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A Transcriptomics Approach to Examining the Effects of Prenatal and Thermal Stress on Developmental Plasticity in Chinook Salmon (Oncorhynchus Tshawytscha)

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A transcriptomics approach to examining the effects of prenatal and thermal stress on developmental plasticity in Chinook salmon (*Oncorhynchus tshawytscha*)

By

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DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

I am the sole author for Chapters 1 and 4 and the primary author on Chapters 2 and 3. Chapters 2 and 3 were co-authored by my co-supervisors Dr. Oliver Love and Dr. Daniel Heath. My collaborators Dr. Christina Semeniuk and Theresa Warriner are co-authors on both Chapters 2 and 3 while my collaborator Dr. Trevor Pitcher is a co-author on Chapter 2 only. In all cases, the key ideas, primary contributions, experimental design, data analysis, interpretation, and writing were performed by the primary author with co-authors providing assistance with experimental design, data analysis, interpretation, logistical support, editing, and funding.

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ABSTRACT

Into the next century, global climate change models predict chronic and acute thermal fluctuations to increase in intensity and frequency, especially in freshwater environments. Recent studies identified that maternal stress signals may alter offspring phenotype, which may confer some adaptive benefit when offspring experience similar stressful environments (Environmental-Match Hypothesis). Using this framework, I investigated if elevated rearing temperature conditions and maternal stress signals produced predictive adaptive responses through modifications to gene transcriptional profiles. I exposed Lake Ontario Chinook salmon (*Oncorhynchus tshawytscha*) eggs to a biologically relevant maternal stress signal (1000ng/mL cortisol bath or control) and divided eggs across two rearing temperatures that mimic current ambient and future (+3°C) thermal environments. We then evaluated gene transcriptional profiles and survival of offspring at three key developmental stages (i.e., eyed-egg, alevin, and fry) and gene transcription profiles in response to an acute, multiday thermal stressor (+9°C/day) later in life (i.e., parr). Overall, we found elevated rearing temperature environments altered transcriptional responses both during development and later in life. Elevated temperatures increased mortality and prenatal cortisol did not rescue survival of offspring reared under elevated temperature conditions. Prenatal cortisol had no significant effect on gene transcriptional profiles for any developmental stage, or in response to an acute thermal stressor later in life under either rearing condition. Therefore, prenatal cortisol may not be an effective inducer of intergenerational plasticity in response to chronic and acute thermal stress.

DEDICATION

To my Mom and Dad, who always supported and believed in me

To my younger brother Mark, who always pushed me to be better

To my immediate and extended family, who always wished the best for me

To my dog Minx, who gave me comfort during long hours of writing

And

To my wife Lisa, who sacrificed so much to help me achieve this goal

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Chapter 1 - GENERAL INTRODUCTION

Environmental Climate Change

According to the Intergovernmental Panel for Climate Change (IPCC), the extent to which mean global average temperatures are increasing cannot be explained by natural forces (such as changes in solar output or volcanic aerosols) alone, but rather are more likely the consequence of continued deposition of anthropogenic greenhouse gases (>90% probability of occurrence) (Rosenzweig et al., 2008). Anthropogenic greenhouse gases have already been well documented for their link to large-scale mean temperature increases over the 20th century (Stott et al., 2010; Tett et al., 1999; Karl & Trenberth, 2003). Using data collected by the IPCC, global models predict warming ranges between 1.7°C to 4.9°C from 1990 to 2100, with a 90% probability interval (Wigley & Raper, 2001); variability arises from uncertainties within the models as well as future anthropogenic emissions. These increases in average temperature are resulting in increased intensity of extreme weather events (Walsh et al., 2016), shifts in biodiversity ranges (Cheung et al., 2009), reduction in global ice coverage (Karl & Trenberth, 2003), increased rates of stratification and hypoxia in aquatic ecosystems (Collingsworth et al., 2017), and many other direct and indirect factors.

