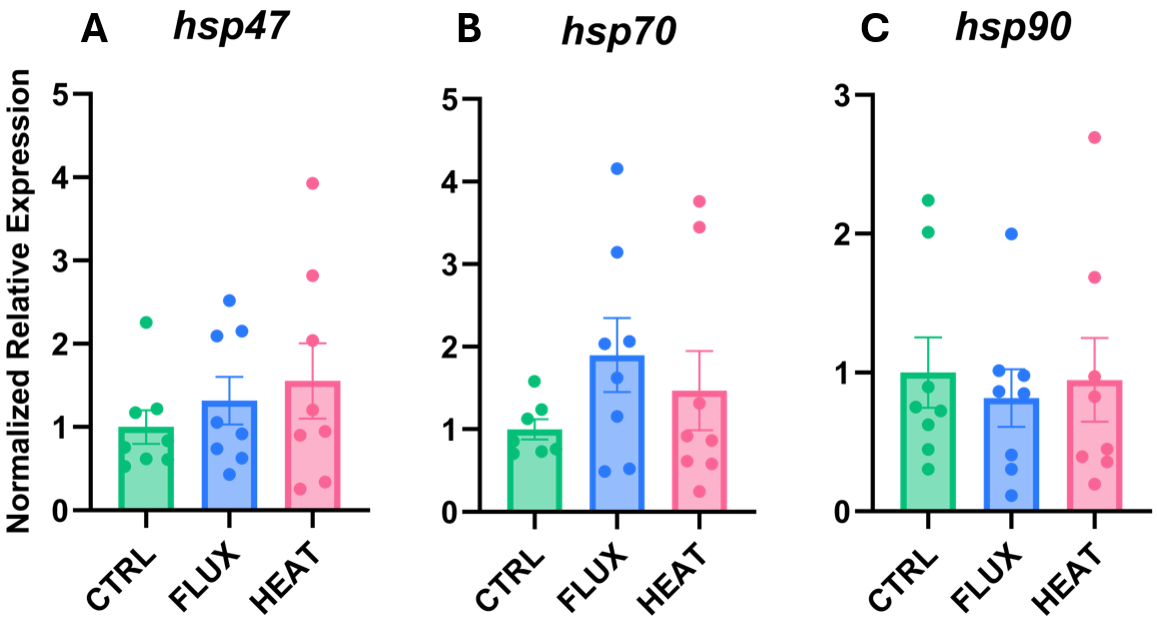


Figure 2.8: Normalized relative expression of the three HSPs measured in zebrafish juveniles at 30 days post fertilization (dpf). The families were either exposed to CTRL, FLUX, or HEAT temperature profiles starting at 1 hpf, and were sampled after use in juvenile respirometry trials. (A) *hsp47*, (B) *hsp70*, and (C) *hsp90*. Bars represent the mean ± SEM of the treatment, and those having different letters are significantly different from each other (One-way ANOVA, Tukey's post hoc comparisons, $p < 0.05$; n = 7-8 families/treatment).

Similar to the larval stage, no significant differences were found between the treatment groups for six out of seven miRNAs measured, including let-7d-5p, miR-103-3p, miR-122, miR-214, miR-301a, and miR-499-5p (One-way ANOVA, $p > 0.05$, Table A.4, Fig. 2.9A-D,F,G). However, a significant effect of treatment was detected for miR-181a (One-way ANOVA, $F_{2,20} = 13.74$, $p = 0.0002$; Fig. 2.9E), as the transcript was approximately twice as abundant in both the FLUX and HEAT groups relative to the CTRL (Tukey post hoc test, $p = 0.0021$ and $p=0.0002$, respectively). The FLUX and HEAT groups did not differ significantly from each other (Tukey post hoc test, $p = 0.722$).

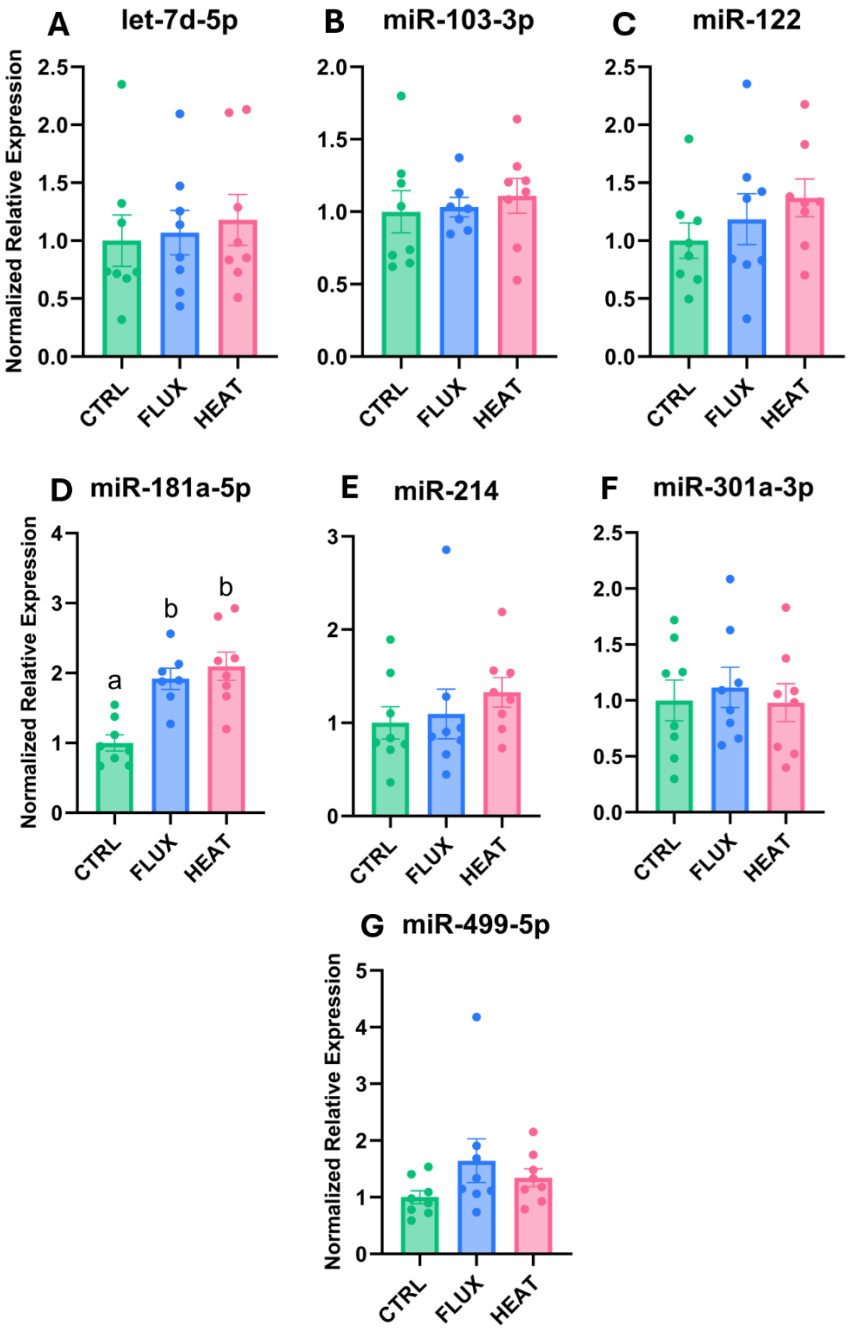


Figure 2.9: Normalized relative expression of the seven miRNAs measured in zebrafish juveniles at 30 days post fertilization (dpf). The families were either exposed to CTRL, FLUX, or HEAT temperature profiles starting at 1 hpf, and were sampled after use in juvenile respirometry trials. (A) let-7d-5p, (B) miR-103-3p, (C) miR-122, (D) miR-181a-5p, (E) miR-214, (F) miR-301a-3p, and (G) miR-499-5p. Bars represent the mean ± SEM of the treatment, and those having different

letters are significantly different from each other (One-way ANOVA, Tukey's post hoc comparisons, $p < 0.05$; n = 7-8 families/treatment).

2.2.6 Larval and juvenile weight

The weight of larvae and juveniles from different treatment groups was compared at the two sampling points using separate one-way ANOVAs. At 5 dpf, a significant effect of developmental thermal regime was observed on the average larval weight (One-way ANOVA, $F_{2,33} = 97.84$, $p < 0.0001$; Fig. 2.10A). On average, the families from the HEAT group had larvae that were 60% larger than those in the CTRL group (Tukey post hoc test, $p < 0.0001$), while the FLUX larvae were 20% smaller than the CTRL ones (Tukey post hoc test, $p < 0.007$). When compared to the FLUX group, the larvae in the HEAT group were nearly twice as large (Tukey post hoc test, $p < 0.0001$).

Significant differences were also detected between treatment groups in the wet weight of the juveniles at 30 dpf (One-way ANOVA, $F_{2,30} = 14.63$, $p < 0.0001$; Fig. 2.10B). As with the larval stage, the HEAT group was significantly larger (by nearly 163%) than the CTRL (Tukey post hoc test, $p < 0.0001$) and FLUX group (Tukey post hoc test, $p = 0.0002$). However, contrary to the larval stage, the weight did not differ significantly between the CTRL and FLUX group (Tukey post hoc, $p = 0.968$).

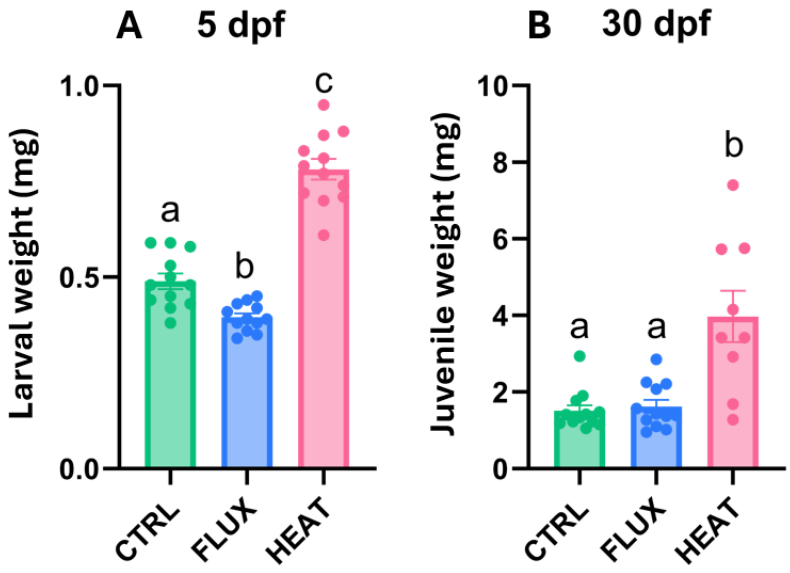


Figure 2.10: Weight of zebrafish reared under different developmental thermal regimes. At (A) 5 days post fertilization (dpf), the larval weight of each family was determined by dividing the total weight of the family by number of larvae (n = 12 families/treatment, 10 larvae per family). At (B) 30

dpf, the average juvenile weight was computed by averaging the weight of juveniles involved in respirometry trials from that family (n = 9 – 12 families/treatment, 3 – 8 juveniles per family). Bars represent the mean ± SEM of the treatment, and those having different letters are significantly different from each other (One-way ANOVA, Tukey's post hoc comparisons, $p < 0.05$).

2.2.7 Adult CT_{max}, T_{ag}, and morphometrics

Linear mixed effects analysis showed that developmental thermal regime had no significant effect on the CT_{max} of adult fish at 6 mpf ($t = -0.5575$, $p = 0.5924$; Fig. 2.11A). Additionally, no significant differences were found between families from the CTRL and FLUX groups with respect to T_{ag} ($t = 1.4462$, $p = 0.1861$; Fig. 2.11B), LSI ($t = 0.607$, $p = 0.561$; Fig. 2.11C), K ($t = 0.149$, $p = 0.885$; Fig. 2.11D), and body weight ($t = 0.801$, $p = 0.447$; Fig. 3.9E). Detailed results from the models are reported in Table A.5.

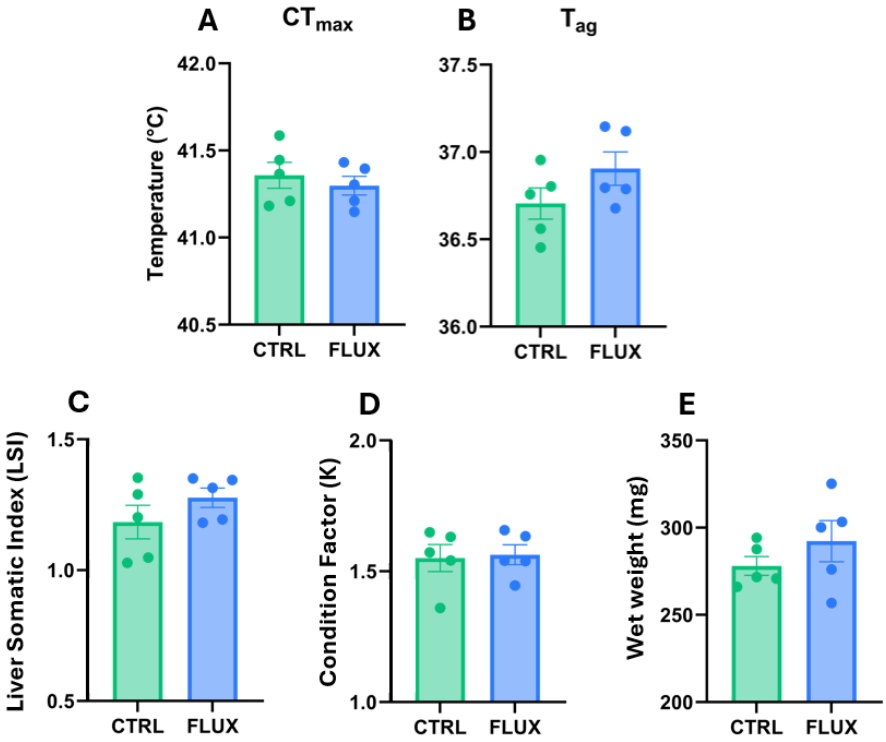


Figure 2.11: Parameters measured during CT_{max} trials of longitudinal zebrafish adults at 6 months post fertilization (mpf) after exposure to different developmental thermal regimes. (A) Critical thermal maximum (CT_{max}) and (B) agitation temperature (T_{ag}) at 6 mpf. Morphometric endpoints like (C) Liver Somatic Index (LSI), (D) Condition Factor (K), and (E) Wet weight (mg) were also measured. Each point indicates the average response of a family (n = 5 families/treatment, with 2 individuals from a family sampled at each timepoint). Bars represent the mean ± SEM of the treatment, and those having different letters are significantly different from each other (LME, Tukey's post hoc contrasts, $p < 0.05$).

2.2.8 Adult brain mRNA and miRNA expression

The effects of treatment and timepoint on the normalized relative expression of three HSPs and five miRNAs measured in adult brains were assessed using LMEs and GAMMs. Although treatment or the interaction between the two factors did not significantly impact the abundance of the HSPs measured, there was a strong temporal effect for all of them, each displaying subtly distinct patterns. More specifically, the expression of *hsp47* was significantly elevated at CT_{max}, but returned to baseline levels upon recovery (LME; Table 2.2, 2.3; Fig. 2.12A). Alternatively, *hsp70* expression was upregulated by 30 times at CT_{max} relative to baseline, and continued to remain elevated even at recovery (GAMM; Table 2.2, 2.3; Fig. 2.12B). Finally, while there was no significant difference between the expression of *hsp90* at CT_{max} compared to baseline, a nearly 200-fold increase in its abundance was detected in the brains of fish at recovery (GAMM; Table 2.2, 2.3; Fig. 2.12C).

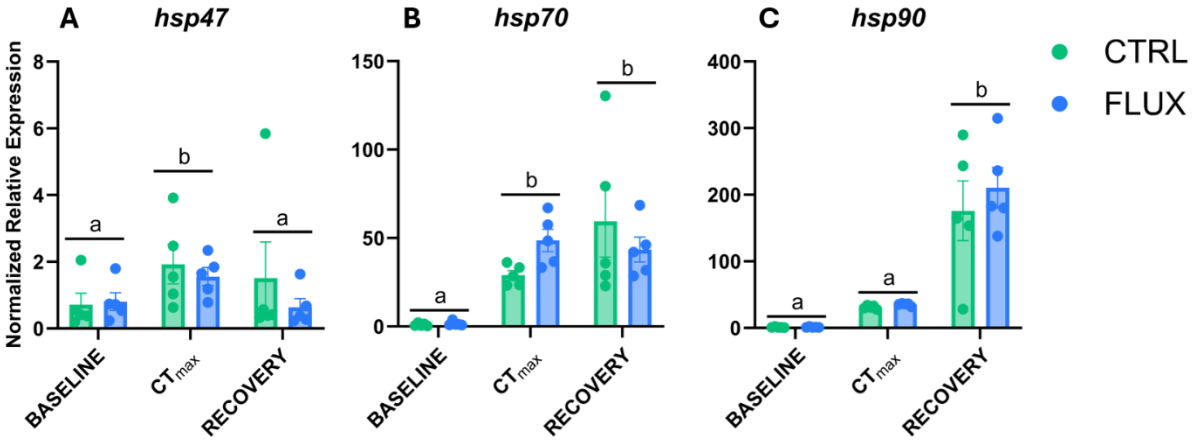


Figure 2.12: Normalized relative expression of the three HSPs measured in the brains of longitudinal zebrafish adults at 6 months post fertilization (mpf) after exposure to different developmental thermal regimes. The fish were sampled at different timepoints throughout the critical thermal maximum (CT_{max}) trial: Baseline (at 28°C prior to thermal challenge), CT_{max} (upon losing equilibrium), and Recovery (2 hr recovery at 28°C after attaining CT_{max}). (A) *hsp47* (B) *hsp70* and (C) *hsp90*. Each point represents the average response of the family (n = 5 families/treatment, with 2 individuals from a family sampled at each timepoint). Bars represent the mean ± SEM of the treatment. Different letters indicate significant differences ($p < 0.05$) between timepoints, and asterisks indicate significant differences between treatment groups within a timepoint (LME used for *hsp47* and GAMM used for *hsp70* and *hsp90*, followed by Tukey's post hoc contrasts).

Of the miRNAs quantified, there was no significant effect of either of the fixed factors (or any interaction terms) on the expression of miR-103-3p, miR-122, and miR-301a-3p (LME; Table 2.2; Fig. 2.13B,C,E). In contrast, the abundance of the let-7d-5p transcript differed significantly across

timepoint, but not treatment (LME; Table 2.2; Fig. 2.13A). Specifically, its expression was downregulated in the brains of fish exposed to the thermal challenge (Table 2.3). Finally, there was a significant main effect of treatment on the expression of miR-181a-5p (LME; Table 2.2; Fig. 2.13D). This impact was driven by differences between treatment groups within timepoints as the transcript's expression was elevated in the brains of the FLUX fish compared to the CTRL ones at baseline and CT_{max} only (Table 2.3).

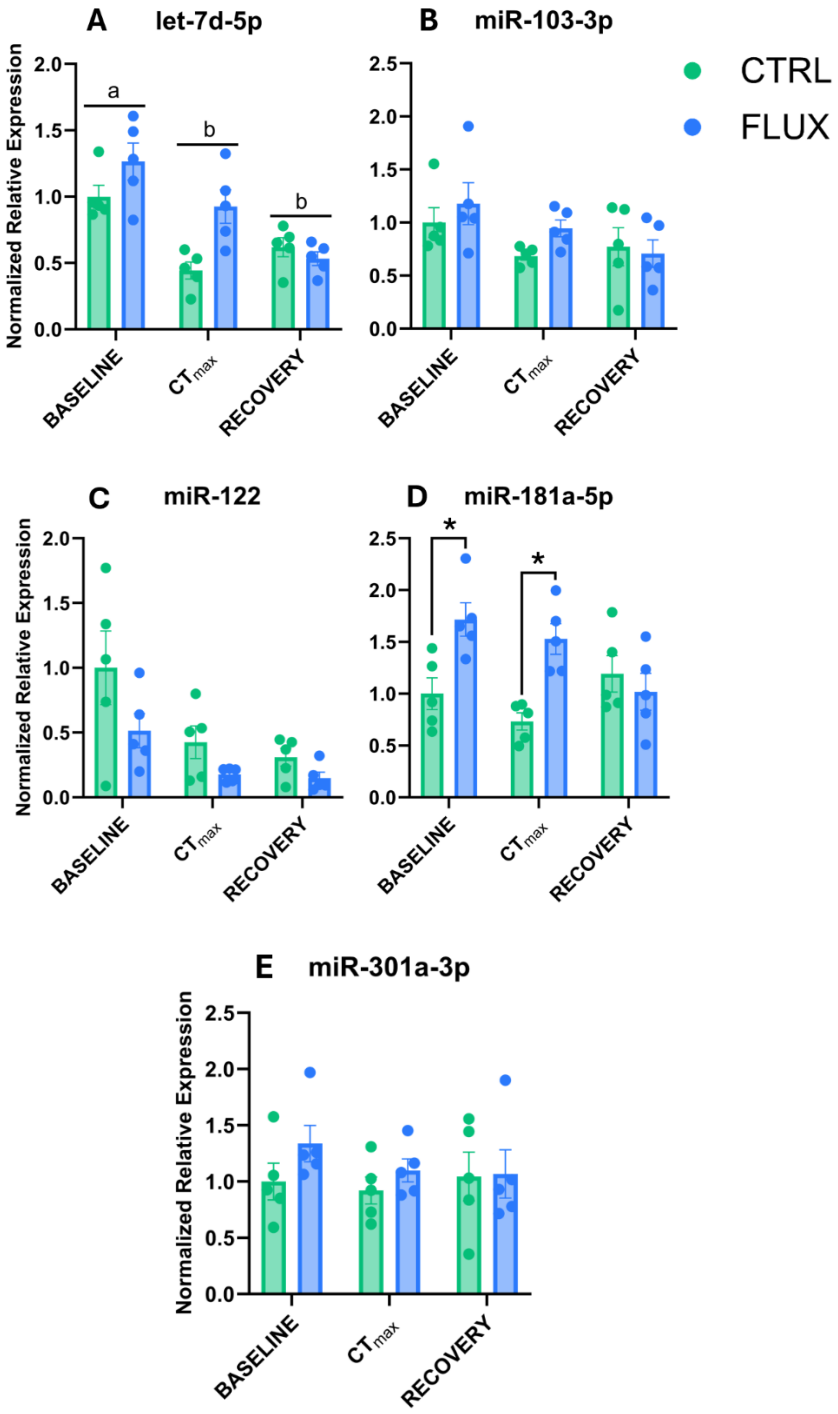


Figure 2.13: Normalized relative expression of the five miRNAs measured in the brains of longitudinal zebrafish adults at 6 months post fertilization (mpf) after exposure to different developmental thermal regimes. The fish were sampled at different timepoints throughout the critical thermal maximum (CT_{max}) trial: Baseline (at 28°C prior to thermal challenge), CT_{max} (upon

losing equilibrium), and Recovery (2 hr recovery at 28°C after attaining CT_{max}). (A) let-7d-5p, (B) miR-103-3p, (C) miR-122, (D) miR-181a-5p, and (E) miR-301a-3p. Each point represents the average response of the family (n = 5 families/treatment, with 2 individuals from a family sampled at each timepoint). Bars represent the mean ± SEM of the treatment. Different letters indicate significant differences ($p < 0.05$) between timepoints, and asterisks indicate significant differences between treatment groups within a timepoint (LME, Tukey's post hoc contrasts).

Table 2.2: Results from models for mRNA and miRNA expression in the brains of zebrafish adults at 6 months post fertilization (mpf) throughout the CTmax trials. An LME was used for all targets, except for hsp70 and hsp90, for which a GAMM was employed. Family was accounted for in the models as a random variable.

Target	Fixed effect	Estimate	Standard error	df	t-value	P value
hsp47	Intercept	−0.8623	0.346	25.55	−2.49	0.0196
	Treatment (FLUX)	0.19	0.490	25.55	0.389	0.701
	Timepoint (CT_{max})	1.148	0.414	46	2.773	0.008
	Timepoint (Recovery)	0.0445	0.414	46	0.108	0.914
	Treatment (FLUX): Timepoint (CT_{max})	−0.201	0.585	46	-0.344	0.732
	Treatment (FLUX): Timepoint (Recovery)	−0.309	0.585	46	-0.528	0.600
hsp70	Intercept	−1.1834	0.1526	N/A	−7.753	< 0.0001
	Treatment (FLUX)	0.226	0.216	N/A	1.046	0.300
	Smooth terms:		**Ref.df**	**edf**	**F-value**	**P value**
	s(Timepoint)		1.979	1.979	112.8	< 0.0001
	s(Timepoint):Treatment		3	2.013 e-06	0.0	0.177
hsp90	Intercept	−2.302	0.0932	N/A	−24.717	< 0.0001
	Treatment (FLUX)	0.141	0.132	N/A	1.071	0.289
	Smooth terms:		**Ref.df**	**edf**	**F-value**	**P value**
	s(Timepoint)		1.973	1.973	521.5	< 0.0001
	s(Timepoint):Treatment		3	1.038 e-08	0.0	0.967
let-7d-5p	Intercept	0.173	0.069	42.46	2.52	0.0158
	Treatment (FLUX)	0.094	0.097	42.46	0.966	0.339
	Timepoint (CT_{max})	−0.363	0.097	46	−3.735	0.0005
	Timepoint (Recovery)	−0.241	0.097	46	-2.481	0.0168
	Treatment (FLUX): Timepoint (CT_{max})	0.246	0.137	46	1.794	0.0794
	Treatment (FLUX): Timepoint (Recovery)	−0.139	0.137	46	−1.014	0.316
miR-103-3p	Intercept	0.499	0.161	42.46	3.113	0.0033
	Treatment (FLUX)	0.168	0.227	42.46	0.741	0.463

	Timepoint (CT_{max})	−0.303	0.227	46	−1.335	0.188
	Timepoint (Recovery)	−0.404	0.227	46	−1.781	0.081
	Treatment (FLUX): Timepoint (CT_{max})	0.087	0.321	46	0.272	0.787
	Treatment (FLUX): Timepoint (Recovery)	−0.081	0.321	46	−0.254	0.800
miR-122	Intercept	1.000	0.0625	42.46	16.004	<0.0001
	Treatment (FLUX)	−0.0243	0.0884	42.46	-0.275	0.785
	Timepoint (CT_{max})	0.0258	0.0884	46	0.292	0.772
	Timepoint (Recovery)	0.0987	0.0884	46	1.118	0.269
	Treatment (FLUX): Timepoint (CT_{max})	0.141	0.125	46	1.130	0.264
	Treatment (FLUX): Timepoint (Recovery)	0.184	0.125	46	1.472	0.148
miR-181a-5p	Intercept	−0.0549	0.0639	42.46	−0.859	0.395
	Treatment (FLUX)	0.233	0.0903	42.46	2.586	0.0132
	Timepoint (CT_{max})	−0.113	0.0903	46	−1.248	0.219
	Timepoint (Recovery)	0.066	0.0903	46	0.736	0.465
	Treatment (FLUX): Timepoint (CT_{max})	0.087	0.128	46	0.681	0.499
	Treatment (FLUX): Timepoint (Recovery)	−0.280	0.128	46	−2.192	0.0335
miR-301a-5p	Intercept	0.0349	0.0675	42.46	0.516	0.608
	Treatment (FLUX)	0.133	0.0955	42.46	1.397	0.17
	Timepoint (CT_{max})	−0.011	0.0955	46	−0.115	0.909
	Timepoint (Recovery)	−0.0049	0.0955	46	−0.052	0.959
	Treatment (FLUX): Timepoint (CT_{max})	−0.0614	0.135	46	−0.454	0.652
	Treatment (FLUX): Timepoint (Recovery)	−0.089	0.135	46	−0.66	0.512

Table 2.3: Post hoc contrasts from the models evaluating the impacts of timepoint and treatment on mRNA and miRNA expression in adults at 6 months post fertilization (mpf). When a significant interaction between fixed factors was present in a model, contrasts involved comparisons between timepoints within a treatment, and between treatments within a timepoint.

Target	Contrast	Estimate	Standard error	Df	t-value	P value
hsp47	**Timepoint**					
	Baseline− CT_{max}	−0.455	0.127	46	−3.577	0.0024
	Baseline−Recovery	0.0476	0.127	46	0.375	0.926
	CT_{max}−Recovery	0.503	0.127	46	3.952	0.0008
hsp70	**Timepoint**					
	Baseline−CT_{max}	−0.944	0.197	46	−4.783	0.0001
	Baseline−Recovery	−1.263	0.197	46	−6.397	<0.0001
	CT_{max}−Recovery	−0.319	0.197	46	−1.614	0.249

hsp90	**Timepoint**					
	Baseline−CT_{max}	−0.182	0.111	46	−1.641	0.239
	Baseline−Recovery	−1.090	0.111	46	−9.804	<0.0001
	CT_{max}−Recovery	−0.907	0.111	46	−8.164	<0.0001
let-7d-5p	**Timepoint**					
	Baseline−CT_{max}	0.239	0.0686	46	3.488	0.0031
	Baseline−Recovery	0.310	0.0686	46	4.523	0.0001
	CT_{max}−Recovery	0.071	0.0686	46	1.035	0.559
miR-181a-5p	**Treatment: Timepoint**					
	Baseline					
	CTRL−FLUX	−0.234	0.0903	42.5	−2.586	0.0132
	CT_{max}					
	CTRL−FLUX	−0.321	0.0903	42.5	−3.549	0.001
	Recovery					
	CTRL−FLUX	0.0465	0.0903	42.5	0.514	0.610
	Timepoint: Treatment					
	CTRL					
	Baseline−CT_{max}	0.113	0.0903	46	1.248	0.432
	Baseline−Recovery	−0.0665	0.0903	46	−0.736	0.743
	CT_{max}−Recovery	−0.179	0.0903	46	−1.984	0.128
	FLUX					
	Baseline−CT_{max}	0.0257	0.0903	46	0.284	0.956
	Baseline−Recovery	0.215	0.0903	46	2.364	0.0571
	CT_{max}−Recovery	0.188	0.0903	46	2.079	0.105

2.2.9 Bioinformatically-predicted mRNA targets of miRNAs

KEGG pathway analysis revealed that of the seven miRNAs measured in this study, no significantly enriched gene pathways were detected for five miRNAs, including, let-7d-5p, miR-122, miR-181a-5p, miR-214, and miR-499. For miR-103-3p, eight significantly enriched pathways were found. The impacted pathways included cellular signaling and biosynthesis pathways involved in key physiological processes such as metabolism, muscular function, and cellular communication. MiR-301a-3p was predicted to target the same number of pathways, encompassing processes such as the metabolism of carbohydrates, antioxidants, porphyrins, and xenobiotics. All pathways for each miRNA in this study are reported in Appendix B, and significantly enriched pathways are listed in Table 2.4.

Table 2.4: Enriched KEGG pathways predicted to be regulated by miR-103-3p and miR-301a-3p. The number of mRNA targets in a pathway targeted by the miRNAs, and the % of the pathway covered by these genes is provided. The Benjamini–Hochberg p values associated with each pathway are also listed.

miRNA	KEGG identifier	KEGG Term	Number of mRNA targeted	% of pathway targeted	P value
miR-103-3p	dre04744	Phototransduction	9	1.4	0.0044
	dre04070	Phosphatidylinositol signaling system	13	2.0	0.0044
	dre04912	GnRH signaling pathway	13	2.0	0.008
	dre04910	Insulin signaling pathway	15	2.3	0.0081
	dre04261	Adrenergic signaling in cardiomyocytes	18	2.8	0.0081
	dre04916	Melanogenesis	13	2.0	0.011
	dre04625	C-type lectin receptor signaling pathway	12	1.9	0.011
	dre04270	Vascular smooth muscle contraction	12	2.0	0.041
miR-301a-3p	dre00040	Pentose and glucuronate interconversions	6	3.8	0.0018
	dre00053	Ascorbate and aldarate metabolism	5	3.2	0.016
	dre00860	Porphyrin metabolism	5	3.2	0.017
	dre00982	Drug metabolism – cytochrome P450	5	3.2	0.017
	dre00980	Metabolism of xenobiotics by cytochrome P450	5	3.2	0.017
	dre00140	Steroid hormone biosynthesis	5	3.2	0.02
	dre00830	Retinol metabolism	5	3.2	0.028
	dre00983	Drug metabolism – other enzymes	5	3.2	0.036

Chapter 3
General discussion and future directions

The significant increase in average temperature and thermal variability of water bodies, driven by climate change and various anthropogenic inputs, has led to numerous studies focusing on the effects of these changes on aquatic organisms. While a majority of these investigations have demonstrated how such changes influence phenotypic plasticity in whole-organism traits throughout ontogeny, little is known about the mechanistic processes underpinning them. To our knowledge, this is the first study to explore the epigenetic modifications associated with plastic responses to environmentally relevant developmental thermal fluctuations throughout the lifespan of a fish. Here I provide evidence that diel thermal fluctuations during development can have lasting impacts on miRNA expression profiles within a generation, although the impacts on physiological processes are less clear. The major results of this thesis are summarized in Fig. 3.1.

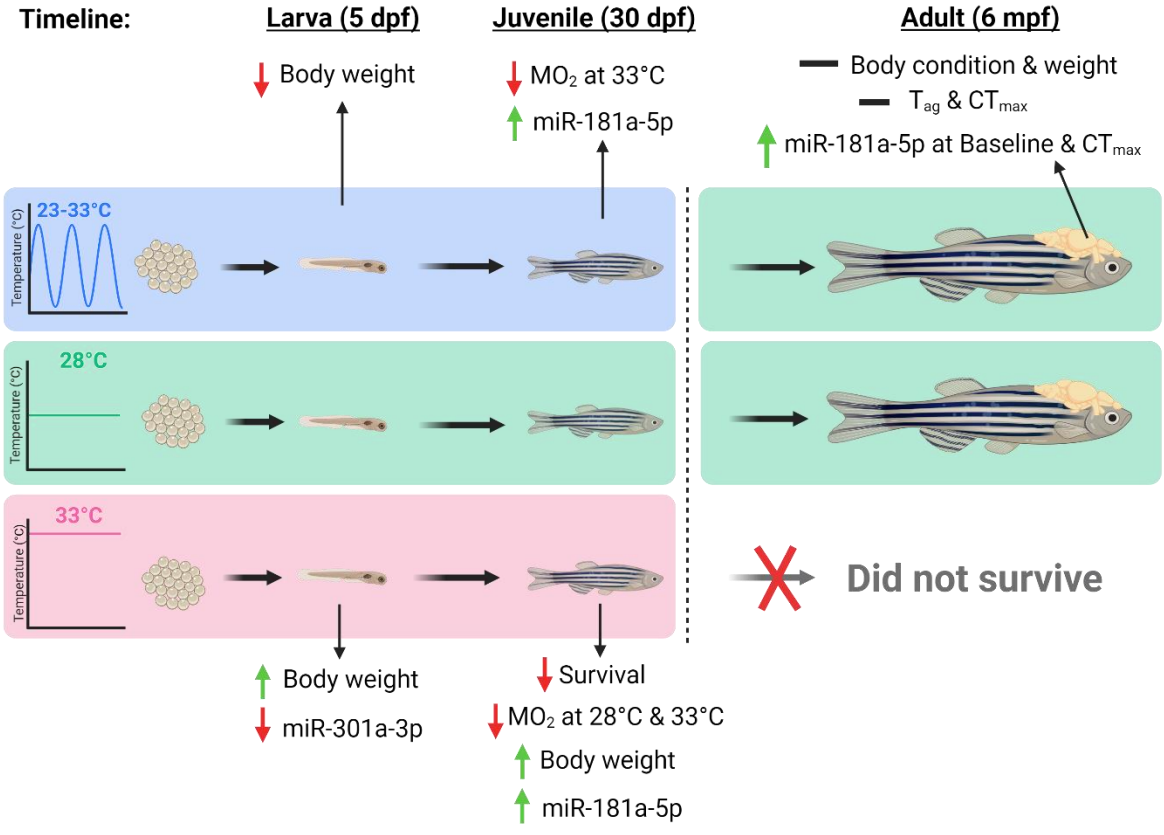


Figure 3.1: Graphical summary of thesis results. The different thermal regimes, sampling points, and takeaways from the thesis are depicted. Created with BioRender.com

3.1 Summary and synthesis of results

3.1.1 Larval stage

At 5 dpf, larvae exposed to constant elevated temperatures exhibited major differences relative to those held at constant control conditions. The *hsp70* and *hsp90* transcripts were upregulated in these fish, which is unsurprising given that there are many reports of the induction of the HSR in fish larvae upon chronic exposure to increased water temperature (Hallare et al., 2005; Li et al., 2015; Politis et al., 2017; Kim et al., 2022). It is important to note though that *hsp47* expression remained unchanged in the larvae from this treatment. This type of HSP is sequestered within the endoplasmic reticulum in cells and is collagen-specific, as opposed to the other two, which are more ubiquitously expressed and perform diverse functions (Lele et al., 1998; Iwama et al., 1998; Zhang et al., 2014). So, while it is possible that the treatment did not induce any changes in *hsp47* expression in these larvae, it is also likely that it resulted in tissue-specific changes which could not be detected in the pooled whole-body samples.

Interestingly, miR-301a-3p was found to be downregulated in the fish from the constant high temperature treatment. A similar change in the expression of this miRNA has been reported in zebrafish gonads (Ikert et al., 2020) and rainbow trout liver (Liu et al., 2022a) upon exposure to elevated temperatures and xenobiotics, indicating its potential as a biomarker for environmentally-induced stress. In the context of our study, this downregulation has several implications. Bioinformatic analysis revealed that miR-301a-3p is predicted to regulate multiple metabolic processes, with pentose and glucuronate interconversions and ascorbate and aldarate metabolism being the most enriched pathways. These pathways are interlinked, and play crucial roles in carbohydrate metabolism, production of precursors like amino acids for other biosynthetic pathways, and energy production (Burns et al., 1960; Bánhegyi, et al., 1997; Luo et al., 2007; Wang et al., 2014; Wu et al., 2024). Schnurr et al. (2014) demonstrated that chronic exposure to high water temperatures accelerates development in zebrafish, a finding confirmed by our study as the larvae in the elevated temperature group were nearly twice as large as those in the constant control regime. Rapid development driven by elevated temperature has been shown to deplete the maternal reserves in the yolk sac more quickly (Wilson, 2012; Hunt von Herbing & Pan, 2022), which would have resulted in an altered energy balance in these larvae. Consequently, these changes appear to have led to a miRNA-mediated activation of pathways related to carbohydrate metabolism in these fish to maintain homeostasis. Indeed, similar changes in these pathways have been documented in fish exposed to

extreme temperatures (Wang et al., 2014; Zhang et al., 2021). The contributions to the amino acid biosynthesis pathway from the interconversions between glucuronate and pentose, in particular, may have great functional significance for these larvae as amino acids are proposed to be the primary energy source for fish experiencing extended periods of heat stress (Nuez-Ortín et al., 2018; Yang et al., 2020).

Besides the changes in metabolism, these pathways could also help buffer the larvae against the cellular stress caused by exposure to elevated temperatures. For instance, metabolites and byproducts from these pathways such as ascorbate can scavenge reactive oxygen species (ROS), thereby counteracting the oxidative stress that fish exposed to elevated temperatures are known to experience (Bánhegyi et al., 1997; Takahashi et al., 2012; Madeira et al., 2013; Birnie-Gauvin et al., 2017). Additionally, Liu et al. (2022a) experimentally verified that *hsp90b2*, the gene that transcribes the HSP90β isoform, is targeted by ssa-miR-301-3p. Given that HSP90β is constitutively expressed (Krone et al., 1997), and higher baseline expression of HSPs is associated with an increase in the temperature required for the activation of the HSR (Dietz and Somero, 1992), this miRNA could play an important role in modulating their thermal tolerance as well. However, this isoform was not identified as a putative target through our bioinformatics analysis, nor was its expression measured through qPCR in this study as the primer utilized detected the α isoform. Given these findings, further research into the precise nature of the relationship between this miRNA and cellular protective mechanisms in early developmental stages is warranted.

On the other hand, the miRNA and mRNA expression in the larvae subjected to diel thermal cycles did not differ from the control larvae and they were marginally smaller. A few explanations can account for these results, though they may not be entirely distinct. Firstly, it is plausible that the HSR was activated at temperatures above the thermal optimum but could not be detected because the larvae were sampled at a single timepoint at ~24.5°C (Table A.1). Indeed, as indicated by Podrabsky and Somero (2004) and Coulter et al. (2015), the expression of mRNAs associated with HSPs and other proteins cycled along with temperature in the tissues of fish subjected to diel thermal fluctuations. Thus, one of the factors contributing to the reduced size could have been the energy-intensive cyclical production of these proteins involved in cellular stress pathways. As highlighted in Chapter 1, the time spent developing at temperatures outside the thermal optimum could have also reduced the overall developmental rate, thus lowering the average weight of these larvae (Dhillon & Fox, 2007). Lastly, it is possible that changes in other pathways may have increased metabolic costs,

thereby channeling energy away from growth or developmental processes. No changes in miR-301a-3p expression were observed, which suggests that the same carbohydrate metabolism pathways altered in the heat group were not impacted here. Instead, other studies have shown that the transcript levels of genes associated with cholesterol metabolism and the turnover of membrane proteins increased greatly in the liver of adult killifish (*Austrofundulus limnaeus*) in fish exposed to wide diel thermal fluctuations (Podrabsky & Somero, 2004). The authors proposed that these modifications were part of a homeoviscous adaptation strategy to maintain the fluidity and structure of plasma membranes in response to these fluctuations, which would likely have been metabolically expensive. Genes associated with the pentose phosphate pathway and pyrimidine metabolism were also variably expressed in fish exposed to cycling temperatures, suggesting a complex interplay of metabolic adjustments in response to thermal fluctuations. Therefore, we need to better understand the changes in various physiological processes, their interactions, and their associated costs to comprehensively describe the effects of thermal variability on larval body size.