In aquatic ecosystems, global surface water temperatures have increased in ocean systems by approximately 0.2°C per decade for the last three decades, similar to model predictions for global climate change from the 1980's (Hansen et al., 2006). The observed ocean trends have been consistent with model predictions due to continued input and refinement but do not accurately represent small regional water bodies such as lakes and rivers (Ficke et al., 2007). Often, lakes and rivers experience temperature fluctuations to a greater extent, on smaller

temporal scales, than ocean systems due to a lower volume of water to mitigate the effects of increasing heat deposition into the system (Austin & Colman, 2007). Concerns escalate when we focus on the disproportionate amount of biodiversity that exists within these systems. Freshwater systems alone can account for approximately 10% of all known species and almost 1/3 of all vertebrates (Strayer & Dudgeon, 2010). A vast majority of aquatic vertebrates are also ectothermic, which means they rely on environmental heat sources to regulate their body temperature and metabolic activity (Gillooly et al., 2001). Given these combined sets of conditions, aquatic ectotherms (like many fish species) are expected to be particularly vulnerable to chronic and acute thermal stressors on both a seasonal and a daily basis (Paaijmans et al, 2013).

Environmental Stress

In vertebrates, one mechanism used in response to environmental variation that alters an organism's functioning (i.e., environmental stressor) is through a physiological cascade to aid in maintaining homeostasis (Creel et al., 2013) until the stressor is removed through an adaptive behavioural response (Monclús et al., 2005; Somero, 2010) or an alteration to the organism's developmental, or life-history strategy (Bateson et al., 2014; Wingfield et al., 1998). The combined direct and indirect effects of global climate change described above can result in behavioural or physiological responses to mitigate the effects of the environmental stressor. What often mediates these responses are hormonal cascades, and one of the hormones most associated with a stress response are glucocorticoids (GCs) (Bonga, 1997; Oakley & Cidlowski, 2013; Schreck & Tort, 2016). Circulating GCs are transiently produced by the body in response to baseline energy demands to maintain natural homeostasis by mobilizing available energy sources through binding to nuclear glucocorticoid receptors in a number of tissues in the body

(Caratti et al., 2015). When homeostasis is disrupted, this is often termed "stress" and GC production/release is increased to make energy sources more readily available to maintain homeostasis while the stressor is present (Vyas et al., 2016). All organisms exposed to environmental stressors, such as chronic and acute thermal fluctuations at current or predicted future temperatures, may elicit a stress response that maintains homeostasic balance to prolong survival until the disruption is removed naturally or through physiological (e.g., gene regulatory mechanisms, hormonal regulations) or behavioural adaptations (e.g. migration, dormancy, microhabitat selection) (Somero, 2010; Williams et al., 2016). In ectothermic fish, the primary GC within the hypothalamic-pituitary-interreanal (HPI) axis is cortisol. As with all steroid hormones, cortisol is a lipophilic molecule, meaning it readily dissolves in fats and lipids and can therefore provide an important means by which females of oviparous (egg-laying) species have the potential to transfer information about environmental stressors to developing offspring (Haussmann et al., 2012; Li & Leatherland, 2012).

Maternal Stress and Developmental Plasticity

Females of oviparous species experiencing a stress response while undergoing oogenesis have been observed to deposit circulating cortisol into the lipid rich ovum which may act as a prenatal signal of a poor environment (Auperin & Geslin, 2008; Hayward & Wingfield, 2004; Love et al., 2009; Sopinka et al., 2015). If the prenatal signal correctly matches the environmental conditions the offspring experience, the subsequent stress-induced phenotypes (i.e., alterations in gene transcription, morphological adaptations, energy management) may be better matched to the stressful post-natal environment (Capelle et al., 2016; Giesing et al., 2011). This paradigm of environmentally-matched stress-induced phenotypes is the basis for the "Environmental-Matching Hypothesis" (Sheriff & Love, 2013) where by receiving a signal that reflects a future

environmental condition, the subsequent stress-induced phenotypes may be adaptive under the context of the post-natal environment. This may result in benefits such as increased survival and enhanced performance under those environmental conditions (Love & Williams, 2008; Sheriff & Love, 2013). A prenatal stress signal that does not actively reflect the experience of the female in the stressful environment generates a mismatch between offspring stress-induced phenotypes and the future environment which can result in a maladaptive response under the context of the post-natal environment (Love & Williams, 2008)

"Developmental plasticity" results from genomic responses to environmental cues, such as thermal and maternal stress, experienced early in development that ultimately generate lasting alterations to phenotypes as well as shaping later life-history traits (Pigliucci, 2006). These lasting alterations to an individual's phenotype can affect their developmental trajectory and alter future biological processes (i.e., metabolism (Le Roy et al., 2017), immune function (Barton, 2002), growth rates (Sinclair et al., 2016) and even stress responses (Brown et al., 2005), etc.) to environmental stressors (Nettle & Bateson, 2015). These alterations in cellular processes and physiological responses are exemplified in freshwater ectotherms (such as fish) since these processes are strongly linked to their thermal environment (van Vliet et al., 2013). Many freshwater fish are physiologically limited in the ranges that they can thrive in and are often times restricted in how far they can migrate to stay within their ideal ranges (Sheldon, 1998).

Developmental Plasticity in Fish

Fish have been well represented in studies of environmentally induced developmental plasticity (Fox et al., 2019; Le Roy et al., 2017; Muñoz et al., 2017; Scott & Johnston, 2012). Elevated water temperature during embryonic development is just one of many environmental cues that

has been shown to induce developmental plasticity in a number of phenotypic (including performance) traits in fish (Donelson et al., 2011; Mccormick, 1998). Likewise, exposure to elevated egg GCs is another environmental cue that has been shown to alter offspring phenotypes in fish (Capelle et al., 2016; Sopinka et al., 2017). When the two environmental cues are compounded, phenotypes such as decreased body mass and length, reduced growth rates, reduced yolk-sac volume at hatching, lower consumed volume of yolk-sac at first exogenous feeding, and morphological malformations can be exhibited (Eriksen et al., 2006; Lorenzon et al., 2001). Some of these phenotypes are often viewed as negative consequences of stress but under the environmental context of a chronically or acutely fluctuating elevated water system, exhibiting these phenotypes, such as reduced body size or energy consumption, reduces an individual's energy demand and therefore might be an adaptive response rather than maladaptive one (Clark et al., 2012; Daufresne et al., 2009; Pörtner et al., 2008). The phenotypes produced in response to the perceived environmental cues are long lasting alterations and produce various developmental trajectories which is often referred to as a "predictive adaptive response" (Bateson et al., 2014).

Transcriptional Phenotypes: Measuring the Degree of Developmental Plasticity

A single genotype can produce multiple phenotypes depending on the environmental conditions driving the variation in gene expressions (Pigliucci, 2006). With variation in gene transcription being the underlying mechanism associated with developmentally plastic traits (Wellband et al., 2018), post-genomic techniques are becoming an important tool for examining phenotypic plasticity and phenotypic variation (Cossins et al., 2006). Transcriptomics has emerged as a widely accepted technique used to examine physiological responses to multiple biotic, abiotic, and anthroprogenic stressors such as, thermal stress, toxicity, predation, etc. (Sagri et al., 2014;

St-Cyr et al., 2008; Tomalty et al., 2015). With advances in tools used to measure functional transcriptomic variation (ex., Openarray, RNA-Seq, microarray), it is becoming more common to utilize transcriptional plasticity to examine physiological acclimation and evolutionary adaptation (Cheviron et al., 2008). These techniques have become important for the characterization of developmental plasticity providing key observations of early and transient effects of gene transcription variation at either individual (candidate) genes or for a group of genes (transcriptional profile) (Aubin-Horth & Renn, 2009). The developmental trajectory taken based on the early environmental cues often results in variable responses from the norm, and now these variations can be quantified through analysis of gene transcription at various developmental stages (i.e. "time series approach").