3.1.2 Juvenile stage

Over the 30-day experimental period, the survival of the fish in the elevated temperature treatment was much lower than those in control conditions. The survival rates of the two groups were comparable throughout the embryonic stage, but declined sharply in the high temperature treatment once larvae began to rely on exogenous food sources (~7 dpf; Verbueken, et al., 2018). Such mass mortality has also been evidenced in other studies (Schnurr et al., 2014), and can be explained by the cessation of feeding that occurs in fish experiencing chronic thermal stress (Shafland & Pestrak, 1982). One mechanism that could explain this result is that high water temperature during development can increase the incidence of mouth deformities in other fish species (Lein et al. 1997; Okamura et al. 2007), leading to the failure of first few feeds (Parra & Yúfera, 2000; Yúfera & Darias, 2007). Though parameters associated with mouth shape and size were not assessed during this study, these changes likely contributed to the high levels of mortality observed in the HEAT group. These detrimental effects on survival were probably compounded further as starvation leads to deformities in and degradation of digestive system structures like the gut (Theilacker, 1978; Yúfera et al., 1993; Gisbert et al., 2004). Lastly, it is also important to consider the role of learning in improving feeding success and efficiency in larvae (Drost, 1988; Coughlin 1991). Fish hone their aim and ability to capture food during early life stages by observing conspecifics and attempting to secure prey items themselves (Drost, 1987; Drost et al., 1988; Lucore & Connaughton, 2021). So, given the

rapid development in the HEAT group, it is also possible that these larvae did not have enough time to burnish their feeding technique in time to meet their energy deficit.

The survival rates of the fluctuating temperature group were not significantly different from the control group. This result is corroborated by results from other species (Li et al., 2021), and highlights the robustness of fish development to thermal variability. Additionally, the reduced body weight of fish from the fluctuating treatment that was observed at 5 dpf did not persist until the juvenile stage. This was likely the result of altered feeding, digestive, and/or growth efficiency as the daily feeds occurred at the lower end of the thermal cycle (~23°C) in this treatment as opposed to 28°C in the constant control conditions. For instance, the specific dynamic action (SDA), which represents the energetic cost associated with processes such as food ingestion, digestion, absorption, and excretion, can vary in fish depending on the water temperature (Luo & Xie, 2008; Tirsgaard et al., 2014). Temperature has also been shown to influence processes such as food detection and intake, digestive enzyme activity, foraging behavior, and appetite in fish (see Volkoff & Rønnestad, 2020 for a comprehensive review).

From the respirometry results, it is evident that the MO_2 of the juveniles reared under the chronic elevated temperature conditions was significantly lower at 28°C relative to the other two groups, which did not differ from each other. As the fish from this group were nearly twice as large as the others, this reduced MO_2 could be at least partially attributed to the well-documented inverse relationship between body weight and mass-specific metabolic rate (Kleiber, 1932; Oikawa & Itazawa, 2003). However, since this relationship is not as definitive during the early developmental stages, it remains uncertain if this was the primary driver of the observed decrease (Oikawa et al., 1993).

A particularly interesting finding was that juveniles reared under both fluctuating and elevated thermal regimes demonstrated a blunted metabolic response to an acute temperature increase. The patterns of compensation, though, were distinct. The fish in the elevated temperature displayed ‘partial’ compensation under Precht’s framework of metabolic compensation, which is commonly observed in warm-acclimated fish (Precht, 1958; Das et al., 2004; Donelson et al., 2011; Pan & Hunt von Herbing, 2017; Schulte, 2015). While fluctuating temperatures were not included in this model, the fish from the fluctuating group had a compensatory profile akin to if they had ‘perfectly’ compensated for the exposure to high temperatures. Massey (2023) conducted a similar experiment in which zebrafish were subjected to either diel thermal fluctuations (27 ± 5°C) or constant control

(27°C) temperature until 30 dpf, after which they were acclimated to control conditions until adulthood. As adults, fish that were developmentally exposed to temperature fluctuations exhibited a lower MO_2 at 27°C relative to those with no such thermal history. However, since the MO_2 was only measured at one temperature, the extent and mechanism of compensation could not be assessed.

One explanation for why the slope of the metabolic rate was negligible for the fluctuating group comes from a principle that contrasts the acute and chronic metabolic responses of fish acclimated to different temperatures (Fig. 3.2; Hill et al., 2012). In this study, as in many others that have evidenced metabolic compensation, the fish were acclimated to a specific temperature for an extended period such as a few weeks, after which their instantaneous MO_2 was measured at multiple test temperatures over a short timespan (i.e. hours). Thus, the measurement would be considered an acute response. In contrast, the chronic response of an organism could be quantified if the organism were acclimated to each test temperature for weeks before its measurement, after which its metabolic rate at each temperature was charted. Predictably, the slope of the chronic response would be much shallower than the acute one, suggesting that they would be less responsive to changes in body temperature. Thus, the instantaneous change in MO_2 observed in the juveniles from the fluctuating treatment in our study closely resembles the chronic acclimatory response proposed by Hill et al. (2012), suggesting that chronic exposure to thermal variability produces similar metabolic effects to acclimating fish at each of these static testing temperatures (Fig. 3.2). These effects may not have been observed by Massey (2023) as the MO_2 was only quantified at a singular temperature, and it was measured in adults after a significant amount of time had elapsed since they experienced thermal variability. Future experiments could focus on understanding the specific impacts of thermal variability on metabolic rate across a range of temperatures, developmental stages, and thermal history.

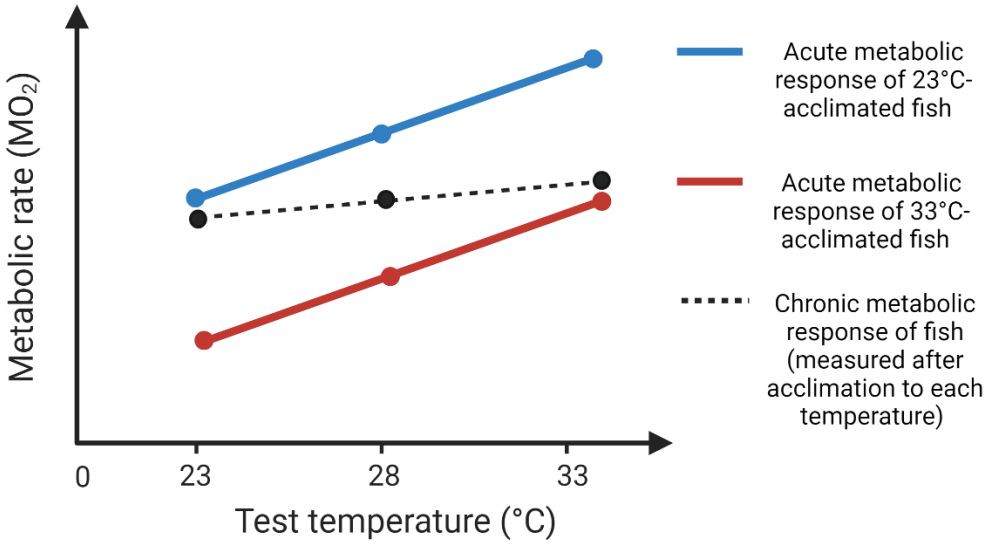


Figure 3.2: Relationship between the metabolic rate and testing temperature contrasting the proposed acute and chronic metabolic responses in fish acclimated to different temperatures. The acute response is quantified by measuring the MO_2 of a fish acclimated to a specific temperature (such as 23°C or 33°C) over multiple testing temperatures in the span of a few hours. The chronic response is quantified by acclimating the fish to each testing temperature for weeks and then measuring their MO_2 at the associated temperature. Adapted from Hill et al. (2012) and created using Biorender.com.

RT-qPCR analyses of juveniles revealed that miR-181a-5p was the only differentially expressed transcript amongst those measured, and was upregulated in both the fluctuating and elevated temperature groups. While this miRNA was predicted to exert regulatory effects on many genes, it was not associated with any specific KEGG pathways. Consequently, the functional analysis prioritized potential gene targets regulated by miR-181a-5p that were involved in mitochondrial structure and function. This emphasis stems from the fact that the compensatory adjustments in whole-animal MO_2 are primarily linked to modifications in the oxidative and respiratory rates of this organelle (Guderley, 2004; Seebacher et al., 2010; Jayasundara et al., 2015; Ekström et al., 2017). The upregulation in miR-181a-5p detected in these juveniles may contribute to these changes as *in-vitro* experiments have shown that this miRNA has an inverse relationship with proteins involved in oxidative phosphorylation like cytochrome B and cytochrome C oxidase II (Zhuang et al., 2020). Additionally, this miRNA is predicted to target several genes involved in the fatty acid biosynthesis and metabolism pathways (Ma et al., 2019), suggesting that it could contribute to adaptive alterations in mitochondrial membrane composition and integrity as discussed previously (Guderley, 2004;

Kraffe et al., 2007; Chung et al., 2018). Finally, recent work conducted with mice indicates that miR-181a-5p plays a pivotal role in regulating mitochondrial biogenesis (Indrieri et al., 2019), a process known to be crucial in fish for acclimation to extreme temperatures, particularly in cold environments (Johnston & Maitland, 1980; Johnston, 1982). Specifically, Indrieri et al. (2019) reported that silencing this miRNA resulted in faster mitochondrial turnover by increasing mitochondrial production and clearance. Thus, the compensatory ability of FLUX and HEAT fish could have been because their mitochondria are more efficient and undergo less frequent turnover. Although the effects of acclimation to high temperatures or fluctuations on mitochondrial turnover are not well-explored, changes in this process may be critical for compensation in this scenario. Future work should aim to elucidate how fish reared under fluctuating and chronic temperature regimes vary in their mitochondrial properties to better understand the differences in their compensatory abilities.

3.1.3 Adult stage

Exposure to diel thermal fluctuations during the embryonic and larval stages had no discernible effect on the body condition, weight, and thermal tolerance of longitudinal adults. Taking the lack of effect on CT_{max} and T_{ag} together with the changes in juvenile MO_2, these results are in line with the 'plastic floors and concrete ceilings' theory which suggests that routine metabolic rates may be more amenable to thermal variability than the upper critical threshold (Sandblom et al., 2016; Morash et al., 2018). It should be noted, however, that these findings are in contrast with previous studies, which have demonstrated that thermal variability during development reduces body size and increases thermal tolerance in adult zebrafish (Schaefer & Ryan, 2006; Massey, 2023). As discussed previously, the differences in feeding temperatures used in this study ameliorated the effects of fluctuations on body weight. Besides minor variations in the ramp rate used for CT_{max} trials, age at testing, and the range of diel thermal fluctuations, there were no significant methodological differences between previous work and the current study. Instead, the discrepancy in heat tolerance may be attributed to factors like sample size and grouping patterns as Massey (2023) employed a larger sample size and a split clutch design with embryos from three pairs, while Schaefer & Ryan (2006) used group-spawned embryos.

During the CT_{max} trials, HSPs exhibited a significant induction in the brains of adult fish, though developmental thermal history had no effect on their expression. Given the well-established correlation between the degree of activation of the HSR and CT_{max} (Iwama et al., 1998; Iwama and

Vijayan, 1999; Saravia et al., 2021; Penman et al., 2023), it is unsurprising that no differences were detected in this measurement and T_{ag} between the treatment groups. Conversely, let-7d-5p showed a different pattern, with it being significantly downregulated at and after CT_{max}. Since this miRNA is known to be repressed under hypoxic conditions in various fish tissues (Kulshreshtha et al., 2008; Lai et al., 2016; Heinrichs-Caldas et al., 2023), our results may provide support for the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis. Under this hypothesis, the localized oxygen deprivation that occurs in tissues, particularly the brain, at high temperatures constrains the upper critical thermal threshold (Pörtner, 2021; Andreassen et al., 2022). Additionally, because data from murine cell lines suggests that let-7d-5p promotes neuronal migration and the differentiation of stem cells into neuronal and glial cells (Zhao et al., 2013), its expression could have been downregulated to divert energy away from these processes to preserve energy for cellular stress mechanisms like the HSR (López-Maury et al., 2008; Jesus et al., 2016). Interestingly, Zhao et al. (2013) also reported that overexpression of the miRNA was associated with enhanced neuronal proliferation. Thus, it would be worthwhile to explore how thermal stress affects neurogenesis and neural differentiation in fish brains and the role let-7d-5p plays in these signaling cascades to understand the functional consequences of changes in its expression.

As with the juvenile stage, the only miRNA whose expression was altered by developmental thermal fluctuations in adult brains was miR-181a-5p, which was elevated at both baseline and CT_{max}. As previously discussed, inhibition of this miRNA has been shown to increase the rates of mitochondrial turnover (Indrieri et al., 2019). Taking into account that acute exposure to extreme temperatures leads to mitochondrial dysfunction in fish (Kobayashi, 2000; Liu et al., 2022b; Liu et al., 2022c), the upregulation of miR-181a-5p in the brains of FLUX fish could suggest that damaged mitochondria are cleared at lower rates, thereby resulting in a greater extent of neuronal death/injury during thermal stress. Indeed, many studies have suggested that increased expression of this miRNA is associated with a greater extent of neuronal damage and apoptosis in the forebrain and cerebrum during ischemia, a condition in which blood flow and, consequently, oxygen supply to the brain are reduced (Stary et al., 2017; Griffiths et al., 2019; Xiaoming Song et al., 2021). However, the regulatory impact of this miRNA on this cell death pathway is not as straightforward, as some studies have found that it represses the expression of apoptotic factors and protects neurons upon recovery from ischemia (Liu et al., 2019). Thus, we can only conclude that this miRNA may alter the extent of neuronal injury that these fish likely experience during the localized hypoxia in the brain at CT_{max} (as

per the OCLTT). As such, the role of this miRNA during thermal stress in fish brains should be clarified to identify if the observed changes in its expression are protective or detrimental.

Taken together, the findings associated with this miRNA indicate a tradeoff, where the same mitochondrial changes which allow for metabolic compensation in FLUX fish, could serve to decrease their ability to cope with and recover from acute thermal stressors. This has been highlighted by studies which have shown that warm-acclimated New Zealand wrasse (*Notolabrus celidotus*) could only make minor adjustments in the mitochondrial properties of their hearts upon exposure to heat stress (Iftikar et al., 2015), while warm-acclimated killifish (*Fundulus heteroclitus*) showed reduced brain and heart mitochondrial performance at temperatures approaching their CT_{max} as opposed to control fish (Chung et al., 2017). As highlighted by Gerber et al. (2020), the lack of improvement in the mitochondrial function of these fish could be because they have utilized most of their acclimatory capacity to minimize the costs associated with their elevated rearing temperatures, leaving little room to acclimate to further thermal changes. However, not all species respond this way. The mitochondria of warm-acclimated Atlantic salmon (*Salmo salar*) functioned better at higher temperatures than controls in parameters such as respiratory capacity and membrane integrity (Gerber et al., 2020). Future inquiries should aim to better understand how mitochondrial function and its relationship to acclimatory potential and thermal tolerance is affected by different rearing temperatures to determine how different species will respond to climate change.

3.1.4 Small effect sizes: Does a little go a long way?

Despite the subtle differences in miRNA expression seen between treatment groups in this study, I posit that these results could still be biologically significant. Although a singular miRNA typically has a modest impact on its target mRNA, often producing less than a 2-fold change in protein levels, there are several ways that the repressive effects of miRNAs can be enhanced (Baek et al., 2008). For instance, mRNAs often have more than one binding site, thus allowing a miRNA to bind to its target at multiple locations to provide stronger regulatory effects (Mayr et al., 2009). Multiple miRNAs can act in concert on a target mRNA to produce an additive effect if sites are well-dispersed, and a synergistic one if the binding sites are located within 8-40 nt of each other (Grimson et al., 2007; Friedman et al., 2009; Bartel, 2009). The effects of changes in miRNA can also be increased when the targets of miRNAs are involved in positive feedback loops as any changes in protein levels can be amplified along the pathway (Iliopoulos et al., 2009). For instance, miRNAs from the let-7 family

have been shown to target genes involved in continuous cell proliferation that occurs via positive feedback in cancerous tissue (Iliopoulos et al., 2009). Finally, as elucidated by the enrichment analyses in this study, a miRNA can target more than one gene involved in a pathway or macromolecular complexes, thereby exerting a significant impact on physiological processes across multiple levels (Ebert & Sharp, 2012). As an example, miR-181a has been found to strongly regulate the sensitivity of T cells by modulating the expression of many phosphatases involved in the signaling cascade (Li et al., 2007).

3.2 Conclusion

3.2.1 Overarching conclusions

This study was one of the first to elucidate the role of epigenetics in regulating the response of fish to thermal variability. More specifically, we see that exposure to cycling temperatures during development results in a greater extent of metabolic compensation than fish exposed to chronically elevated temperatures. These changes were also associated with an increase in miR-181a-5p in both groups, which could regulate mitochondrial processes critical for compensation. Although the increase in the levels of this persisted until adulthood, the impact of these changes on the ability of adults to cope with thermal stress remains ambiguous as the tolerance to high temperatures was not affected in these fish. Research from mammalian models (Griffiths et al., 2019; Liu et al., 2019) suggests that the upregulation of this miRNA could result in greater neuronal injury following hypoxic conditions in the brain. Thus, it seems that chronic exposure to thermal variability results in a tradeoff, as fish acclimated to such conditions have lower energetic expenditure under a broad range of temperatures but fare worse upon encountering acute heat stress. Together, these results warrant further exploration into how miRNAs and their associated gene networks are altered by acute and chronic temperature changes, and how they regulate mitochondrial performance during these scenarios. Overall, this study highlights that diel thermal fluctuations confer a pattern of plasticity that is distinct from that caused by constant elevated temperatures or constant control conditions. This highlights the need to incorporate thermal variability into experimental designs to accurately understand the physiological processes in fish from natural environments. From a broader perspective, this study also lays the groundwork for understanding how fish in natural environments will react to thermal challenges such as heat waves.

3.2.2 Limitations

In the larval and juvenile stages, the expression of five out of the seven miRNAs evaluated was unchanged by exposure to diel thermal fluctuations. This result is equally as intriguing as the significant changes in miRNA expression and could be the product of many underlying factors. As highlighted earlier, one of the drawbacks of whole-body sampling is that tissue-specific responses are diluted. Given that miRNAs control critical processes like body axis patterning and cell differentiation during development, their expression may have been altered in specific regions and bypassed detection (Bhattacharya et al., 2016). Moreover, less than 0.01% of the total miRNA sequences catalogued for zebrafish were evaluated in this study (Cao et al., 2023). This limitation becomes quite apparent in the context of the chronic heat-exposed group, wherein only one miRNA was altered at the 5 dpf stage, yet survival was drastically impacted following this stage. Thus, it is almost certain that there were changes in the abundance of other miRNAs that were not detected in this study. A more comprehensive picture of changes in miRNA transcriptome could be obtained using methods such as small RNA sequencing (Cadonic et al., 2023). Such a technique would also be beneficial because it would allow for correlations between miRNA expression and their predicted mRNA targets, improving the understanding of the regulatory impacts of miRNAs.

In a similar vein, this study sampled the fish at timepoints that marked important transitions between life stages because I wanted to understand the effects of chronic exposure to diel thermal fluctuations. However, several other critical phases were missed. The developmental landscape is known for its rapid changes, which miRNAs are known to govern (Bhattacharya et al., 2016). An example of such a crucial developmental phase that was not captured here includes organogenesis, which occurs before 5 dpf (Kimmel et al., 1995; Verbueken et al., 2018). Additionally, the diel thermal variation introduces further complexity, offering numerous points along the thermal cycle to sample from. Thus, it may be worthwhile to not only sample at more developmental stages, but also more points throughout the cycle to capture acute changes in mRNA. Lastly, it is important to note that miRNAs themselves are part of much larger and complex gene regulatory networks. As an example, miRNA biosynthesis has been found to be regulated by molecular chaperones like HSP90 and Aha1 (Liu et al., 2022d). Therefore, it is possible that the lack of an effect on the miRNAs in this study is due to mechanisms that maintain their expression to bolster the robustness of the developmental program. While robustness of a biological process can be achieved by an insensitivity of these pathways to environmental change, it is more commonly attained through redundancy and

regulation (Bateson & Gluckman, 2012). Lastly, the different feeding temperatures likely also impacted the final results of this study. Thus, I recommend that a standard feeding and rearing protocol is determined for fluctuating temperature studies going forward.

3.2.3 Future Directions

This thesis has shed some light on the role of epigenetics in the response of fish to natural thermal variation, and future research could build on the current findings through various avenues. Firstly, conducting similar experiments using other fish species as well as those with diverse evolutionary histories may offer valuable insights. In this study, zebrafish were used due to their rapid development and ease of husbandry. However, since zebrafish are a tropical species and can tolerate a wide range of temperatures, these results may have limited applicability to stenothermal fish native to Canada like the rainbow trout. Given that the effects of global warming are expected to be more pronounced at higher latitudes (Rizzo & Wiken, 1992), it is crucial to determine how temperate species respond to rapid changes in temperature so appropriate remediation measures can be implemented. It is also important to note that this study utilized lab-reared strains, which have been shown to differ greatly in their genetic background and potential for plasticity as compared to their wild counterparts due to generations of being maintained under optimal conditions (Whiteley et al., 2011; Morgan et al., 2019). Therefore, the results from this study may not be relevant for wild-caught fish which often experience more selective pressure. Another way researchers could better capture natural conditions is by incorporating seasonal and stochastic variation in their experiments. This could be achieved by collecting data from streams and rivers using iButton data loggers and replicating the thermal profiles in the laboratory. As suggested by Turko et al. (2023), experiments that aim to characterize the effects of multiple stressors on fish physiology should utilize ‘natural’ conditions including diel thermal and oxygen fluctuations as the control to accurately describe the effects of stressors of interest like contaminants on fish physiology. Lastly, while this thesis only explored within-generational plastic responses to environmental variability, many studies have demonstrated that these changes can be transmitted across generations (Lim & Bernier, 2022; Massey & Dalziel, 2023; Heinrichs-Caldas et al., 2023). So, future studies could aim to delineate maternal and paternal miRNA contributions to their offspring to better understand the role of epigenetics in transgenerational thermal plasticity.