While it is important to identify the pleiotropic effects environmental conditions can trigger for a single gene due to the complex interaction of an external stimuli and intracellular signaling (Moignard et al., 2013), an organism is made up of entire systems interacting with each other based on these variations as well. Using post-transcriptional techniques to quantify individual gene transcriptional phenotypes for multiple genes simultaneously, we can identify significant relationships of genes within, and across, multiple biological systems (ex., growth, metabolism, immune function, stress response, etc.) to get a more holistic understanding of how an environmental stressor results in a complex physiological response (Le et al., 2005; Martyniuk et al., 2013). With the advances in post-genomic techniques and tools, a better understanding of how gene-interaction relationships play a key role in biological responses to environmental stressors is now becoming more apparent, and the ability to quantify these changes allow us to identify the degree to which these stressors are impacting these systems (Smolen et al., 2000).

Model System

Pacific salmon species are increasingly being used as models for examining the effects of thermal and maternal stress (Scheuerell & Williams, 2005; Sopinka, et al., 2017; Taylor et al., 2016; Taylor, 2008). Two interesting characteristics that all Pacific salmon share is that they are oviparous and semelparous. As oviparous species, females can deposit circulating cortisol into the developing eggs before releasing the eggs into the environment for fertilization and incubation. Manipulation of this interaction between female and egg allows us to examine if an environmentally relevant maternal stress indicator adaptively modifies a transcriptional response for individuals reared in a thermally stressful environment. By examining transcriptional responses across multiple developmental stages, we may be able characterize developmentally plastic traits responding to the effects of thermal and maternal stress.

In semelparous species, females only have one chance to reproduce, which emphasizes the need to produce offspring that are able to withstand current, and future, environmental stressors (Kuijper & Hoyle, 2015). As such, in semelparous species whose offspring share the same rearing environment as their mothers, matching offspring phenotype to environmental conditions (i.e., the basis for the Environmental-Matching Hypothesis) becomes even more of an important means to maximize offspring performance or fitness. There is a greater emphasis on receiving an honest indicator about the offspring's future environment because these early environmental cues result in lasting alterations to an individual's phenotype and affects the developmental trajectory that the individuals then take during development (Nettle & Bateson, 2015). Given these combined sets of conditions, Pacific salmon are a strong model system to examine the effects of temperature and maternal stress through transcriptional phenotypes. For my thesis, I chose to examine the transcriptomic outcomes of possible maternal matching in

Chinook salmon (*Oncorhynchus tshawytscha*) due to their significant decline in total population numbers, as a suspected consequence of climate change effects (Crozier, 2016; Crozier et al., 2008), as well as their important ecological (Gresh et al., 2000; Rutherford et al., 2005) and economic (Gislason et al., 2017; Melstrom & Lupi, 2013) value in both their native and naturalized ranges.

North America's Northwest coast populations of Chinook salmon, like many other Pacific salmon, are declining in total numbers with some populations being designated as endangered or threatened (Northwest Fisheries Science Center, 2015; COSEWIC, 2018; Albaugh et al., 2011). While there are habitat restoration projects being implemented to aid in population recovery in depleted river basins, models indicate that the negative impact of climate change on habitat deterioration will limit the level to which the populations can recover (Battin et al., 2007). Due to concerns with how Pacific salmon populations are responding to increasing climate variation (Beamish & Mahnken, 2001; Crossin et al., 2008; Whitney, Hinch, & Patterson, 2013), Chinook salmon, as well as other Pacific salmon species, are becoming key models for testing hypotheses, such as Environmental-Matching, to identify whether prenatal signals of environmental quality can induce the types of phenotypic plasticity in offspring that will help to rescue populations from the effects of climate change. Since offspring and mothers share overlapping spatial environments (i.e., natal stream), signals from the mother during migration and spawning become important ques to communicate to the offspring about their future environmental conditions. Migration itself is stressful and elicits an increase in glucocorticoid production in female salmon (Baker & Vynne, 2014), but females have been shown to still remain sensitive to environmental stressors throughout this process and may elevate their glucocorticoid levels even further in response (Cook et al., 2014). Importantly, this continued

elevation in circulating glucocorticoids in the plasma during migration and spawning entering the lipid rich eggs may generate further effects on offspring phenotypes that enhance performance and survival in these overlapping spatial conditions both female and offspring share (Gagliano & Mccormick, 2009; Love & Williams, 2008).