References

Andreassen, A. H., Hall, P., Khatibzadeh, P., Jutfelt, F. & Kermen, F. (2022). Brain dysfunction during warming is linked to oxygen limitation in larval zebrafish. *Proceedings of the National Academy of Sciences, 119*(39), e2207052119.

Angilletta Jr., M. J. (2009). *Thermal Adaptation: A Theoretical and Empirical Synthesis.* Oxford, UK: Oxford University Press.

Arroyo, J. D., Chevillet, J. R., Kroh, E. M., Ruf, I. K., Pritchard, C. C., Gibson, D. F., Mitchell, P. S., Bennett, C. F., Pogosova-Agadjanyan, E. L., Stirewalt, D. L. & Tait, J. F. (2011). Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proceedings of the National Academy of Sciences, 108*(12), 5003–5008.

Bánhegyi, G., Braun, L., Csala, M., Puskás, F. & Mandl, J. (1997). Ascorbate metabolism and its regulation in animals. *Free Radical Biology and Medicine, 23*(5), 793–803.

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

Bateson, P. & Gluckman, P. (2012). Plasticity and robustness in development and evolution. *International Journal of Epidemiology, 41*(1), 219–223.

Bateson, P., Gluckman, P. & Hanson, M. (2014). The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *The Journal of Physiology, 592*(11), 2357–368.

Becker, C. D. & Genoway, R. G. (1979). Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environmental Biology of Fishes, 4*, 245–256.

Beldade, P., Mateus, A. R. A. & Keller, R. A. (2011). Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular ecology, 20*(7), 1347–1363.

Bhattacharya, M., Sharma, A. R., Sharma, G., Patra, B. C., Nam, J. S., Chakraborty, C. & Lee, S. S. (2017). The crucial role and regulations of miRNAs in zebrafish development. *Protoplasma, 254*, 17–31.

Birnie-Gauvin, K., Costantini, D., Cooke, S. J. & Willmore, W. G. (2017). A comparative and evolutionary approach to oxidative stress in fish: a review. *Fish and Fisheries, 18*(5), 928–942.

Bizuayehu, T. T. & Babiak, I. (2014). MicroRNA in teleost fish. *Genome biology and evolution, 6*(8), 1911–1937.

Bizuayehu, T. T., Johansen, S. D., Puvanendran, V., Toften, H. & Babiak, I. (2015). Temperature during early development has long-term effects on microRNA expression in Atlantic cod. *BMC genomics, 16*, 305.

Boutilier, R. G., Heming, T. A. & Iwama, G. K. (1984). Physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology* (eds. W. S. Hoar, D. J. Randall), vol. 10, pp. 403–430. Cambridge, MA: Academic Press.

Bozinovic, F., Bastías, D. A., Boher, F., Clavijo-Baquet, S., Estay, S. A. & Angilletta Jr, M. J. (2011). The mean and variance of environmental temperature interact to determine physiological tolerance and fitness. *Physiological and Biochemical Zoology, 84*(6), 543–552.

Brauner, C. J. & Richards, J. G. (2020). Physiological performance in aquaculture: Using physiology to help define optimal conditions for growth and environmental tolerance. In *Fish physiology* (ed. T. J. Benfey, A. P. Farrell, C. J. Brauner), vol. 38, pp. 83–121. Cambridge, MA: Academic Press.

Burggren, W. (2018). Developmental phenotypic plasticity helps bridge stochastic weather events associated with climate change. *Journal of Experimental Biology, 221*(9), jeb161984.

Burns, J. J., Conney, A. H., Dayton, P. G., Evans, C., Martin, G.R. & Taller, D. (1960). Observations on the drug-induced synthesis of D-glucuronic, L-gulonic and L-ascorbic acids in rats. *Journal of Pharmacology and Experimental Therapeutics, 129*(2), 132–138.

Cadonic, I. G., Heath, J. W., Dixon, B. & Craig, P. M. (2023). Diploid and triploid Chinook salmon (*Oncorhynchus tshawytscha*) have altered microRNA responses in immune tissues after infection with *Vibrio anguillarum*. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 48,* 101121.

Cadonic, I. G., Ikert, H. & Craig, P. M. (2020). Acute air exposure modulates the microRNA abundance in stress responsive tissues and circulating extracellular vesicles in rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 34,* 100661.

Cai, Y., Yu, X., Hu, S. & Yu, J. (2009). A brief review on the mechanisms of miRNA regulation. *Genomics, Proteomics and Bioinformatics, 7*(4), 147–154.

Caklec, J. (2023). Investigating the impacts of diurnal temperature and venlafaxine exposure in zebrafish. Master's thesis, University of Waterloo, Waterloo, ON.

Calisi, R. M. & Bentley, G. E. (2009). Lab and field experiments: are they the same animal?. *Hormones and behavior, 56*(1), 1–10.

Campos, C., Sundaram, A. Y., Valente, L. M., Conceição, L. E., Engrola, S. & Fernandes, J. M. (2014). Thermal plasticity of the miRNA transcriptome during Senegalese sole development. *BMC Genomics, 15*(1), 525.

Cao, Q., Zhang, H., Li, T., He, L., Zong, J., Shan, H., Huang, L., Zhang, Y., Liu, H. & Jiang, J., 2023. Profiling miRNAs of teleost fish in responses to environmental stress: A review. *Biology, 12*(3), 388.

Champroux, A., Cocquet, J., Henry-Berger, J., Drevet, J. R. & Kocer, A. (2018). A decade of exploring the mammalian sperm epigenome: Paternal epigenetic and transgenerational inheritance. *Frontiers of Cellular and Developmental Biology, 6*, 50.

Cheng, L. Y., Bailey, A. P., Leevers, S. J., Ragan, T. J., Driscoll, P. C. & Gould, A. P. (2011). Anaplastic lymphoma kinase spares organ growth during nutrient restriction in Drosophila. *Cell, 146*, 435–447.

Chung, D. J., Bryant, H. J., & Schulte, P. M. (2017). Thermal acclimation and subspecies-specific effects on heart and brain mitochondrial performance in a eurythermal teleost (*Fundulus heteroclitus*). *Journal of Experimental Biology, 220*(8), 1459–1471.

Chung D. J., Sparagna, G. C., Chicco, A. J. & Schulte, P. M. (2018). Patterns of mitochondrial membrane remodeling parallel functional adaptations to thermal stress. *Journal of Experimental Biology, 221*, jeb174458.

Claireaux, G. & Lefrançois, C. (2007). Linking environmental variability and fish performance: integration through the concept of scope for activity. *Philosophical Transactions of the Royal Society B: Biological Sciences, 362*(1487), 2031–2041.

Colinet, H., Sinclair, B. J., Vernon, P. & Renault, D. (2015). Insects in fluctuating thermal environments. *Annual Review Of Entomology, 60*, 123–140.

Coughlin, D. J. (1991). Ontogeny of feeding behavior of first-feeding Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences, 48*, 1896-1904.

Coulter, D. P., Höök, T. O., Mahapatra, C. T., Guffey, S. C. & Sepúlveda, M. S. (2015). Fluctuating water temperatures affect development, physiological responses and cause sex reversal in fathead minnows. *Environmental Science & Technology, 49*(3), 1921–1928.

Craig, P. M., Trudeau, V. L. & Moon, T. W. (2014). Profiling hepatic microRNAs in zebrafish: fluoxetine exposure mimics a fasting response that targets AMP-activated protein kinase (AMPK). *PLoS One, 9*(4), e95351.

Dahlke, F. T., Wohlrab, S., Butzin, M. & Pörtner, H.-O. (2020). Thermal bottlenecks in the life cycle define climate vulnerability of fish. *Science, 369*(6499), 65–70.

Dai, Y. J., Jia, Y. F., Chen, N., Bian, W. P., Li, Q. K., Ma, Y. B., Chen, Y. L. & Pei, D. S. (2014). Zebrafish as a model system to study toxicology. *Environmental toxicology and chemistry, 33*(1), 11–17.

Das, T., Pal, A. K., Chakraborty, S. K., Manush, S. M., Chatterjee, N. & Mukherjee, S. C. (2004). Thermal tolerance and oxygen consumption of Indian Major Carps acclimated to four temperatures. *Journal of Thermal Biology, 29*(3), 157–163.

David, P., Hingle, A., Greig, D., Rutherford, A., Pomiankowski, A. & Fowler, K. (1998). Male sexual ornament size but not asymmetry reflects condition in stalk–eyed flies. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 265*(1411), 2211–2216.

de Alba, G., López-Olmeda, J. F. & Sánchez-Vázquez, F. J. (2021). Rearing temperature conditions (constant vs. thermocycle) affect daily rhythms of thermal tolerance and sensing in zebrafish. *Journal of Thermal Biology, 97*, 102880.

de Waal, E., Yamazaki, Y., Ingale, P., Bartolomei, M., Yanagimachi, R. & McCarrey, J. R. (2012). Primary epimutations introduced during intracytoplasmic sperm injection (ICSI) are corrected by germline-specific epigenetic reprogramming. *Proceedings of the National Academy of Sciences of the United States of America, 109*(11), 4163–4168.

Denli, A.M., Tops, B.B.J., Plasterk, R.H.A., Ketting, R.F. & Hannon, G.J. (2004). Processing of primary microRNAs by the microprocessor complex. *Nature, 432*, 231–235.

Dhillon, R. S. & Fox, M. G. (2007). Growth-independent effects of a fluctuating thermal regime on the life-history traits of the Japanese medaka (*Oryzias latipes*). *Ecology of Freshwater Fish, 16*(3), 425–431.

Dietz, T. J. & Somero, G. N. (1992). The threshold induction temperature of the 90-kDa heat shock protein is subject to acclimatization in eurythermal goby fishes (genus *Gillichthys*). *Proceedings of the National Academy of Sciences of the United States of America, 89*, 3389-3393.

Dillon, M. E., Woods, H. A., Wang, G., Fey, S. B., Vasseur, D. A., Telemeco, R. S., Marshall, K. & Pincebourde, S. (2016). Life in the frequency domain: the biological impacts of changes in climate variability at multiple time scales. *Integrative and Comparative Biology, 56*(1), 14–30.

Donelson, J. M., Munday, P. L., Mccormick, M. I. & Nilsson, G. E. (2011). Acclimation to predicted ocean warming through developmental plasticity in a tropical reef fish. *Global Change Biology, 17*, 1712–1719.

Dowd, W. W., King, F. A. & Denny, M. W. (2015). Thermal variation, thermal extremes and the physiological performance of individuals. *Journal of Experimental Biology, 218*(12), 1956–1967.

Drake, J. E., Tjoelker, M.G., Vårhammar, A., Medlyn, B. E., Reich, P. B., Leigh, A., Pfautsch, S., Blackman, C. J., López, R., Aspinwall, M. J. & Crous, K. Y. (2018). Trees tolerate an extreme heatwave via sustained transpirational cooling and increased leaf thermal tolerance. *Global Change Biology, 24*(6), 2390–2402.

Drost, M. R. (1987). Relation between aiming and catch success in larval fishes. *Canadian Journal of Fisheries and Aquatic Sciences, 44*, 304–315.

Drost, M. R., Osse, J. W. M. & Muller, M. (1988). Prey capture by fish larvae, waterflow patterns and the effect of escape movements of prey. *Netherlands Journal of Zoology, 38*, 23–45.

Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R. & Mearns, L. O. (2000). Climate extremes: Observations, modeling, and impacts. *Science, 289*(5487), 2068–2074.

Ebert, M. S. & Sharp, P. A. (2012). Roles for microRNAs in conferring robustness to biological processes. *Cell, 149*(3), 515–524.

Eirin-Lopez, J. M. & Putnam, H. M. (2019). Marine environmental epigenetics. *Annual Review of Marine Sciences, 11*, 335–369.

Eme, J., Mueller, C.A., Lee, A.H., Melendez, C., Manzon, R.G., Somers, C.M., Boreham, D.R. & Wilson, J.Y. (2018). Daily, repeating fluctuations in embryonic incubation temperature alter metabolism and growth of Lake whitefish (*Coregonus clupeaformis*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 226*, 49–56.

Enright, A., John, B., Gaul, U., Tuschl, T., Sander, C. & Marks, D. (2003). MicroRNA targets in Drosophila. *Genome biology, 4*, 1–27.

Ern, R., Andreassen, A. H. & Jutfelt, F. (2023). Physiological mechanisms of acute upper thermal tolerance in fish. *Physiology, 38*(3), 141–158.

Estay, S. A., Clavijo-Baquet, S., Lima, M. & Bozinovic, F. (2011). Beyond average: an experimental test of temperature variability on the population dynamics of *Tribolium confusum*. *Population Ecology, 53*, 53–58.

Fangue, N. A., Hofmeister, M. & Schulte, P. M. (2006). Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. *Journal of Experimental Biology, 209*(15), 2859–2872.

Feldmeth, C. R., Stone, E. A. & Brown, J. H. (1974). An increased scope for thermal tolerance upon acclimating pupfish (Cyprinodon) to cycling temperatures. *Journal of Comparative Physiology, 89*(1), 39–44.

Feugere, L., Scott, V. F., Rodriguez-Barucg, Q., Beltran-Alvarez, P. & Wollenberg Valero, K. C. (2021). Thermal stress induces a positive phenotypic and molecular feedback loop in zebrafish embryos. *Journal of Thermal Biology, 102*, 103114.

Flodmark, L. E. W., VØllestad, L. A. & Forseth, T. (2004). Performance of juvenile brown trout exposed to fluctuating water level and temperature. *Journal of Fish Biology, 65*(2), 460–470.

Friedman, R. C., Farh, K. K. H., Burge, C. B. & Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome research, 19*(1), 92–105.

Georgakilas, G., Vlachos, I. S., Paraskevopoulou, M. D., Yang, P., Zhang, Y., Economides, A. N. & Hatzigeorgiou, A. G. (2014). microTSS: accurate microRNA transcription start site

identification reveals a significant number of divergent pri-miRNAs. *Nature communications*, *5*(1), 5700.

Gerber, L., Clow, K. A., Mark, F. C. & Gamperl, A. K. (2020). Improved mitochondrial function in salmon (*Salmo salar*) following high temperature acclimation suggests that there are cracks in the proverbial 'ceiling'. *Scientific Reports, 10*(1), 21636.

Gisbert, E., Conklin, D. B. & Piedrahita, R. H. (2004). Effects of delayed first feeding on the nutritional condition and mortality of California halibut larvae. *Journal of Fish Biology, 64*(1), 116–132.

Gilchrist, G. W. (1995). Specialists and generalists in changing environments. I. Fitness landscapes of thermal sensitivity. *The American Naturalist, 146*(2), 252–270.

Grenchik, M. K., Donelson, J. M., Munday, P. L. (2013). Evidence for developmental thermal acclimation in the damselfish, *Pomacentrus moluccensis*. *Coral Reefs, 32*, 85–90.

Griffiths, B. B., Ouyang, Y. B., Xu, L., Sun, X., Giffard, R. G. & Stary, C. M. (2019). Postinjury inhibition of miR-181a promotes restoration of hippocampal CA1 neurons after transient forebrain ischemia in rats. *Eneuro, 6*(4), 0002.

Grimson, A., Farh, K. K. H., Johnston, W. K., Garrett-Engele, P., Lim, L. P. & Bartel, D. P. (2007). MicroRNA targeting specificity in mammals: Determinants beyond seed pairing. *Molecular cell, 27*(1), 91–105.

Guderley H. (2004). Metabolic responses to low temperature in fish muscle. *Biological Reviews, 79*, 409–427.

Guo, H., Ingolia, N. T., Weissman, J. S. & Bartel, D. P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature, 466*(7308), 835–840.

Ha, M. & Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nature reviews Molecular cell biology, 15*(8), 509–524.

Hallare, A. V., Schirling, M., Luckenbach, T., Köhler, H. R. & Triebskorn, R. (2005). Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. *Journal of Thermal Biology, 30*(1), 7–17.

Heinrichs-Caldas, W., Ikert, H., Almeida-Val, V. M. F. & Craig, P. M. (2023). Sex matters: Gamete-specific contribution of microRNA following parental exposure to hypoxia in zebrafish. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 47*, 101090.

Hill, R. W., Wyse, G. A., Anderson, M. & Anderson, M. (2012). Thermal Relations. In *Animal physiology* (ed. A. Sinauer), vol. 2, pp. 419-454. Sunderland, MA: Sinauer associates.

Hochachka, P. W. & Somero, G. N. (2002). *Biochemical adaptation: Mechanism and process in physiological evolution.* Oxford, UK: Oxford University Press.

Hunt von Herbing, I. & Pan, F. T. (2022). Multiple stressors, allostasis and metabolic scaling in developing zebrafish. *Journal of Experimental Biology, 225*(20), jeb244095.

Iannaccone, M., Cosenza, G., Pauciullo, A., Garofalo, F., Proroga, Y. T., Capuano, F. & Capparelli, R. (2018). Milk microRNA-146a as a potential biomarker in bovine tuberculosis. *Journal Of Dairy Research, 85*(2), 178–180.

Iftikar, F. I., Morash, A. J., Cook, D. G., Herbert, N. A. & Hickey, A. J. R. (2015). Temperature acclimation of mitochondria function from the hearts of a temperate wrasse (*Notolabrus celidotus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 184*, 46–55.

Ikert, H. & Craig, P. M. (2020). Chronic exposure to venlafaxine and increased water temperature reversibly alters microRNA in zebrafish gonads (*Danio rerio*). *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *33*, 100634.

Ikert, H. (2021). Impacts of acute and anthropogenic stress on fish microRNA. PhD thesis, University of Waterloo, Waterloo, ON.

Ikert, H., Lynch, M. D., Doxey, A. C., Giesy, J. P., Servos, M. R., Katzenback, B. A. & Craig, P. M. (2021). High throughput sequencing of microRNA in rainbow trout plasma, mucus, and surrounding water following acute stress. *Frontiers in Physiology, 11*, 588313.

Iliopoulos, D., Hirsch, H. A. & Struhl, K. (2009). An epigenetic switch involving NF-κB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell, 139*(4), 693–706.

Imholt, C., Malcolm, I. A., Bacon, P. J., Gibbins, C. N., Soulsby, C., Miles, M. & Fryer, R. J. (2011). Does diurnal temperature variability affect growth in juvenile Atlantic salmon *Salmo salar*? *Journal of Fish Biology, 78*(2), 436–448.

Imsland, A. K., Sunde, L. M., Folkvord, A. & Stefansson, S. O. (1996). The interaction of temperature and fish size on growth of juvenile turbot. *Journal of Fish Biology, 49*, 926–940.

Indrieri, A., Carrella, S., Romano, A., Spaziano, A., Marrocco, E., Fernandez-Vizarra, E., Barbato, S., Pizzo, M., Ezhova, Y., Golia, F.M. & Ciampi, L. (2019). miR-181a/b downregulation exerts a protective action on mitochondrial disease models. *EMBO Molecular Medicine, 11*(5), e8734.

Intergovernmental Panel on Climate Change (IPCC). (2021). *Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*. New York (NY): Cambridge University Press.

Iqbal, K., Tran, D. A., Li, A. X., Warden, C., Bai, A. Y., Singh, P., Wu, X., Pfeifer, G. P. & Szabó, P. E. (2015). Deleterious effects of endocrine disruptors are corrected in the mammalian germline by epigenome reprogramming. *Genome Biology, 16*(1), 59.

Iwama, G. K., Thomas, P. T., Forsyth, R. B. & Vijayan, M. M. (1998). Heat shock protein expression in fish. *Reviews in Fish Biology and Fisheries, 8*, 35–56.

Jayasundara, N., Tomanek, L., Dowd, W. W. & Somero, G. N. (2015). Proteomic analysis of cardiac response to thermal acclimation in the eurythermal goby fish *Gillichthys mirabilis*. *Journal of Experimental Biology,* 218, 1359–1372.

Jesus, T. F., Grosso, A. R., Almeida-Val, V. M. F. & Coelho, M. M. (2016). Transcriptome profiling of two Iberian freshwater fish exposed to thermal stress. *Journal of Thermal Biology, 55*, 54–61.

Jeyachandran, S., Chellapandian, H., Park, K. & Kwak, I. S. (2023). A review on the involvement of heat shock proteins (extrinsic chaperones) in response to stress conditions in aquatic organisms. *Antioxidants, 12*(7), 1444.

Jiang, L., Zhang, J., Wang, J.-J., Wang, L., Zhang, L., Li, G., Yang, X., Ma, X., Sun, X., Cai, J., Zhang, J., Huang, X., Yu, M., Wang, X., Liu, F., Wu, C.-I., He, C., Zhang, B., Ci, W. & Liu, J. (2013). Sperm, but not oocyte, DNA methylome is inherited by zebrafish early embryos. *Cell, 153*(4), 773–784.

Jodar, M., Selvaraju, S., Sendler, E., Diamond, M. P. & Krawetz, S. A. (2013). The presence, role and clinical use of spermatozoal RNAs. *Human reproduction update, 19(*6), 604–624.

Johnston, I. A. & Maitland, B. (1980). Temperature acclimation in crucian carp, Carassius carassius L., morphometric analyses of muscle fibre ultrastructure. *Journal of Fish Biology, 17,* 113–125.

Johnston, I. A. (1982). Capillarisation, oxygen diffusion distances and mitochondrial content of carp muscles following acclimation to summer and winter temperatures. *Cell and Tissue Research, 222,* 325–337.

Johnston, I. A., Lee, H.-T., Macqueen, D. J., Paranthaman, K., Kawashima, C., Anwar, A., Kinghorn, J. R. & Dalmay, T. (2009). Embryonic temperature affects muscle fibre recruitment in adult zebrafish: Genome-wide changes in gene and microRNA expression associated with the transition from hyperplastic to hypertrophic growth phenotypes. *Journal of Experimental Biology, 212*(12), 1781–1793.

Kefford, B. J., Ghalambor, C. K., Dewenter, B., Poff, N. L., Hughes, J., Reich, J. & Thompson, R. (2022). Acute, diel, and annual temperature variability and the thermal biology of ectotherms. *Global Change Biology, 28*(23), 6872–6888.

Kern, P., Cramp, R. L. & Franklin, C. E. (2015). Physiological responses of ectotherms to daily temperature variation. *Journal of Experimental Biology, 218*(19), 3068–3076.

Khelifa, R., Blanckenhorn, W. U., Roy, J., Rohner, P. T. & Mahdjoub, H. (2019). Usefulness and limitations of thermal performance curves in predicting ectotherm development under climatic variability. *Journal of Animal Ecology, 88*(12), 1901–1912.

Kim, S. S., Lee, C. J., Yoo, H. K., Choi, J., Byun, S. G., Kim, W. J., Lim, H. J. & Park, J. S. (2022). Effect of water temperature on walleye pollock (*Gadus chalcogrammus*) embryos, larvae and juveniles: survival, HSP70 expression, and physiological responses. *Aquaculture, 554*, 738136.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental dynamics, 203*(3), 253–310.

Kingsolver, J. G., Higgins, J. K. & Augustine, K. E. (2015). Fluctuating temperatures and ectotherm growth: distinguishing non-linear and time-dependent effects. *Journal of Experimental Biology, 218*(14), 2218–2225.

Kinouchi, T., Yagi, H. & Miyamoto, M. (2007). Increase in stream temperature related to anthropogenic heat input from urban wastewater. *Journal of Hydrology, 335*(1), 78–88.

Kleiber, M. (1932). Body size and metabolism. *Hilgardia, 6*, 315–353.

Kobayashi, T., Goto, K. & Miyazaki, T. (2000). Pathological changes caused by cold-water stress in Japanese eel *Anguilla japonica. Diseases of aquatic organisms, 40*(1), 41–50.

Kraffe E., Marty, Y. & Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids and their fatty acid compositions. *Journal of Experimental Biology, 210,* 149–165.

Kristensen, T. N., Ketola, T. & Kronholm, I. (2020). Adaptation to environmental stress at different timescales. *Annals of the New York Academy of Sciences, 1476*(1), 5–22.

Krone, P. H., Lele, Z. & Sass, J. B. (1997). Heat shock genes and the heat shock response in zebrafish embryos. *Biochemistry and Cell Biology, 75*, 487-497.

Lai, K. P., Li, J. W., Tse, A. C. K., Chan, T. F. & Wu, R. S. S. (2016). Hypoxia alters steroidogenesis in female marine medaka through miRNAs regulation. *Aquatic Toxicology, 172*, 1–8.

Łaszewski, M. (2017). Human impact on spatial water temperature variability in lowland rivers: A case study from central poland. *Polish Journal of Environmental Studies, 27*(1), 191–200.

Lau, K., Lai, K. P., Bao, J. Y. J., Zhang, N., Tse, A., Tong, A., Li, J. W., Lok, S., Kong, R. Y. C., Lui, W. Y. & Wu, R. S. S. (2014). Identification and expression profiling of microRNAs in the brain, liver and gonads of marine medaka (*Oryzias melastigma*) and in response to hypoxia. *PloS One, 9*(10), e110698.

Lee, R. C., Feinbaum, R. L. & Ambros, V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell, 75*, 843–854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Rådmark, O., Kim, S. & Kim, V.N. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature, 425*(6956), 415–419.

Lee, Y., Jeon, K., Lee, J. T., Kim, S. & Kim, V. N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. *The EMBO journal, 21*, 4663–4670.

Lein, I., Holmefjord, I. & Rye, M. (1997). Effects of temperature on yolk sac larvae of Atlantic halibut (*Hippoglossus hippoglossus L*.). *Aquaculture, 157*, 123–135.

Lele, Z., Engel, S. & Krone, P. H. (1997). hsp47 and hsp70 gene expression is differentially regulated in a stress-and tissue-specific manner in zebrafish embryos. *Developmental genetics, 21*(2), 123–133.

Lenth, R., Singmann, H., Love, J., Buerkner, P. & Herve, M. (2018). Package "Emmeans". R Package Version 4.0-3. http://cran.r-project.org/package=emmeans

Leung, A. K. L., & Sharp, P. A. (2010). MicroRNA Functions in Stress Responses. *Molecular Cell, 40*(2), 205–215.

Li, A. J., Leung, P. T., Bao, V. W., Lui, G. C., & Leung, K. M. (2015). Temperature-dependent physiological and biochemical responses of the marine medaka *Oryzias melastigma* with consideration of both low and high thermal extremes. *Journal Of Thermal Biology, 54*, 98–105.

Li, Q.J., Chau, J., Ebert, P.J., Sylvester, G., Min, H., Liu, G., Braich, R., Manoharan, M., Soutschek, J., Skare, P. & Klein, L.O. (2007). miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell, 129*(1), 147–161.

Li, S., Guo, H., Chen, Z., Jiang, Y., Shen, J., Pang, X. & Li, Y. (2021). Effects of acclimation temperature regime on the thermal tolerance, growth performance and gene expression of a cold-water fish, *Schizothorax prenanti*. *Journal of Thermal Biology, 98*, 102918.

Li, X., Cassidy, J. J., Reinke, C. A., Fischboeck, S. & Carthew, R. W. (2009). A microRNA imparts robustness against environmental fluctuation during development. *Cell, 137*(2), 273–282.

Lim, M. Y. T. & Bernier, N. J. (2022). Zebrafish parental progeny investment in response to cycling thermal stress and hypoxia: deposition of heat shock proteins but not cortisol. *Journal of Experimental Biology, 225*(21), jeb244715.

Lindeman, L. C., Andersen, I. S., Reiner, A. H., Li, N., Aanes, H., Østrup, O., Winata, C., Mathavan, S., Müller, F., Aleström, P. & Collas, P. (2011). Prepatterning of developmental gene expression by modified histones before zygotic genome activation. *Developmental Cell, 21*(6), 993–1004.

Lindquist, S. & Craig, E. A. (1988). The heat-shock proteins. *Annual review of genetics, 22*(1), 631–677.

Liu, X., Hong, L., Peng, W., Jiang, J., Peng, Z. & Yang, J. (2019). The Neuroprotective Effect of miR-181a After Oxygen–Glucose Deprivation/Reperfusion and the Associated Mechanism. *Journal of Molecular Neuroscience, 68*, 261-274.

Liu, Q., Yang, J., Gong, Y., Cai, J., Zheng, Y., Zhang, Y., Yu, D. & Zhang, Z. (2020). MicroRNA profiling identifies biomarkers in head kidneys of common carp exposed to cadmium. *Chemosphere, 247*, 125901.

Liu, E., Zhao, X., Li, C., Wang, Y., Li, L., Zhu, H. & Ling, Q. (2022b). Effects of acute heat stress on liver damage, apoptosis and inflammation of pikeperch (*Sander lucioperca*). *Journal of thermal biology, 106*, 103251.

Liu, R., Liu, R., Song, G., Li, Q., Cui, Z., & Long, Y. (2022c). Mitochondria dysfunction and cell apoptosis limit resistance of nile tilapia (*Oreochromis niloticus*) to lethal cold stress. *Animals, 12*(18), 2382.

Liu, X., Yang, Y. Y. & Wang, Y. (2022d). HSP90 and Aha1 modulate microRNA maturation through promoting the folding of Dicer1. *Nucleic Acids Research, 50*(12), 6990-7001.

Liu, Z., Ma, F., Kang, Y. & Liu, X. (2022a). Gene ssa-miR-301a-3p improves rainbow trout (*Oncorhynchus mykiss*) resistance to heat stress by targeting hsp90b2. *PeerJ, 10*, e13476.

López-Maury, L., Marguerat, S. & Bähler, J. (2008). Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. *Nature Reviews Genetics, 9*(8), 583-593.

Lucore, E. C. & Connaughton, V. P. (2021). Observational learning and irreversible starvation in first-feeding zebrafish larvae: is it okay to copy from your friends? *Zoology, 145*, 125896.

Luo, B., Groenke, K., Takors, R., Wandrey, C. & Oldiges, M. (2007). Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography–mass spectrometry. *Journal of chromatography A, 1147*(2), 153-164.

Luo, Y. & Xie, X. (2008). Effects of temperature on the specific dynamic action of the southern catfish, *Silurus meridionalis*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 149*(2), 150-156.

Ma, Y., Zang, L., Wang, D., Jiang, J., Wang, C., Wang, X., Fang, F. & Wang, H. (2019). Effects of miR-181a-5p abnormal expression on zebrafish (*Danio rerio*) vascular development following triclosan exposure. *Chemosphere, 223*, 523-535.

Madeira, D., Narciso, L., Cabral, H. N., Vinagre, C. & Diniz, M. S. (2013). Influence of temperature in thermal and oxidative stress responses in estuarine fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 166*(2), 237–243.

Madeira, D., Vinagre, C., Costa, P. M. & Diniz, M. S. (2014). Histopathological alterations, physiological limits, and molecular changes of juvenile *Sparus aurata* in response to thermal stress. *Marine Ecology Progress Series*, *505*, 253–266.

Major, K.M., DeCourten, B. M., Li, J., Britton, M., Settles, M. L., Mehinto, A. C., Connon, R. E. & Brander, S. M. (2020). Early life exposure to environmentally relevant levels of endocrine disruptors drive multigenerational and transgenerational epigenetic changes in a fish model. *Frontiers in Marine Science, 7*, 471.

Martin, L. B., Liebl, A. L., Trotter, J. H., Richards, C. L., McCoy, K. & McCoy, M. W. (2011). Integrators: physiological determinants of phenotypic plasticity. *Integrative and Comparative Biology, 51*, 514–27.

Martin, S. W., Meek, A. H. & Willeberg, P. (1987). Measurement of disease frequency and production. In *Veterinary epidemiology: Principles and methods*, pp. 73–76. Ames, IA: Iowa State University Press.

Massey, M. D. & Dalziel, A. C. (2023). Parental early life environments drive transgenerational plasticity of offspring metabolism in a freshwater fish (*Danio rerio*). *Biology Letters*, *19*(10), 20230266.

Massey, M. D. & Hutchings, J. A. (2021). Thermal variability during ectotherm egg incubation: a synthesis and framework. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *335*(1), 59–71.

Massey, M. D. B. (2023). Phenotypic plasticity in response to thermal variability within and across generations. PhD thesis, Dalhousie University, Halifax, NS.

Massey, M. D., Fredericks, M. K., Malloy, D., Arif, S. & Hutchings, J. A. (2022). Differential reproductive plasticity under thermal variability in a freshwater fish (*Danio rerio*). *Proceedings of the Royal Society B, 289*(1982), 20220751.

Mayr, C., Hemann, M. T. & Bartel, D. P. (2007). Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science, 315*(5818), 1576-1579.

McGeary, S. E., Lin, K. S., Shi, C. Y., Pham, T. M., Bisaria, N., Kelley, G. M. & Bartel, D. P. (2019). The biochemical basis of microRNA targeting efficacy. *Science, 366*(6472), eaav1741.

Meehl, G. A. & Tebaldi, C. (2004). More intense, more frequent, and longer lasting heat waves in the 21st century. *Science, 305*(5686), 994–997.

Mhanni, A. A. & McGowan, R. A. (2004). Global changes in genomic methylation levels during early development of the zebrafish embryo. *Development Genes and Evolution, 214*(8), 412–417.

Morash, A. J., Neufeld, C., MacCormack, T. J. & Currie, S. (2018). The importance of incorporating natural thermal variation when evaluating physiological performance in wild species. *Journal of Experimental Biology, 221*(14), jeb164673.

Morash, A. J., Speers-Roesch, B., Andrew, S. & Currie, S. (2021). The physiological ups and downs of thermal variability in temperate freshwater ecosystems. *Journal of Fish Biology, 98,* 1524–1535.

Morgan, R., Finnøen, M.H. & Jutfelt, F. (2018). CTmax is repeatable and doesn't reduce growth in zebrafish. *Scientific reports, 8*(1), 7099.

Morgan, R., Sundin, J., Finnøen, M. H., Dresler, G., Vendrell, M. M., Dey, A., Sarkar, K. & Jutfelt, F. (2019). Are model organisms representative for climate change research? Testing thermal tolerance in wild and laboratory zebrafish populations. *Conservation Physiology, 7,* 1–11.

Niehaus, A. C., Angilletta Jr, M. J., Sears, M. W., Franklin, C. E. & Wilson, R. S. (2012). Predicting the physiological performance of ectotherms in fluctuating thermal environments. *Journal of Experimental Biology, 215*(4), 694–701.

Nuez-Ortín, W. G., Carter, C. G., Nichols, P. D., Cooke, I. R., Wilson, R. (2018). Liver proteome response of pre-harvest Atlantic salmon following exposure to elevated temperature. *BMC Genomics, 19,* 1–13.

O'Dea, R. E., Noble, D. W. A., Johnson, S. L., Hesselson, D. & Nakagawa, S. (2016). The role of non-genetic inheritance in evolutionary rescue: Epigenetic buffering, heritable bet hedging and epigenetic traps. *Environmental Epigenetics, 2,* dvv014.

Oikawa, S., & Itazawa, Y. (2003). Relationship between summated tissue respiration and body size in a marine teleost, the porgy *Pagrus major*. *Fisheries science, 69*(4), 687–694.

Oikawa, S., Mendez, A. J., Oram, J. F., Bierman, E. L. & Cheung, M. C. (1993). Effects of high-density lipoprotein particles containing apo AI, with or without apo A-II, on intracellular cholesterol efflux. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 1165*(3), 327–334.

Okamura, A., Yamada, Y., Horie, N., Utoh, T., Mikawa, N., Tanaka, S. & Tsukamoto, K. (2007). Effects of water temperature on early development of Japanese eel *Anguilla japonica*. *Fisheries Science, 73,* 1241–1248.

Paaijmans, K. P., Heinig, R. L., Seliga, R. A., Blanford, J. I., Blanford, S., Murdock, C. C. & Thomas, M. B. (2013). Temperature variation makes ectotherms more sensitive to climate change. *Global Change Biology, 19,* 2373–2380.

Pan, T. C. F. & Hunt von Herbing, I. (2017). Metabolic plasticity in development: Synergistic responses to high temperature and hypoxia in zebrafish, *Danio rerio*. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology, 327*(4), 189–199.

Pankhurst, N. W. & Munday, P. L. (2011). Effects of climate change on fish reproduction and early life history stages. *Marine and Freshwater Research, 62*(9), 1015–1026.

Paradis, A. R., Pepin, P. & Brown, J. A. (1996). Vulnerability of fish eggs and larvae to predation: review of the influence of the relative size of prey and predator. *Canadian Journal of Fisheries and Aquatic Sciences, 53*(6), 1226–1235.

Parra, G. & Yúfera, M. (2000). Feeding, physiology and growth responses in first-feeding gilthead seabream (*Sparus aurata L.*) larvae in relation to prey density. *Journal of Experimental Marine Biology and Ecology, 243*(1), 1–15.

Penman, R. J., Bugg, W., Rost-Komiya, B., Earhart, M. L. & Brauner, C. J. (2023). Slow heating rates increase thermal tolerance and alter mRNA HSP expression in juvenile white sturgeon (*Acipenser transmontanus*). *Journal of Thermal Biology, 115*, 103599.

Pfennig, D. W., Wund, M. A., Snell-Rood, E. C., Cruickshank, T., Schlichting, C. D. & Moczek, A. P. (2010). Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology & Evolution, 25*, 459–467.

Podrabsky, J. E. & Somero, G. N. (2004). Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus. Journal of Experimental Biology, 207*(13), 2237–2254.

Politis, S.N., Mazurais, D., Servili, A., Zambonino-Infante, J.L., Miest, J.J., Sørensen, S.R., Tomkiewicz, J. & Butts, I.A. (2017). Temperature effects on gene expression and morphological development of European eel, *Anguilla anguilla* larvae. *PLoS One, 12*(8), e0182726.

Porter, S. M. & Bailey, K. M. (2007). The effect of early and late hatching on the escape response of walleye pollock (*Theragra chalcogramma*) larvae. *Journal Of Plankton Research, 29*(3), 291–300.

Pörtner, H.O. (2021). Climate impacts on organisms, ecosystems and human societies: Integrating OCLTT into a wider context. *Journal of Experimental Biology, 224*, jeb238360.

Pottier, P., Burke, S., Zhang, R. Y., Noble, D. W., Schwanz, L. E., Drobniak, S. M. & Nakagawa, S. (2022). Developmental plasticity in thermal tolerance: Ontogenetic variation, persistence, and future directions. *Ecology Letters, 25*(10), 2245–2268.

Precht, H. (1958). Concepts of the temperature adaptation of unchanging reaction systems of cold-blooded animals. In *Physiological adaptation* (ed. C. L. Prosser), pp. 50–77. Washington, DC: American Physiological Society.

Qiang, J., Tao, Y. F., He, J., Xu, P., Bao, J. W. & Sun, Y. L. (2017). miR-122 promotes hepatic antioxidant defense of genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*) exposed to cadmium by directly targeting a metallothionein gene. *Aquatic Toxicology, 182*, 39–48.

Radford, E. J., Ito, M., Shi, H., Corish, J. A., Yamazawa, K., Isganaitis, E., Seisenberger, S., Hore, T. A., Reik, W., Erkek, S., Peters, A. H. F. M., Patti, M.-E. & Ferguson-Smith, A. C. (2014). In utero undernourishment perturbs the adult sperm methylome and is linked to metabolic disease transmission. *Science, 345*(6198), 1255903.

Raptis, C. E., van Vliet, M. T. & Pfister, S. (2016). Global thermal pollution of rivers from thermoelectric power plants. *Environmental Research Letters, 11*(10), 104011.

Raza, S. H. A., Abdelnour, S. A., Alotaibi, M. A., AlGabbani, Q., Naiel, M. A., Shokrollahi, B., Noreldin, A. E., Jahejo, A. R., Shah, M. A., Alagawany, M. & Zan, L. (2022). MicroRNAs mediated environmental stress responses and toxicity signs in teleost fish species. *Aquaculture, 546*, 737310.

Reed, T. E., Waples, R. S., Schindler, D. E., Hard, J. J. & Kinnison, M. T. (2010). Phenotypic plasticity and population viability: the importance of environmental predictability. *Proceedings of the Royal Society B: Biological Sciences, 277*(1699), 3391–3400.

Rizzo, B. & Wiken, E. D. (1992). Assessing the sensitivity of Canada's ecosystems to climatic change. *Climatic Change, 21*(1), 37–55.

Robjant, D. (2014). Nauseating Flux: Iris Murdoch on Sartre and Heraclitus. *European Journal of Philosophy, 22*(4), 633–652.

Roznik, E. A. & Alford, R. A. (2012). Does waterproofing Thermochron iButton dataloggers influence temperature readings? *Journal of Thermal Biology, 37*(4), 260–264.

Ruel, J. J. & Ayres, M. P. (1999). Jensen's inequality predicts effects of environmental variation. *Trends in Ecology & Evolution, 14*(9), 361–366.

Rummukainen, M. (2012). Changes in climate and weather extremes in the 21st century. *Wiley Interdisciplinary Reviews: Climate Change, 3*(2), 115–129.

Sandblom, E., Clark, T.D., Gräns, A., Ekström, A., Brijs, J., Sundström, L.F., Odelström, A., Adill, A., Aho, T. & Jutfelt, F. (2016). Physiological constraints to climate warming in fish follow principles of plastic floors and concrete ceilings. *Nature communications, 7*(1), 11447.

Sandersfeld, T., Mark, F.C. & Knust, R. (2017). Temperature-dependent metabolism in Antarctic fish: Do habitat temperature conditions affect thermal tolerance ranges?. *Polar Biology, 40*, 141–149.

Saravia, J., Paschke, K., Oyarzún-Salazar, R., Cheng, C. C., Navarro, J. M. & Vargas-Chacoff, L. (2021). Effects of warming rates on physiological and molecular components of response to CTMax heat stress in the Antarctic fish *Harpagifer antarcticus*. *Journal of Thermal Biology, 99*, 103021.

Schaefer, J. & Ryan, A. (2006). Developmental plasticity in the thermal tolerance of zebrafish *Danio rerio. Journal of Fish Biology, 69*(3), 722–734.

Schirone, R. C. & Gross, L. (1968). Effect of temperature on early embryological development of the zebra fish, *Brachydanio rerio*. *Journal of Experimental Zoology, 169*, 43–52.

Schnurr, M. E., Yin, Y. & Scott, G. R. (2014). Temperature during embryonic development has persistent effects on metabolic enzymes in the muscle of zebrafish. *Journal of Experimental Biology, 217*, 1370–1380.

Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *Journal of Experimental Biology, 218*(12), 1856-1866.

Scott, G. R. & Johnston, I. A. (2012). Temperature during embryonic development has persistent effects on thermal acclimation capacity in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 14247–14252.