The specific population of Chinook salmon examined here are a naturalized, potamodromous population that had major stockings in the 1960's in the Laurentian Great Lakes, and stocking continues to this day through the efforts of the Ontario Ministry of Natural Resources and Fisheries (OMNRF). For both the Northwest Coast, and the Laurentian Great Lakes populations, the types of environmental stressors which spawning mothers and freshwater offspring are facing are similar, making the Laurentian Great Lakes population a viable model for examining how Chinook salmon in general may respond to thermally elevated freshwater ecosystems. Climate change is one of the highest ranking environmental stress factors for its impact on biodiversity in the Laurentian Great Lakes and also plays an indirect role in the most impactful stressor affecting the Great Lakes ecosystem (i.e., invasive and nuisance species) (Smith et al., 2015). As mentioned previously, lakes and rivers experience chronic and acute temperature fluctuations, on a shorter temporal scale, to a greater extent than do larger bodies of water, like oceans (Austin & Colman, 2007). Through examination of a population that experiences these chronic and acute thermal variations at a higher frequency of occurrence, we may be able to identify how a maternal stress indicator modifies transcriptional phenotypes of offspring to enable them to cope with climate change induced habitat deterioration. As a result, these investigations can shed light on possible adaptive responses to common environmentally stressful conditions that both populations experience. Given these combined thermal and temporal conditions in smaller bodies of water, we would expect there would be some naturally

selected maternal effect mechanism to aid offspring to match to their expected future environment (Narum & Campbell, 2015; Salinas & Munch, 2012). This mechanism should become even more honed in a species that cannot continue to provide input throughout development and only have one chance to produce offspring that are able to adapt to environmental variation. Through examination of a species that can only provide maternal care through prenatal preparation to a future environment, we can identify the effect of prenatal stress on developmental plasticity in these future thermally stressful environments.

Thesis Objectives

The overall goal of this thesis is to identify how developmental plasticity (identified through transcriptional response) of juvenile Chinook salmon responds to thermal and maternal stress, and whether these responses appear to match or mismatch fish for future thermally stressful environments. As previously mentioned, when the two environmental cues of thermal and maternal stress are compounded, phenotypes often develop that alter an individual's physiological response to the environmental conditions they are experiencing (Eriksen et al., 2006; Lorenzon et al., 2001). For Chapter 2, we took an applied approach to examine the Environmental-Matching Hypothesis to determine whether individuals reared at elevated water temperatures or exposed to a maternal stress signal show plasticity in specific functional systems (i.e., growth, metabolism, immune function, and stress response) that may give some indication of developmentally plastic responses over multiple developmental stages (i.e., eyed-egg, alevin, and fry). We also examined whether the interaction of the two treatments could rescue offspring mortality in response to the chronically elevated thermal conditions. To do this, a 2x2 experimental design was developed to examine all possible combinations of thermal and maternal stress (**Figure 1.1**). This design allows for the comparison in transcriptional profiles

across groups that received a maternal stress signal matching the thermal environment they developed under (i.e., control:current and dosed:elevated) and those which received a signal incorrectly indicating the offspring's future thermal environment (i.e., control:elevated and dosed:current). Transcriptional analysis was performed on whole body samples. We examined 26 candidate genes associated with growth, metabolism, immune function, and stress response because the literature often focuses on these groups when examining some form of stress response (Toews et al., 2019; Tomalty et al., 2015; Wellband et al., 2018). What is not as frequently discussed is maternal stress as a possible modifier of gene transcription in association with some other form of environmental stressor. Through examination of plastic gene transcriptional responses, we can use the framework of the Environmental-Match Hypothesis to identify if maternal or thermal stress have any capacity to alter transcriptional phenotypes, indicating their ability to modulate molecular processes (Cossins et al., 2006; Gracey et al., 2004; Wellband et al., 2018). We also examined if prenatal cortisol had some capacity to rescue these early developmental stages from the negative effects of the elevated rearing temperatures and reduce mortality under these environmental conditions.

For Chapter 3, we aim to identify whether individuals which were reared in the predicted future elevated conditions and received a maternal stress signal exhibited adaptive, compensatory, or detrimental transcriptional responses to an acute thermal challenge during a later life history stage (i.e., parr). Using the same 2x2 experimental design, individuals from all treatment groups were exposed to either a temperature spike of 9°C over a 9 hr period for 3 consecutive days or maintained at a constant temperature (control) for the same period of time. Transcriptional analysis of the candidate genes then be used in hierarchical approach to analyze the rapid response of hepatic and muscle tissue at varying levels of transcriptional profiles to the

acute thermal stress, to give an indication of possible developmental phenotypes that might have persisted into later life history stages (Komoroske et al., 2015; Wiseman et al., 2007). The candidate genes being examined fall under the same functional gene categories (i.e., growth, metabolism, immune, and stress) as in Chapter 2.

Combined together, these chapters identify if a maternal stress indicator can rescue or reduce the negative impacts of chronic and acute thermal stress on developing embryos in combination with possible predictive-adaptive responses elevated rearing conditions might produce through modification of gene transcriptional phenotypes. Temperatures have significantly increased in the Laurentian Great Lakes over the past 50 years and are expected to continue to increase in average temperatures over the next 50 years (Dobiesz & Lester, 2009; Trumpickas et al., 2009). Sea temperatures (NOAA, 2019), and freshwater ecosystems in North America's Northwest Coast (Melack et al., 1997) are also experiencing these continued thermal increases. These effects are reducing the nutrients deposited into the ecosystems, through decomposition of deceased post-spawning males and females, and affected the overall composition of biodiversity (Schindler et al., 2016). If maternal stress is a mechanism that can modify transcriptional phenotypes that might confer some adaptive benefit under future climate predictions, and this can be quantified through functional genomics, we may be able to implement these practices in developing conservation techniques as well as when rearing hatchery fish that will be used for stocking efforts geared towards current, and future environmental variation. This experiment is important because it adds to a growing database of transcriptional profiles related to elevated thermal conditions expected under climate change. Building larger transcriptomic databases increases the efficacy of models that try to relate transcriptional variations to the phenotype through examination of which genes, or functional

groups of genes, are exhibiting variation when that phenotype is present (Aubin-Horth & Renn, 2009).