Seebacher, F., Brand, M. D., Else, P. L., Guderley, H., Hulbert, A. J. & Moyes, C. D. (2010). Plasticity of oxidative metabolism in variable climates: molecular mechanisms. *Physiological and Biochemical Zoology, 83*(5), 721–732.

Seebacher, F., White, C. & Franklin, C. (2015). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nature Climate Change, 5*, 61–66.

Shafland, P. L. & Pestrak, J. M. (1982). Lower lethal temperatures for fourteen non-native fishes in Florida. *Environmental Biology Of Fishes, 7*, 149–156.

Skinner, M. K. (2011). Role of epigenetics in developmental biology and transgenerational inheritance. *Birth Defects Research Part C: Embryo Today: Reviews, 93*(1), 51–55.

Song, X., Xue, Y. & Cai, H. (2021). Down-regulation of miR-181a-5p prevents cerebral ischemic injury by upregulating En2 and activating Wnt/β-catenin pathway. *Journal of Stroke and Cerebrovascular Diseases, 30*(3), 105485.

Spence, R., Gerlach, G., Lawrence, C. & Smith, C. (2008). The behavior and ecology of the zebrafish, *Danio rerio. Biological reviews of the Cambridge Philosophical Society, 83*, 13–34.

Stary, C. M., Xu, L., Li, L., Sun, X., Ouyang, Y. B., Xiong, X., Zhao, J. & Giffard, R. G. (2017). Inhibition of miR-181a protects female mice from transient focal cerebral ischemia by targeting astrocyte estrogen receptor-α. *Molecular and Cellular Neuroscience, 82*, 118–125.

Steel, E. A. & Lange, I. A. (2007). Using wavelet analysis to detect changes in water temperature regimes at multiple scales: Effects of multi-purpose dams in the Willamette River basin. *River Research and Applications, 23*(4), 351–359.

Stefanovic, D. I., Manzon, L. A., McDougall, C. S., Boreham, D. R., Somers, C. M., Wilson, J. Y. & Manzon, R. G. (2016). Thermal stress and the heat shock response in embryonic and young of the year juvenile lake whitefish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 193*, 1–10.

Takahashi, S., Izawa, Y. & Suzuki, N. (2012). Astroglial pentose phosphate pathway rates in response to high-glucose environments. *ASN neuro, 4*(2), AN20120002.

Tang, H. Y., Smith-Caldas, M. S., Driscoll, M. V., Salhadar, S. & Shingleton, A. W. (2011). FOXO regulates organ-specific phenotypic plasticity in Drosophila. *PLoS genetics, 7*(11), e1002373.

Terblanche, J. S. & Chown, S. L. (2006). The relative contributions of developmental plasticity and adult acclimation to physiological variation in the tsetse fly, *Glossina pallidipes* (Diptera, Glossinidae). *Journal of Experimental Biology, 209*, 1064–1073.

Thatcher, E. J., Bond, J., Paydar, I. & Patton, J. G. (2008). Genomic Organization of Zebrafish microRNAs. *BMC Genomics, 9*(1), 253.

Theilacker, G. H. (1978). Effect of starvation on the histological and morphological characteristics of jack mackerel, *Trachurus symmetricus*, larvae. *Fishery bulletin, 76*(2), 403–414.

Tirsgaard, B., Svendsen, J. C. & Steffensen, J. F. (2015). Effects of temperature on specific dynamic action in Atlantic cod *Gadus morhua*. *Fish physiology and biochemistry, 41*, 41–50.

Turchinovich, A., Tonevitsky, A. G. & Burwinkel, B. (2016). Extracellular miRNA: a collision of two paradigms. *Trends in biochemical sciences, 41*(10), 883–892.

Turko, A. J., Firth, B. L., Craig, P. M., Eliason, E. J., Raby, G. D. & Borowiec, B. G. (2023). Physiological differences between wild and captive animals: a century-old dilemma. *Journal of Experimental Biology, 226*(23), jeb246037.

Vagner, M., Zambonino-Infante, J. L. & Mazurais, D. (2019). Fish facing global change: are early stages the lifeline? *Marine Environmental Research, 147*, 159–178.

van Gelderen, T. A., Montfort, J., Álvarez-Dios, J. A., Thermes, V., Piferrer, F., Bobe, J. & Ribas, L. (2022). Deciphering sex-specific miRNAs as heat-recorders in zebrafish. *Scientific Reports, 12*(1), 18722.

Vasseur, D. A., DeLong, J. P., Gilbert, B., Greig, H. S., Harley, C. D. G., McCann, K. S., Savage, V., Tunney, T. D. & O'Connor, M. I. (2014). Increased temperature variation poses a greater risk to species than climate warming. *Proceedings of the Royal Society B: Biological Sciences, 281*(1779), 20132612.

Vazquez, D., Gianoli, E., Morris, W. & Bozinovic, F. (2017). Evolutionary and ecological impacts of increased climatic variability. *Biological Reviews, 92*, 22–420.

Venney, C.J., Cayuela, H., Rougeux, C., Laporte, M., Mérot, C., Normandeau, E., Leitwein, M., Dorant, Y., Præbel, K., Kenchington, E. & Clément, M. (2023). Genome-wide DNA methylation predicts environmentally driven life history variation in a marine fish. *Evolution, 77*(1), 186–198.

Verbueken, E., Bars, C., Ball, J.S., Periz-Stanacev, J., Marei, W.F., Tochwin, A., Gabriëls, I.J., Michiels, E.D., Stinckens, E., Vergauwen, L. and Knapen, D., 2018. From mRNA expression of drug disposition genes to in vivo assessment of CYP-mediated biotransformation during zebrafish embryonic and larval development. International journal of molecular sciences, 19(12), 3976.

Verheyen, J. & Stoks, R. (2019). Temperature variation makes an ectotherm more sensitive to global warming unless thermal evolution occurs. *Journal of Animal Ecology, 88*(4), 624–636.

Volkoff, H. & Rønnestad, I. (2020). Effects of temperature on feeding and digestive processes in fish. *Temperature, 7*(4), 307–320.

Wang, C. & Liu, H. (2022). Factors influencing degradation kinetics of mRNAs and half-lives of microRNAs, circRNAs, lncRNAs in blood in vitro using quantitative PCR. *Scientific Reports, 12*, 7259.

Wang, Q., Tan, X., Jiao, S., You, F. & Zhang, P. J. (2014). Analyzing cold tolerance mechanism in transgenic zebrafish (*Danio rerio*). *PloS One, 9*(7), p.e102492.

Wang, T., Wen, X., Hu, Y., Zhang, X., Wang, D. & Yin, S. (2019). Copper nanoparticles induced oxidation stress, cell apoptosis and immune response in the liver of juvenile *Takifugu fasciatus*. *Fish & Shellfish Immunology, 84*, 648–655.

Wang, X., Yin, D., Li, P., Yin, S., Wang, L., Jia, Y. & Shu, X. (2015). MicroRNA-sequence profiling reveals novel osmoregulatory microRNA expression patterns in catadromous eel *Anguilla marmorata*. *PLoS One, 10*, e136383

Weber, A. V., Firth, B. L., Cadonic, I. G. & Craig, P. M. (2023). Interactive effects of venlafaxine and thermal stress on zebrafish (Danio rerio) inflammatory and heat shock responses. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 269*, 109620.

Weiland, M., Gao, X. H., Zhou, L. & Mi, Q. S. (2012). Small RNAs have a large impact: circulating microRNAs as biomarkers for human diseases. *RNA biology, 9*(6), 850–859.

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. Oxford, UK: Oxford University Press.

West-Eberhard, M. J. (2005). Developmental plasticity and the origin of species differences. *Proceedings of the National Academy of Sciences, 102*(1), 6543–6549.

Whiteley, A. R., Bhat, A., Martins, E. P., Mayden, R. L., Arunachalam, M., Uusi-Heikkilä, S., Ahmed, A. T. A., Shrestha, J., Clark, M., Stemple, D. & Bernatchez, L. (2011). Population genomics of wild and laboratory zebrafish (*Danio rerio*). *Molecular ecology*, *20*(20), 4259–4276.

Wightman, B., Ha, I. & Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell*, *75*, 855–862.

Wilson, C. (2012). Aspects of larval rearing. *ILAR Journal*, *53*, 169–178.

Wu, S.-F., Zhang, H. & Cairns, B. R. (2011). Genes for embryo development are packaged in blocks of multivalent chromatin in zebrafish sperm. *Genome Research*, *21*(4), 578–589.

Wu, Y., Xia, Y., Hu, A., Xiong, G., Wu, W., Shi, L., Chen, L., Guo, X., Qiao, Y., Liu, C. & Yin, T. (2024). Difference in muscle metabolism caused by metabolism disorder of rainbow trout liver exposed to ammonia stress. *Science of The Total Environment*, *924*, 171576.

Yan, K., Liu, P., Wu, C. A., Yang, G. D., Xu, R., Guo, Q. H., Huang, J. G. & Zheng, C. C. (2012). Stress-induced alternative splicing provides a mechanism for the regulation of microRNA processing in *Arabidopsis thaliana*. *Molecular cell*, *48*(4), 521–531.

Yang, S. S., Zhao, T. T., Ma, A. J., Huang, Z. H., Liu, Z. F., Cui, W. X., Zhang, J., Zhu, C., Guo, X. & Yuan, C. (2020). Metabolic responses in *Scophthalmus maximus* kidney subjected to thermal stress. *Fish and Shellfish Immunology*, *103*, 37–46.

Yao, H., Li, X., Tang, L., Wang, H., Wang, C., Mu, C. & Shi, C. (2020). Metabolic mechanism of the mud crab (*Scylla paramamosain*) adapting to salinity sudden drop based on GC-MS technology. *Aquaculture reports*, *18*, 100533.

Yi, R., Qin, Y., Macara, I. G. & Cullen, B. R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development*, *17*(24), 3011–3016.

Yin, C., Salloum, F. N. & Kukreja, R. C. (2009). A novel role of microRNA in late preconditioning: Upregulation of endothelial nitric oxide synthase and heat shock protein 70. *Circulation Research*, *104*(5), 572–575.

Yúfera, M., Pascual, E., Polo, A. & Sarasquete, M. C. (1993). Effect of starvation on the feeding ability of gilthead seabream (*Spams aurata* L.) larvae at first feeding. *Journal of Experimental Marine Biology and Ecology*, *169*(2), 259–272.

Yúfera, M. & Darias, M. J. (2007). The onset of exogenous feeding in marine fish larvae. *Aquaculture*, *268*(1-4), 53–63.

Žák, J. & Reichard, M. (2020). Fluctuating temperatures extend median lifespan, improve reproduction and reduce growth in turquoise killifish. *Experimental Gerontology*, *140*, 111073.

Zhang, C. N., Tian, H. Y., Li, X. F., Zhu, J., Cai, D. S., Xu, C., Wang, F., Zhang, D. D. & Liu, W.B. (2014). The effects of fructooligosaccharide on the immune response, antioxidant capability and HSP70 and HSP90 expressions in blunt snout bream (*Megalobrama amblycephala Yih*) under high heat stress. *Aquaculture, 433,* 458–466.

Zhang, Z., Zhou, C., Fan, K., Zhang, L., Liu, Y. & Liu, P. F. (2021). Metabolomics analysis of the effects of temperature on the growth and development of juvenile European seabass (*Dicentrarchus labrax*). *Science of the Total Environment, 769,* 145155.

Zhao, C. Sun, G., Ye, P., Li, S. & Shi, Y. (2013). MicroRNA let-7d regulates the TLX/microRNA-9 cascade to control neural cell fate and neurogenesis. *Scientific Reports, 3,* 1329.

Zhuang, X., Chen, Y., Wu, Z., Xu, Q., Chen, M., Shao, M., Cao, X., Zhou, Y., Xie, M., Shi, Y. & Zeng, Y. (2020). Mitochondrial miR-181a-5p promotes glucose metabolism reprogramming in liver cancer by regulating the electron transport chain. *Carcinogenesis, 41*(7), 972-983.

Zimmer, A. M., Mandic, M., Rourke, K. M. & Perry, S. F. (2020). Breathing with fins: do the pectoral fins of larval fishes play a respiratory role? *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 318*(1), R89–R97.

Appendices

Appendix A
Additional information from statistical tests

Table A.1: Temperature metrics from iButton loggers for each treatment until 30 dpf. The average temperature of each water bath over the duration of the study is listed below, along with the temperature during larval sampling, and the minimum and maximum temperature.

		Temperature (°C)			
Treatment	**Water bath**	**Average over experimental period**	**5 dpf sampling**	**Minimum**	**Maximum**
CTRL	1	27.2	27.5	26.5	28.5
	2	27.7	27.5	26.5	28.5
	3	27.0	27	26.5	27.5
FLUX	1	28.0	24.5	22.5	33.5
	2	28.1	24.5	22.5	33.5
	3	27.9	25	22.5	33.5
HEAT	1	32.1	32	31.5	33.0
	2	32.3	32.5	31.0	33.5
	3	32.5	32.5	32.0	33.0

Table A.2: Dissolved Oxygen (DO) concentration over the 30-day experimental period. The average DO concentration of tanks in each water bath over the duration of the study are listed below, along with the minimum and maximum for each treatment

	DO concentration (mg/L)		
Treatment	**Average over experimental period**	**Minimum**	**Maximum**
CTRL	6.0	5.2	7.2
FLUX	6.0	5.0	7.4
HEAT	5.9	5.0	6.9

Table A.3: One-way ANOVA statistics on mRNA and miRNA expression in zebrafish larvae at 5 days post fertilization (dpf). DFn and DFd represent degrees of freedom numerator and denominator, respectively.

	Treatment effect	
Target	**F(DFn, DFd)**	**P value**
hsp47	F(2,20) = 1.19	0.325
hsp70	F(2,20) = 3.684	0.0434
hsp90	F(2,20) = 5.97	0.0093
let-7d-5p	F(2,21) = 6.187	0.0077
miR-103-3p	F(2,21) = 3.192	0.0620
miR-122	F(2,21) = 0.981	0.391
miR-181a-5p	F(2,21) = 3.956	0.0348
miR-214	F(2,21) = 0.555	0.582

miR-301a-3p	F(2,21) = 9.29	0.0013
miR-499-5p	F(2,21) = 0.829	0.45

Table A.4: One-way ANOVA statistics on mRNA and miRNA expression in zebrafish juveniles at 30 days post fertilization (dpf). DFn and DFd represent degrees of freedom numerator and denominator, respectively.

	Treatment effect	
Target	**F(DFn, DFd)**	**P value**
hsp47	F(2,21) = 0.708	0.504
hsp70	F(2,20) = 0.866	0.436
hsp90	F(2,21) = 0.234	0.794
let-7d-5p	F(2,21) = 0.283	0.756
miR-103-3p	F(2,20) = 0.228	0.798
miR-122	F(2,21) = 1.04	0.371
miR-181a-5p	F(2,20) = 13.74	0.0002
miR-214	F(2,21) = 1.226	0.314
miR-301a-3p	F(2,21) = 0.173	0.842
miR-499-5p	F(2,21) = 2.187	0.137

Table A.5: Results from linear mixed effects (LME) models evaluating the impact of treatment on adult morphometrics and endpoints from critical thermal maxima (CT_{max}) trials at 6 months post fertilization (mpf). Parameters evaluated included condition factor (K), Liver Somatic Index (LSI), body weight of zebrafish, CT_{max}, and agitation temperature (T_{ag}). Family was accounted for in the models as a random variable.

Parameter	**Fixed effect**	**Estimate**	**Standard error**	**df**	**t-value**	**P value**
CT_{max}	Intercept	41.37	0.075	30	551.63	< 0.0001
	Treatment (FLUX)	−0.073	0.106	8	-0.688	0.5107
T_{ag}	Intercept	36.71	0.098	30	376.30	< 0.0001
	Treatment (FLUX)	0.199	0.138	8	1.446	0.5107
LSI	Intercept	1.184	0.108	50	10.91	< 0.0001
	Treatment (FLUX)	0.093	0.153	8	0.607	0.561
K	Intercept	1.551	0.0589	50	26.351	< 0.0001
	Treatment (FLUX)	0.0124	0.083	8	0.149	0.885
Body weight	Intercept	278.07	12.571	50	22.12	< 0.0001
	Treatment (FLUX)	14.233	17.778	8	0.00	0.447

Appendix B
KEGG pathways targeted by miRNAs in this study

Table B.1: Pathways targeted by let-7d-5p. The predicted mRNA targets were derived through miRanda, and inputted into DAVID to group them into functional clusters.

KEGG Identifier	Pathway	Number of mRNA targeted	% of pathway targeted
dre01100	Metabolic pathways	40	7.1
dre04020	Calcium signaling pathway	14	2.5
dre04010	MAPK signaling pathway	14	2.5
dre04810	Regulation of actin cytoskeleton	13	2.3
dre04148	Efferocytosis	12	2.1
dre04510	Focal adhesion	12	2.1
dre04144	Endocytosis	11	2.0
dre05132	Salmonella infection	10	1.8
dre04145	Phagosome	9	1.6
dre04080	Neuroactive ligand-receptor interaction	9	1.6
dre04012	ErbB signaling pathway	8	1.4
dre04270	Vascular smooth muscle contraction	8	1.4
dre04110	Cell cycle	8	1.4
dre04261	Adrenergic signaling in cardiomyocytes	8	1.4
dre04625	C-type lectin receptor signaling pathway	7	1.2
dre04141	Protein processing in endoplasmic reticulum	7	1.2
dre04310	Wnt signaling pathway	7	1.2
dre04530	Tight junction	7	1.2
dre04512	ECM-receptor interaction	6	1.1
dre04912	GnRH signaling pathway	6	1.1
dre04137	Mitophagy - animal	6	1.1
dre04068	FoxO signaling pathway	6	1.1
dre04140	Autophagy - animal	6	1.1
dre00310	Lysine degradation	5	0.9
dre04540	Gap junction	5	0.9
dre04520	Adherens junction	5	0.9
dre04910	Insulin signaling pathway	5	0.9
dre04150	mTOR signaling pathway	5	0.9
dre04814	Motor proteins	5	0.9
dre04370	VEGF signaling pathway	4	0.7

dre04914	Progesterone-mediated oocyte maturation	4	0.7
dre01200	Carbon metabolism	4	0.7
dre03040	Spliceosome	4	0.7
dre04916	Melanogenesis	4	0.7
dre04114	Oocyte meiosis	4	0.7
dre04350	TGF-beta signaling pathway	4	0.7
dre04120	Ubiquitin mediated proteolysis	4	0.7
dre00230	Purine metabolism	4	0.7
dre04514	Cell adhesion molecules	4	0.7
dre04371	Apelin signaling pathway	4	0.7
dre04210	Apoptosis	4	0.7
dre04218	Cellular senescence	4	0.7
dre05168	Herpes simplex virus 1 infection	4	0.7
dre00280	Valine, leucine and isoleucine degradation	3	0.5
dre03420	Nucleotide excision repair	3	0.5
dre01212	Fatty acid metabolism	3	0.5
dre04115	p53 signaling pathway	3	0.5
dre03015	mRNA surveillance pathway	3	0.5
dre01232	Nucleotide metabolism	3	0.5
dre04070	Phosphatidylinositol signaling system	3	0.5
dre03082	ATP-dependent chromatin remodeling	3	0.5
dre04260	Cardiac muscle contraction	3	0.5
dre00190	Oxidative phosphorylation	3	0.5
dre04217	Necroptosis	3	0.5
dre04060	Cytokine-cytokine receptor interaction	3	0.5
dre00650	Butanoate metabolism	2	0.4
dre00640	Propanoate metabolism	2	0.4
dre04136	Autophagy - other	2	0.4
dre03020	RNA polymerase	2	0.4
dre00500	Starch and sucrose metabolism	2	0.4
dre00051	Fructose and mannose metabolism	2	0.4
dre01250	Biosynthesis of nucleotide sugars	2	0.4
dre00250	Alanine, aspartate and glutamate metabolism	2	0.4
dre02010	ABC transporters	2	0.4
dre00565	Ether lipid metabolism	2	0.4
dre00071	Fatty acid degradation	2	0.4
dre00514	Other types of O-glycan biosynthesis	2	0.4
dre00520	Amino sugar and nucleotide sugar metabolism	2	0.4
dre00240	Pyrimidine metabolism	2	0.4

dre00600	Sphingolipid metabolism	2	0.4
dre00010	Glycolysis / Gluconeogenesis	2	0.4
dre04330	Notch signaling pathway	2	0.4
dre00830	Retinol metabolism	2	0.4
dre04920	Adipocytokine signaling pathway	2	0.4
dre00562	Inositol phosphate metabolism	2	0.4
dre04146	Peroxisome	2	0.4
dre03013	Nucleocytoplasmic transport	2	0.4
dre04620	Toll-like receptor signaling pathway	2	0.4
dre03010	Ribosome	2	0.4
dre04621	NOD-like receptor signaling pathway	2	0.4
dre04142	Lysosome	2	0.4
dre01240	Biosynthesis of cofactors	2	0.4
dre00531	Glycosaminoglycan degradation	1	0.2
dre00730	Thiamine metabolism	1	0.2
dre00790	Folate biosynthesis	1	0.2
dre03050	Proteasome	1	0.2
dre00630	Glyoxylate and dicarboxylate metabolism	1	0.2
dre00100	Steroid biosynthesis	1	0.2
dre00450	Selenocompound metabolism	1	0.2
dre00532	Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	1	0.2
dre04623	Cytosolic DNA-sensing pathway	1	0.2
dre00380	Tryptophan metabolism	1	0.2
dre00061	Fatty acid biosynthesis	1	0.2
dre00900	Terpenoid backbone biosynthesis	1	0.2
dre00740	Riboflavin metabolism	1	0.2
dre03320	PPAR signaling pathway	1	0.2
dre03271	Virion - Rotavirus	1	0.2
dre00270	Cysteine and methionine metabolism	1	0.2
dre04622	RIG-I-like receptor signaling pathway	1	0.2
dre00052	Galactose metabolism	1	0.2
dre00920	Sulfur metabolism	1	0.2
dre00030	Pentose phosphate pathway	1	0.2
dre04130	SNARE interactions in vesicular transport	1	0.2
dre00785	Lipoic acid metabolism	1	0.2
dre00524	Neomycin, kanamycin and gentamicin biosynthesis	1	0.2
dre03018	RNA degradation	1	0.2

dre00760	Nicotinate and nicotinamide metabolism	1	0.2
dre00513	Various types of N-glycan biosynthesis	1	0.2
dre00510	N-Glycan biosynthesis	1	0.2
dre00564	Glycerophospholipid metabolism	1	0.2
dre00983	Drug metabolism - other enzymes	1	0.2
dre01210	2-Oxocarboxylic acid metabolism	1	0.2
dre00512	Mucin type O-glycan biosynthesis	1	0.2
dre00620	Pyruvate metabolism	1	0.2
dre04340	Hedgehog signaling pathway	1	0.2
dre00410	beta-Alanine metabolism	1	0.2
dre01230	Biosynthesis of amino acids	1	0.2
dre03060	Protein export	1	0.2
dre03083	Polycomb repressive complex	1	0.2

Table B.2: Pathways targeted by miR-103-3p. The predicted mRNA targets were derived through miRanda, and inputted into DAVID to group them into functional clusters.

KEGG Identifier	Pathway	Number of mRNA targeted	% of pathway targeted
dre01100	Metabolic pathways	50	7.8
dre04261	Adrenergic signaling in cardiomyocytes	18	2.8
dre04020	Calcium signaling pathway	18	2.8
dre04010	MAPK signaling pathway	16	2.5
dre04910	Insulin signaling pathway	15	2.3
dre04070	Phosphatidylinositol signaling system	13	2.0
dre04912	GnRH signaling pathway	13	2.0
dre04916	Melanogenesis	13	2.0
dre04270	Vascular smooth muscle contraction	13	2.0
dre04310	Wnt signaling pathway	13	2.0
dre04625	C-type lectin receptor signaling pathway	12	1.9
dre04371	Apelin signaling pathway	12	1.9
dre04218	Cellular senescence	12	1.9
dre04114	Oocyte meiosis	11	1.7
dre04150	mTOR signaling pathway	10	1.6
dre04148	Efferocytosis	10	1.6
dre04140	Autophagy - animal	10	1.6
dre04744	Phototransduction	9	1.4
dre04012	ErbB signaling pathway	9	1.4
dre04530	Tight junction	9	1.4
dre04510	Focal adhesion	9	1.4

dre04810	Regulation of actin cytoskeleton	9	1.4
dre04080	Neuroactive ligand-receptor interaction	9	1.4
dre04068	FoxO signaling pathway	8	1.3
dre05132	Salmonella infection	8	1.3
dre04144	Endocytosis	8	1.3
dre03015	mRNA surveillance pathway	7	1.1
dre04350	TGF-beta signaling pathway	7	1.1
dre04814	Motor proteins	7	1.1
dre03040	Spliceosome	6	0.9
dre04260	Cardiac muscle contraction	6	0.9
dre04621	NOD-like receptor signaling pathway	6	0.9
dre05168	Herpes simplex virus 1 infection	6	0.9
dre00330	Arginine and proline metabolism	5	0.8
dre04370	VEGF signaling pathway	5	0.8
dre03320	PPAR signaling pathway	5	0.8
dre00562	Inositol phosphate metabolism	5	0.8
dre04520	Adherens junction	5	0.8
dre04110	Cell cycle	5	0.8
dre00561	Glycerolipid metabolism	4	0.6
dre00600	Sphingolipid metabolism	4	0.6
dre04115	p53 signaling pathway	4	0.6
dre01230	Biosynthesis of amino acids	4	0.6
dre04920	Adipocytokine signaling pathway	4	0.6
dre00564	Glycerophospholipid metabolism	4	0.6
dre04914	Progesterone-mediated oocyte maturation	4	0.6
dre04540	Gap junction	4	0.6
dre04142	Lysosome	4	0.6
dre04217	Necroptosis	4	0.6
dre04210	Apoptosis	4	0.6
dre04141	Protein processing in endoplasmic reticulum	4	0.6
dre04060	Cytokine-cytokine receptor interaction	4	0.6
dre00410	beta-Alanine metabolism	3	0.5
dre00513	Various types of N-glycan biosynthesis	3	0.5
dre00380	Tryptophan metabolism	3	0.5
dre00071	Fatty acid degradation	3	0.5
dre00280	Valine, leucine and isoleucine degradation	3	0.5
dre00514	Other types of O-glycan biosynthesis	3	0.5
dre04216	Ferroptosis	3	0.5

dre04622	RIG-I-like receptor signaling pathway	3	0.5
dre03250	Viral life cycle - HIV-1	3	0.5
dre00010	Glycolysis / Gluconeogenesis	3	0.5
dre00310	Lysine degradation	3	0.5
dre04330	Notch signaling pathway	3	0.5
dre04620	Toll-like receptor signaling pathway	3	0.5
dre04137	Mitophagy - animal	3	0.5
dre00230	Purine metabolism	3	0.5
dre01240	Biosynthesis of cofactors	3	0.5
dre00770	Pantothenate and CoA biosynthesis	2	0.3
dre00340	Histidine metabolism	2	0.3
dre00532	Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	2	0.3
dre00534	Glycosaminoglycan biosynthesis - heparan sulfate / heparin	2	0.3
dre04136	Autophagy - other	2	0.3
dre03020	RNA polymerase	2	0.3
dre00512	Mucin type O-glycan biosynthesis	2	0.3
dre04130	SNARE interactions in vesicular transport	2	0.3
dre01250	Biosynthesis of nucleotide sugars	2	0.3
dre00250	Alanine, aspartate and glutamate metabolism	2	0.3
dre00620	Pyruvate metabolism	2	0.3
dre00053	Ascorbate and aldarate metabolism	2	0.3
dre00510	N-Glycan biosynthesis	2	0.3
dre00520	Amino sugar and nucleotide sugar metabolism	2	0.3
dre00240	Pyrimidine metabolism	2	0.3
dre04623	Cytosolic DNA-sensing pathway	2	0.3
dre04340	Hedgehog signaling pathway	2	0.3
dre00980	Metabolism of xenobiotics by cytochrome P450	2	0.3
dre03008	Ribosome biogenesis in eukaryotes	2	0.3
dre00983	Drug metabolism - other enzymes	2	0.3
dre03013	Nucleocytoplasmic transport	2	0.3
dre01232	Nucleotide metabolism	2	0.3
dre03010	Ribosome	2	0.3
dre00190	Oxidative phosphorylation	2	0.3
dre04120	Ubiquitin mediated proteolysis	2	0.3
dre04514	Cell adhesion molecules	2	0.3

dre00590	Arachidonic acid metabolism	1	0.2
dre03018	RNA degradation	1	0.2
dre00500	Starch and sucrose metabolism	1	0.2
dre00565	Ether lipid metabolism	1	0.2
dre00515	Mannose type O-glycan biosynthesis	1	0.2
dre03083	Polycomb repressive complex	1	0.2
dre00220	Arginine biosynthesis	1	0.2
dre00785	Lipoic acid metabolism	1	0.2
dre04145	Phagosome	1	0.2
dre00480	Glutathione metabolism	1	0.2
dre00270	Cysteine and methionine metabolism	1	0.2
dre00591	Linoleic acid metabolism	1	0.2
dre00061	Fatty acid biosynthesis	1	0.2
dre00052	Galactose metabolism	1	0.2
dre02010	ABC transporters	1	0.2
dre00640	Propanoate metabolism	1	0.2
dre00061	Fatty acid metabolism	1	0.2
dre00360	Phenylalanine metabolism	1	0.2
dre01210	2-Oxocarboxylic acid metabolism	1	0.2
dre03082	ATP-dependent chromatin remodeling	1	0.2
dre03430	Mismatch repair	1	0.2
dre01200	Carbon metabolism	1	0.2
dre00603	Glycosphingolipid biosynthesis - globo and isoglobo series	1	0.2
dre00592	alpha-Linolenic acid metabolism	1	0.2
dre00830	Retinol metabolism	1	0.2
dre00511	Other glycan degradation	1	0.2
dre03050	Proteasome	1	0.2
dre00760	Nicotinate and nicotinamide metabolism	1	0.2
dre00350	Tyrosine metabolism	1	0.2
dre03410	Base excision repair	1	0.2
dre04146	Peroxisome	1	0.2
dre00260	Glycine, serine and threonine metabolism	1	0.2
dre00982	Drug metabolism - cytochrome P450	1	0.2
dre00790	Folate biosynthesis	1	0.2
dre00750	Vitamin B6 metabolism	1	0.2
dre00120	Primary bile acid biosynthesis	1	0.2

Table B.3: Pathways targeted by miR-122. The predicted mRNA targets were derived through miRanda, and inputted into DAVID to group them into functional clusters.

KEGG Identifier	Pathway	Number of mRNA targeted	% of pathway targeted
dre01100	Metabolic pathways	85	8.2
dre04144	Endocytosis	22	2.1
dre04080	Neuroactive ligand-receptor interaction	22	2.1
dre04810	Regulation of actin cytoskeleton	20	1.9
dre05132	Salmonella infection	19	1.8
dre04020	Calcium signaling pathway	18	1.7
dre04010	MAPK signaling pathway	17	1.6
dre04060	Cytokine-cytokine receptor interaction	15	1.5
dre04140	Autophagy - animal	15	1.5
dre04510	Focal adhesion	15	1.5
dre04142	Lysosome	14	1.4
dre04141	Protein processing in endoplasmic reticulum	13	1.3
dre04310	Wnt signaling pathway	13	1.3
dre04145	Phagosome	12	1.2
dre05168	Herpes simplex virus 1 infection	12	1.2
dre04148	Efferocytosis	12	1.2
dre04621	NOD-like receptor signaling pathway	11	1.1
dre00830	Retinol metabolism	10	1.0
dre00983	Drug metabolism - other enzymes	10	1.0
dre04137	Mitophagy - animal	10	1.0
dre04114	Oocyte meiosis	10	1.0
dre04218	Cellular senescence	10	1.0
dre04261	Adrenergic signaling in cardiomyocytes	10	1.0
dre04070	Phosphatidylinositol signaling system	9	0.9
dre04350	TGF-beta signaling pathway	9	0.9
dre04910	Insulin signaling pathway	9	0.9
dre04217	Necroptosis	9	0.9
dre04530	Tight junction	9	0.9
dre00053	Ascorbate and aldarate metabolism	8	0.8
dre00140	Steroid hormone biosynthesis	8	0.8
dre04012	ErbB signaling pathway	8	0.8
dre04912	GnRH signaling pathway	8	0.8
dre00230	Purine metabolism	8	0.8
dre04110	Cell cycle	8	0.8

dre01240	Biosynthesis of cofactors	8	0.8
dre04150	mTOR signaling pathway	8	0.8
dre00040	Pentose and glucuronate interconversions	7	0.7
dre00860	Porphyrin metabolism	7	0.7
dre00982	Drug metabolism - cytochrome P450	7	0.7
dre00980	Metabolism of xenobiotics by cytochrome P450	7	0.7
dre01212	Fatty acid metabolism	7	0.7
dre00310	Lysine degradation	7	0.7
dre04146	Peroxisome	7	0.7
dre04270	Vascular smooth muscle contraction	7	0.7
dre04514	Cell adhesion molecules	7	0.7
dre04210	Apoptosis	7	0.7
dre00062	Fatty acid elongation	6	0.6
dre04330	Notch signaling pathway	6	0.6
dre03083	Polycomb repressive complex	6	0.6
dre04512	ECM-receptor interaction	6	0.6
dre01232	Nucleotide metabolism	6	0.6
dre04914	Progesterone-mediated oocyte maturation	6	0.6
dre00564	Glycerophospholipid metabolism	6	0.6
dre04625	C-type lectin receptor signaling pathway	6	0.6
dre03082	ATP-dependent chromatin remodeling	6	0.6
dre04120	Ubiquitin mediated proteolysis	6	0.6
dre04371	Apelin signaling pathway	6	0.6
dre02010	ABC transporters	5	0.5
dre04744	Phototransduction	5	0.5
dre03420	Nucleotide excision repair	5	0.5
dre00330	Arginine and proline metabolism	5	0.5
dre04340	Hedgehog signaling pathway	5	0.5
dre00010	Glycolysis / Gluconeogenesis	5	0.5
dre04115	p53 signaling pathway	5	0.5
dre00562	Inositol phosphate metabolism	5	0.5
dre03013	Nucleocytoplasmic transport	5	0.5
dre04540	Gap junction	5	0.5
dre01200	Carbon metabolism	5	0.5
dre03040	Spliceosome	5	0.5
dre04916	Melanogenesis	5	0.5
dre04068	FoxO signaling pathway	5	0.5
dre00534	Glycosaminoglycan biosynthesis - heparan sulfate / heparin	4	0.4

dre01040	Biosynthesis of unsaturated fatty acids	4	0.4
dre04130	SNARE interactions in vesicular transport	4	0.4
dre00071	Fatty acid degradation	4	0.4
dre00280	Valine, leucine and isoleucine degradation	4	0.4
dre04622	RIG-I-like receptor signaling pathway	4	0.4
dre00600	Sphingolipid metabolism	4	0.4
dre04370	VEGF signaling pathway	4	0.4
dre03018	RNA degradation	4	0.4
dre04620	Toll-like receptor signaling pathway	4	0.4
dre04520	Adherens junction	4	0.4
dre04814	Motor proteins	4	0.4
dre00603	Glycosphingolipid biosynthesis - globo and isoglobo series	3	0.3
dre00100	Steroid biosynthesis	3	0.3
dre00515	Mannose type O-glycan biosynthesis	3	0.3
dre00640	Propanoate metabolism	3	0.3
dre00601	Glycosphingolipid biosynthesis - lacto and neolacto series	3	0.3
dre03440	Homologous recombination	3	0.3
dre00051	Fructose and mannose metabolism	3	0.3
dre03410	Base excision repair	3	0.3
dre00513	Various types of N-glycan biosynthesis	3	0.3
dre00620	Pyruvate metabolism	3	0.3
dre00565	Ether lipid metabolism	3	0.3
dre00514	Other types of O-glycan biosynthesis	3	0.3
dre00561	Glycerolipid metabolism	3	0.3
dre03008	Ribosome biogenesis in eukaryotes	3	0.3
dre01230	Biosynthesis of amino acids	3	0.3
dre03015	mRNA surveillance pathway	3	0.3
dre03010	Ribosome	3	0.3
dre00190	Oxidative phosphorylation	3	0.3
dre00232	Caffeine metabolism	2	0.2
dre03450	Non-homologous end-joining	2	0.2
dre00604	Glycosphingolipid biosynthesis - ganglio series	2	0.2
dre00450	Selenocompound metabolism	2	0.2
dre03430	Mismatch repair	2	0.2
dre00531	Glycosaminoglycan degradation	2	0.2

dre00030	Pentose phosphate pathway	2	0.2
dre00020	Citrate cycle (TCA cycle)	2	0.2
dre03030	DNA replication	2	0.2
dre00512	Mucin type O-glycan biosynthesis	2	0.2
dre03022	Basal transcription factors	2	0.2
dre00380	Tryptophan metabolism	2	0.2
dre00270	Cysteine and methionine metabolism	2	0.2
dre00590	Arachidonic acid metabolism	2	0.2
dre04216	Ferroptosis	2	0.2
dre00510	N-Glycan biosynthesis	2	0.2
dre00520	Amino sugar and nucleotide sugar metabolism	2	0.2
dre00970	Aminoacyl-tRNA biosynthesis	2	0.2
dre00240	Pyrimidine metabolism	2	0.2
dre00480	Glutathione metabolism	2	0.2
dre03250	Viral life cycle - HIV-1	2	0.2
dre04920	Adipocytokine signaling pathway	2	0.2
dre04672	Intestinal immune network for IgA production	1	0.1
dre00750	Vitamin B6 metabolism	1	0.1
dre03320	PPAR signaling pathway	1	0.1
dre00591	Linoleic acid metabolism	1	0.1
dre00630	Glyoxylate and dicarboxylate metabolism	1	0.1
dre04623	Cytosolic DNA-sensing pathway	1	0.1
dre00790	Folate biosynthesis	1	0.1
dre00533	Glycosaminoglycan biosynthesis - keratan sulfate	1	0.1
dre03060	Protein export	1	0.1
dre00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	1	0.1
dre00920	Sulfur metabolism	1	0.1
dre00760	Nicotinate and nicotinamide metabolism	1	0.1
dre03266	Virion - Herpesvirus	1	0.1
dre04122	Sulfur relay system	1	0.1
dre00410	beta-Alanine metabolism	1	0.1
dre00532	Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	1	0.1
dre04260	Cardiac muscle contraction	1	0.1
dre01210	2-Oxocarboxylic acid metabolism	1	0.1
dre00430	Taurine and hypotaurine metabolism	1	0.1
dre03265	Virion - Ebolavirus and Lyssavirus	1	0.1

dre00511	Other glycan degradation	1	0.1
dre03460	Fanconi anemia pathway	1	0.1
dre00340	Histidine metabolism	1	0.1
dre04136	Autophagy - other	1	0.1
dre00052	Galactose metabolism	1	0.1
dre00592	alpha-Linolenic acid metabolism	1	0.1
dre00061	Fatty acid biosynthesis	1	0.1

Table B.4: Pathways targeted by miR-181a-5p. The predicted mRNA targets were derived through miRanda, and inputted into DAVID to group them into functional clusters.

KEGG Identifier	Pathway	Number of mRNA targeted	% of pathway targeted
dre01100	Metabolic pathways	50	7.6
dre04810	Regulation of actin cytoskeleton	13	2.0
dre04144	Endocytosis	12	1.8
dre04010	MAPK Signaling pathway	11	1.7
dre04080	Neuroactive ligand-receptor interaction	11	1.7
dre04020	Calcium signaling pathway	10	1.5
dre04371	Apelin signaling pathway	9	1.4
dre04218	Cellular senescence	9	1.4
dre04310	Wnt signaling pathway	9	1.4
dre04530	Tight junction	9	1.4
dre04510	Focal adhesion	9	1.4
dre05168	Herpes simplex virus 1 infection	8	1.2
dre04140	Autophagy - animal	8	1.2
dre04120	Ubiquitin mediated proteolysis	7	1.2
dre04060	Cytokine-cytokine receptor interaction	7	1.1
dre04261	Adrenergic signaling in cardiomyocytes	7	1.1
dre05132	Salmonella infection	7	1.1
dre00564	Glycerophospholipid metabolism	6	0.9
dre04070	Phosphatidylinositol signaling system	6	0.9
dre04137	Mitophagy - animal	6	0.9
dre04142	Lysosome	6	0.9
dre04210	Apoptosis	6	0.9
dre04150	mTOR signaling pathway	6	0.9
dre04814	Motor proteins	6	0.9
dre00480	Glutathione metabolism	5	0.8
dre03013	Nucleocytoplasmic transport	5	0.8
dre04625	C-type lectin receptor signaling pathway	5	0.8
dre01200	Carbon metabolism	5	0.8

dre04260	Cardiac muscle contraction	5	0.8
dre04270	Vascular smooth muscle contraction	5	0.8
dre04910	Insulin signaling pathway	5	0.8
dre04514	Cell adhesion molecules	5	0.8
dre04110	Cell cycle	5	0.8
dre04216	Ferroptosis	4	0.6
dre00330	Arginine and proline metabolism	4	0.6
dre00600	Sphingolipid metabolism	4	0.6
dre01212	Fatty acid metabolism	4	0.6
dre04330	Notch signaling pathway	4	0.6
dre03018	RNA degradation	4	0.6
dre03083	Polycomb repressive complex	4	0.6
dre04115	p53 signaling pathway	4	0.6
dre01230	Biosynthesis of amino acids	4	0.6
dre03015	mRNA surveillance pathway	4	0.6
dre04512	ECM-receptor interaction	4	0.6
dre01232	Nucleotide metabolism	4	0.6
dre04520	Adherens junction	4	0.6
dre04114	Oocyte meiosis	4	0.6
dre04350	TGF-beta signaling pathway	4	0.6
dre00230	Purine metabolism	4	0.6
dre01240	Biosynthesis of cofactors	4	0.6
dre04136	Autophagy - other	3	0.5
dre03020	RNA polymerase	3	0.5
dre00020	Citrate cycle (TCA cycle)	3	0.5
dre01210	2-Oxocarboxylic acid metabolism	3	0.5
dre04130	SNARE interactions in vesicular transport	3	0.5
dre00760	Nicotinate and nicotinamide metabolism	3	0.5
dre00260	Glycine, serine and threonine metabolism	3	0.5
dre00071	Fatty acid degradation	3	0.5
dre00514	Other types of O-glycan biosynthesis	3	0.5
dre04622	RIG-I-like receptor signaling pathway	3	0.5
dre00240	Pyrimidine metabolism	3	0.5
dre04623	Cytosolic DNA-sensing pathway	3	0.5
dre00982	Drug metabolism - cytochrome P450	3	0.5
dre00561	Glycerolipid metabolism	3	0.5
dre03008	Ribosome biogenesis in eukaryotes	3	0.5
dre00310	Lysine degradation	3	0.5
dre00562	Inositol phosphate metabolism	3	0.5

dre04012	ErbB signaling pathway	3	0.5
dre04620	Toll-like receptor signaling pathway	3	0.5
dre04914	Progesterone-mediated oocyte maturation	3	0.5
dre03082	ATP-dependent chromatin remodeling	3	0.5
dre03040	Spliceosome	3	0.5
dre04621	NOD-like receptor signaling pathway	3	0.5
dre04068	FoxO signaling pathway	3	0.5
dre04148	Efferocytosis	3	0.5
dre00360	Phenylalanine metabolism	2	0.3
dre00785	Lipoic acid metabolism	2	0.3
dre00061	Fatty acid biosynthesis	2	0.3
dre00340	Histidine metabolism	2	0.3
dre00512	Mucin type O-glycan biosynthesis	2	0.3
dre00350	Tyrosine metabolism	2	0.3
dre04672	Intestinal immune network for IgA production	2	0.3
dre00250	Alanine, aspartate and glutamate metabolism	2	0.3
dre04744	Phototransduction	2	0.3
dre00380	Tryptophan metabolism	2	0.3
dre00565	Ether lipid metabolism	2	0.3
dre00270	Cysteine and methionine metabolism	2	0.3
dre04340	Hedgehog signaling pathway	2	0.3
dre03250	Viral life cycle - HIV-1	2	0.3
dre00010	Glycolysis / Gluconeogenesis	2	0.3
dre03320	PPAR signaling pathway	2	0.3
dre04920	Adipocytokine signaling pathway	2	0.3
dre04146	Peroxisome	2	0.3
dre04540	Gap junction	2	0.3
dre04912	GnRH signaling pathway	2	0.3
dre04916	Melanogenesis	2	0.3
dre00190	Oxidative phosphorylation	2	0.3
dre04145	Phagosome	2	0.3
dre04217	Necroptosis	2	0.3
dre04141	Protein processing in endoplasmic reticulum	2	0.3
dre03410	Base excision repair	2	0.2
dre00470	D-Amino acid metabolism	1	0.2
dre03266	Virion - Herpesvirus	1	0.2
dre00515	Mannose type O-glycan biosynthesis	1	0.2
dre00790	Folate biosynthesis	1	0.2

dre03022	Basal transcription factors	1	0.2
dre00520	Amino sugar and nucleotide sugar metabolism	1	0.2
dre03440	Homologous recombination	1	0.2
dre00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	1	0.2
dre00900	Terpenoid backbone biosynthesis	1	0.2
dre00280	Valine, leucine and isoleucine degradation	1	0.2
dre00620	Pyruvate metabolism	1	0.2
dre00830	Retinol metabolism	1	0.2
dre00062	Fatty acid elongation	1	0.2
dre00052	Galactose metabolism	1	0.2
dre03010	Ribosome	1	0.2
dre00983	Drug metabolism - other enzymes	1	0.2
dre01250	Biosynthesis of nucleotide sugars	1	0.2
dre00220	Arginine biosynthesis	1	0.2
dre00970	Aminoacyl-tRNA biosynthesis	1	0.2
dre00410	beta-Alanine metabolism	1	0.2
dre02010	ABC transporters	1	0.2
dre04370	VEGF signaling pathway	1	0.2
dre00860	Porphyrin metabolism	1	0.2
dre00450	Selenocompound metabolism	1	0.2
dre00140	Steroid hormone biosynthesis	1	0.2
dre00531	Glycosaminoglycan degradation	1	0.2
dre00980	Metabolism of xenobiotics by cytochrome P450	1	0.2
dre03420	Nucleotide excision repair	1	0.2
dre00650	Butanoate metabolism	1	0.2
dre03267	Virion - Adenovirus	1	0.2
dre00640	Propanoate metabolism	1	0.2

Table B.5: Pathways targeted by miR-214. The predicted mRNA targets were derived through miRanda, and inputted into DAVID to group them into functional clusters.

KEGG Identifier	Pathway	Number of mRNA targeted	% of pathway targeted
dre01100	Metabolic pathways	87	7.5
dre048106	Regulation of actin cytoskeleton	29	2.5
dre04020	Calcium signaling pathway	25	2.1
dre04144	Endocytosis	23	2.0
dre04010	MAPK signaling pathway	23	2.0
dre04510	Focal adhesion	20	1.7
dre05132	Salmonella infection	19	1.6
dre04910	Insulin signaling pathway	18	1.5
dre04218	Cellular senescence	18	1.5

dre04141	Protein processing in endoplasmic reticulum	17	1.5
dre04080	Neuroactive ligand-receptor interaction	17	1.5
dre04110	Cell cycle	16	1.4
dre04148	Efferocytosis	16	1.4
dre04114	Oocyte meiosis	15	1.3
dre04371	Apelin signaling pathway	15	1.3
dre04261	Adrenergic signaling in cardiomyocytes	15	1.3
dre04820	Cytoskeleton in muscle cells	15	1.3
dre04625	C-type lectin receptor signaling pathway	14	1.2
dre04520	Adherens junction	13	1.1
dre04270	Vascular smooth muscle contraction	13	1.1
dre04310	Wnt signaling pathway	13	1.1
dre04150	mTOR signaling pathway	12	1.0
dre04140	Autophagy - animal	12	1.0
dre04012	ErbB signaling pathway	11	0.9
dre04070	Phosphatidylinositol signaling system	11	0.9
dre00514	Other types of O-glycan biosynthesis	10	0.9
dre04916	Melanogenesis	10	0.9
dre04068	FoxO signaling pathway	10	0.9
dre04514	Cell adhesion molecules	10	0.9
dre05168	Herpes simplex virus 1 infection	10	0.9
dre04060	Cytokine-cytokine receptor interaction	10	0.9
dre04530	Tight junction	10	0.9
dre03015	mRNA surveillance pathway	9	0.8
dre03013	Nucleocytoplasmic transport	9	0.8
dre04912	GnRH signaling pathway	9	0.8
dre04350	TGF-beta signaling pathway	9	0.8
dre00230	Purine metabolism	9	0.8
dre03083	Polycomb repressive complex	8	0.7
dre04512	ECM-receptor interaction	8	0.7
dre01232	Nucleotide metabolism	8	0.7
dre04145	Phagosome	8	0.7
dre04142	Lysosome	8	0.7
dre01240	Biosynthesis of cofactors	8	0.7
dre04210	Apoptosis	8	0.7
dre04814	Motor proteins	8	0.7
dre00512	Mucin type O-glycan biosynthesis	7	0.6
dre00565	Ether lipid metabolism	7	0.6
dre01230	Biosynthesis of amino acids	7	0.6

dre04914	Progesterone-mediated oocyte maturation	7	0.6
dre03082	ATP-dependent chromatin remodeling	7	0.6
dre04120	Ubiquitin mediated proteolysis	7	0.6
dre04621	NOD-like receptor signaling pathway	7	0.6
dre00513	Various types of N-glycan biosynthesis	6	0.5
dre00270	Cysteine and methionine metabolism	6	0.5
dre00510	N-Glycan biosynthesis	6	0.5
dre04340	Hedgehog signaling pathway	6	0.5
dre04370	VEGF signaling pathway	6	0.5
dre03018	RNA degradation	6	0.5
dre00564	Glycerophospholipid metabolism	6	0.5
dre01200	Carbon metabolism	6	0.5
dre01210	2-Oxocarboxylic acid metabolism	5	0.4
dre00310	Lysine degradation	5	0.4
dre04115	p53 signaling pathway	5	0.4
dre00562	Inositol phosphate metabolism	5	0.4
dre04540	Gap junction	5	0.4
dre00260	Glycine, serine and threonine metabolism	4	0.3
dre02010	ABC transporters	4	0.3
dre00380	Tryptophan metabolism	4	0.3
dre03420	Nucleotide excision repair	4	0.3
dre00240	Pyrimidine metabolism	4	0.3
dre04623	Cytosolic DNA-sensing pathway	4	0.3
dre03250	Viral life cycle - HIV-1	4	0.3
dre04330	Notch signaling pathway	4	0.3
dre04920	Adipocytokine signaling pathway	4	0.3
dre04620	Toll-like receptor signaling pathway	4	0.3
dre03010	Ribosome	4	0.3
dre04137	Mitophagy - animal	4	0.3
dre03040	Spliceosome	4	0.3
dre04260	Cardiac muscle contraction	4	0.3
dre00190	Oxidative phosphorylation	4	0.3
dre00515	Mannose type O-glycan biosynthesis	3	0.3
dre00534	Glycosaminoglycan biosynthesis - heparan sulfate / heparin	3	0.3
dre00020	Citrate cycle (TCA cycle)	3	0.3
dre03020	RNA polymerase	3	0.3
dre04130	SNARE interactions in vesicular transport	3	0.3

dre00250	Alanine, aspartate and glutamate metabolism	3	0.3
dre04744	Phototransduction	3	0.3
dre00071	Fatty acid degradation	3	0.3
dre00330	Arginine and proline metabolism	3	0.3
dre00480	Glutathione metabolism	3	0.3
dre00561	Glycerolipid metabolism	3	0.3
dre00600	Sphingolipid metabolism	3	0.3
dre03008	Ribosome biogenesis in eukaryotes	3	0.3
dre03320	PPAR signaling pathway	3	0.3
dre04146	Peroxisome	3	0.3
dre04217	Necroptosis	3	0.3
dre00430	Taurine and hypotaurine metabolism	2	0.2
dre00650	Butanoate metabolism	2	0.2
dre00340	Histidine metabolism	2	0.2
dre00532	Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	2	0.2
dre00220	Arginine biosynthesis	2	0.2
dre00790	Folate biosynthesis	2	0.2
dre04136	Autophagy - other	2	0.2
dre00601	Glycosphingolipid biosynthesis - lacto and neolacto series	2	0.2
dre03030	DNA replication	2	0.2
dre00350	Tyrosine metabolism	2	0.2
dre04672	Intestinal immune network for IgA production	2	0.2
dre00051	Fructose and mannose metabolism	2	0.2
dre01250	Biosynthesis of nucleotide sugars	2	0.2
dre03410	Base excision repair	2	0.2
dre00040	Pentose and glucuronate interconversions	2	0.2
dre03050	Proteasome	2	0.2
dre00280	Valine, leucine and isoleucine degradation	2	0.2
dre00590	Arachidonic acid metabolism	2	0.2
dre00520	Amino sugar and nucleotide sugar metabolism	2	0.2
dre04622	RIG-I-like receptor signaling pathway	2	0.2
dre01212	Fatty acid metabolism	2	0.2
dre00010	Glycolysis / Gluconeogenesis	2	0.2
dre00970	Aminoacyl-tRNA biosynthesis	1	0.1
dre03266	Virion - Herpesvirus	1	0.1

dre00760	Nicotinate and nicotinamide metabolism	1	0.1
dre00630	Glyoxylate and dicarboxylate metabolism	1	0.1
dre03450	Non-homologous end-joining	1	0.1
dre04980	Cobalamin transport and metabolism	1	0.1
dre00860	Porphyrin metabolism	1	0.1
dre00982	Drug metabolism - cytochrome P450	1	0.1
dre03430	Mismatch repair	1	0.1
dre00410	beta-Alanine metabolism	1	0.1
dre00450	Selenocompound metabolism	1	0.1
dre00591	Linoleic acid metabolism	1	0.1
dre00533	Glycosaminoglycan biosynthesis - keratan sulfate	1	0.1
dre00900	Terpenoid backbone biosynthesis	1	0.1
dre00360	Phenylalanine metabolism	1	0.1
dre00785	Lipoic acid metabolism	1	0.1
dre00730	Thiamine metabolism	1	0.1
dre00920	Sulfur metabolism	1	0.1
dre00670	One carbon pool by folate	1	0.1
dre00440	Phosphonate and phosphinate metabolism	1	0.1
dre00980	Metabolism of xenobiotics by cytochrome P450	1	0.1
dre00531	Glycosaminoglycan degradation	1	0.1
dre04216	Ferroptosis	1	0.1
dre00052	Galactose metabolism	1	0.1
dre00592	alpha-Linolenic acid metabolism	1	0.1
dre03060	Protein export	1	0.1
dre00603	Glycosphingolipid biosynthesis - globo and isoglobo series	1	0.1
dre00830	Retinol metabolism	1	0.1
dre00053	Ascorbate and aldarate metabolism	1	0.1
dre00030	Pentose phosphate pathway	1	0.1
dre03264	Virion - Flavivirus	1	0.1
dre00511	Other glycan degradation	1	0.1

Table B.6: Pathways targeted by miR-301a-3p. The predicted mRNA targets were derived through miRanda, and inputted into DAVID to group them into functional clusters.

KEGG Identifier	Pathway	Number of mRNA targeted	% of pathway targeted
dre01100	Metabolic pathways	16	10.2

dre00040	Pentose and glucuronate interconversions	6	3.8
dre01240	Biosynthesis of cofactors	6	3.8
dre00053	Ascorbate and aldarate metabolism	5	3.2
dre00982	Drug metabolism - cytochrome P450	5	3.2
dre00980	Metabolism of xenobiotics by cytochrome P450	5	3.2
dre00860	Porphyrin metabolism	5	3.2
dre00140	Steroid hormone biosynthesis	5	3.2
dre00830	Retinol metabolism	5	3.2
dre00983	Drug metabolism - other enzymes	5	3.2
dre05168	Herpes simplex virus 1 infection	4	2.5
dre04310	Wnt signaling pathway	4	2.5
dre04080	Neuroactive ligand-receptor interaction	4	2.5
dre03013	Nucleocytoplasmic transport	3	1.9
dre04141	Protein processing in endoplasmic reticulum	3	1.9
dre04148	Efferocytosis	3	1.9
dre04261	Adrenergic signaling in cardiomyocytes	3	1.9
dre04810	Regulation of actin cytoskeleton	3	1.9
dre04144	Endocytosis	3	1.9
dre04623	Cytosolic DNA-sensing pathway	2	1.3
dre03018	RNA degradation	2	1.3
dre03083	Polycomb repressive complex	2	1.3
dre03015	mRNA surveillance pathway	2	1.3
dre04620	Toll-like receptor signaling pathway	2	1.3
dre04625	C-type lectin receptor signaling pathway	2	1.3
dre01200	Carbon metabolism	2	1.3
dre04916	Melanogenesis	2	1.3
dre04621	NOD-like receptor signaling pathway	2	1.3
dre04110	Cell cycle	2	1.3
dre04217	Necroptosis	2	1.3
dre04218	Cellular senescence	2	1.3
dre04820	Cytoskeleton in muscle cells	2	1.3
dre05132	Salmonella infection	2	1.3
dre04010	MAPK signaling pathway	2	1.3
dre01230	Biosynthesis of amino acids	1	0.6
dre00565	Ether lipid metabolism	1	0.6
dre04520	Adherens junction	1	0.6
dre00230	Purine metabolism	1	0.6

dre04330	Notch signaling pathway	1	0.6
dre00030	Pentose phosphate pathway	1	0.6
dre00190	Oxidative phosphorylation	1	0.6
dre04540	Gap junction	1	0.6
dre00480	Glutathione metabolism	1	0.6
dre00510	N-Glycan biosynthesis	1	0.6
dre04012	ErbB signaling pathway	1	0.6
dre00051	Fructose and mannose metabolism	1	0.6
dre03250	Viral life cycle - HIV-1	1	0.6
dre04370	VEGF signaling pathway	1	0.6
dre03020	RNA polymerase	1	0.6
dre04020	Calcium signaling pathway	1	0.6
dre04510	Focal adhesion	1	0.6
dre04130	SNARE interactions in vesicular transport	1	0.6
dre03420	Nucleotide excision repair	1	0.6
dre00010	Glycolysis / Gluconeogenesis	1	0.6
dre00564	Glycerophospholipid metabolism	1	0.6
dre04114	Oocyte meiosis	1	0.6
dre00790	Folate biosynthesis	1	0.6
dre04210	Apoptosis	1	0.6
dre03320	PPAR signaling pathway	1	0.6
dre04622	RIG-I-like receptor signaling pathway	1	0.6
dre00513	Various types of N-glycan biosynthesis	1	0.6
dre04120	Ubiquitin mediated proteolysis	1	0.6
dre00350	Tyrosine metabolism	1	0.6
dre04115	p53 signaling pathway	1	0.6
dre04530	Tight junction	1	0.6
dre00240	Pyrimidine metabolism	1	0.6
dre00561	Glycerolipid metabolism	1	0.6
dre04350	TGF-beta signaling pathway	1	0.6

Table B.7: Pathways targeted by miR-499-5p. The predicted mRNA targets were derived through miRanda, and inputted into DAVID to group them into functional clusters.

KEGG Identifier	Pathway	Number of mRNA targeted	% of pathway targeted
dre01100	Metabolic pathways	16	10.4
dre04020	Calcium signaling pathway	6	3.9
dre04530	Tight junction	5	3.2
dre04510	Focal adhesion	4	2.6
dre05132	Salmonella infection	4	2.6
dre04820	Cytoskeleton in muscle cells	4	2.6
dre04144	Endocytosis	4	2.6

dre04080	Neuroactive ligand-receptor interaction	4	2.6
dre03050	Proteasome	3	1.9
dre03018	RNA degradation	3	1.9
dre01230	Biosynthesis of amino acids	3	1.9
dre04912	GnRH signaling pathway	3	1.9
dre04350	TGF-beta signaling pathway	3	1.9
dre04120	Ubiquitin mediated proteolysis	3	1.9
dre04270	Vascular smooth muscle contraction	3	1.9
dre04371	Apelin signaling pathway	3	1.9
dre04141	Protein processing in endoplasmic reticulum	3	1.9
dre00220	Arginine biosynthesis	2	1.3
dre00020	Citrate cycle (TCA cycle)	2	1.3
dre00620	Pyruvate metabolism	2	1.3
dre00565	Ether lipid metabolism	2	1.3
dre04340	Hedgehog signaling pathway	2	1.3
dre00600	Sphingolipid metabolism	2	1.3
dre01200	Carbon metabolism	2	1.3
dre03040	Spliceosome	2	1.3
dre04114	Oocyte meiosis	2	1.3
dre04910	Insulin signaling pathway	2	1.3
dre04068	FoxO signaling pathway	2	1.3
dre01240	Biosynthesis of cofactors	2	1.3
dre04148	Efferocytosis	2	1.3
dre04810	Regulation of actin cytoskeleton	2	1.3
dre04010	MAPK signaling pathway	2	1.3
dre00630	Glyoxylate and dicarboxylate metabolism	1	0.6
dre00250	Alanine, aspartate and glutamate metabolism	1	0.6
dre04520	Adherens junction	1	0.6
dre03410	Base excision repair	1	0.6
dre04920	Adipocytokine signaling pathway	1	0.6
dre00770	Pantothenate and CoA biosynthesis	1	0.6
dre03010	Ribosome	1	0.6
dre04261	Adrenergic signaling in cardiomyocytes	1	0.6
dre04012	ErbB signaling pathway	1	0.6
dre04070	Phosphatidylinositol signaling system	1	0.6
dre00970	Aminoacyl-tRNA biosynthesis	1	0.6
dre04310	Wnt signaling pathway	1	0.6
dre03460	Fanconi anemia pathway	1	0.6
dre00330	Arginine and proline metabolism	1	0.6

dre04814	Motor proteins	1	0.6
dre04218	Cellular senescence	1	0.6
dre04620	Toll-like receptor signaling pathway	1	0.6
dre04136	Autophagy - other	1	0.6
dre03015	mRNA surveillance pathway	1	0.6
dre04145	Phagosome	1	0.6
dre04512	ECM-receptor interaction	1	0.6
dre04115	p53 signaling pathway	1	0.6
dre03083	Polycomb repressive complex	1	0.6
dre04140	Autophagy - animal	1	0.6
dre04142	Lysosome	1	0.6
dre01210	2-Oxocarboxylic acid metabolism	1	0.6
dre03022	Basal transcription factors	1	0.6
dre05168	Herpes simplex virus 1 infection	1	0.6
dre00130	Ubiquinone and other terpenoid-quinone biosynthesis	1	0.6
dre00562	Inositol phosphate metabolism	1	0.6
dre00510	N-Glycan biosynthesis	1	0.6
dre01212	Fatty acid metabolism	1	0.6
dre00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	1	0.6
dre04623	Cytosolic DNA-sensing pathway	1	0.6
dre04260	Cardiac muscle contraction	1	0.6
dre04110	Cell cycle	1	0.6
dre00860	Porphyrin metabolism	1	0.6
dre01040	Biosynthesis of unsaturated fatty acids	1	0.6
dre04217	Necroptosis	1	0.6
dre00062	Fatty acid elongation	1	0.6
dre04216	Ferroptosis	1	0.6
dre03082	ATP-dependent chromatin remodeling	1	0.6
dre00270	Cysteine and methionine metabolism	1	0.6
dre00410	beta-Alanine metabolism	1	0.6
dre00430	Taurine and hypotaurine metabolism	1	0.6
dre04621	NOD-like receptor signaling pathway	1	0.6
dre00230	Purine metabolism	1	0.6