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Figures

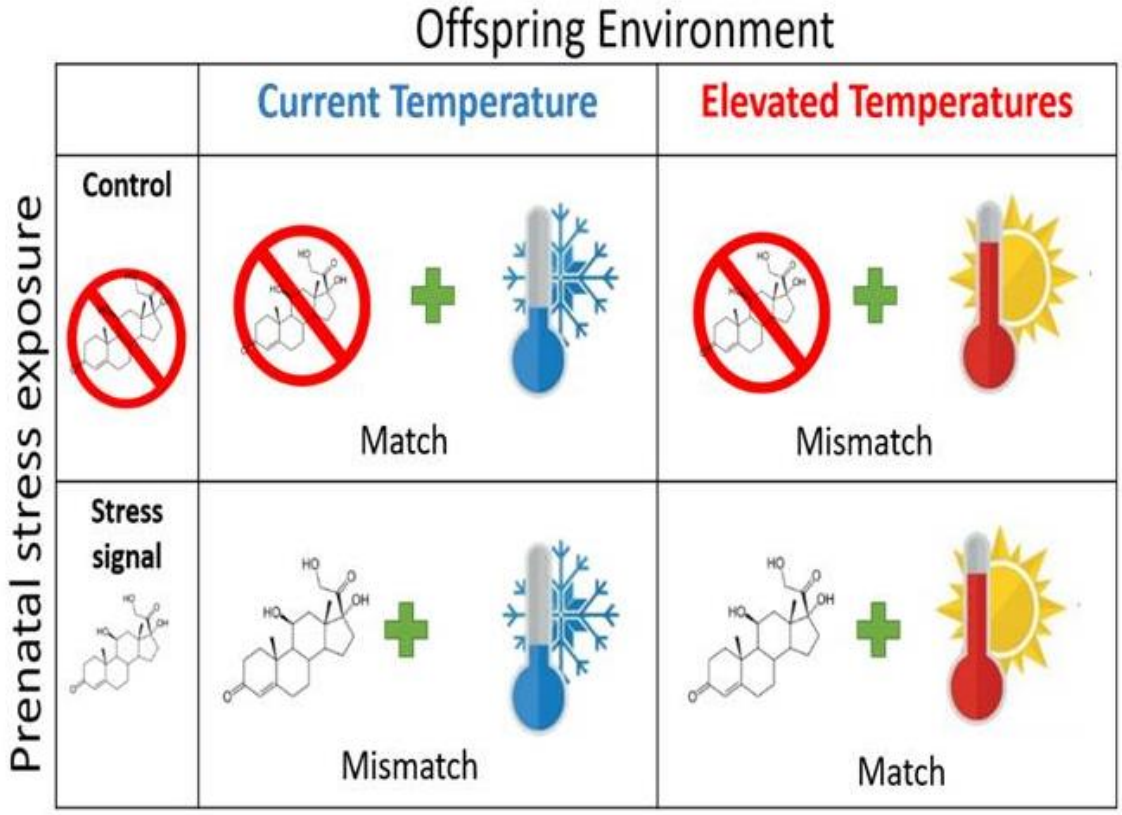

Figure 1.1 - Overall experimental design for Chapters 2 and 3 showing the 2x2 prenatal and thermal stress interactions. In a benign, current thermal environment, receiving no prenatal stress signal would correctly match to the offspring's environment. The opposite is true for the elevated environment, whereby receiving a prenatal stress signal correctly matches the environmentally stressful conditions the offspring are experiencing. These interactions often result in varying phenotypes that may be adaptive in the context of the environmental conditions. This is the concept of the "Environmental-Matching Hypothesis". The image of the experimental design came from Theresa Warriner's M.Sc. thesis (2019).

Chapter 2 – EFFECTS OF PRENATAL CORTISOL AND CHRONIC THERMAL ELEVATIONS ON TRANSCRIPTIONAL PROFILES DURING EARLY DEVELOPMENT IN CHINOOK SALMON

Introduction

Human Induced Rapid Environmental Change (HIREC) is creating multiple novel stressors, as well as combinations of stressors, at extremes that wildlife have not experienced in their evolutionary history (Sih et al., 2011). Anthropogenic impacts are directly, and indirectly, affecting habitat degradation (Poff et al., 2002), species invasions (Salo et al., 2007), pollution (Dudgeon et al., 2006), and of course global climate change (Romero-Lankoa et al., 2014). According to the Intergovernmental Panel on Climate Change (IPCC), anthropogenic factors have already had a serious impact on global temperature changes over all continents for more than a 50-year period (Bindoff et al., 2019). In aquatic ecosystems as a whole, average global surface water temperatures are increasing (Wigley & Raper, 2001; NOAA, 2019) and these sustained increases are having a diversity of abiotic and biotic impacts, including increases in the intensity of extreme weather events (Walsh et al., 2016), habitat degradation (both ecologically and chemically; Butchart et al., 2010; Mallin et al., 2006; Vollenweider et al., 1974), reducing annual ice coverage (Karl & Trenberth, 2003), resulting in shifts in biodiversity ranges (Cheung et al., 2009). Alarmingly, in smaller bodies of fresh water, such as lakes and rivers, decadal increases in average water temperatures are more than twice that of the oceans (Kintisch, 2015). The result is shorter periods before lake stratification occurs and higher summer temperatures in these bodies of water, which will lead to increased thermal stress on organisms in these environments (Austin & Colman, 2007). Within freshwater ecosystems, these elevated temperatures stand to impose a significant risk to a large proportion of the ectothermic organisms

within them, given that their body temperatures and metabolic activity are directly tied to environmental temperature (Gillooly et al., 2001). As a result, many fish species are now facing chronic and acute temperature fluctuations beyond their species' preferred temperature ranges (Ficke et al., 2007), and these fluctuations are expected to become more frequent as climate change persists (Jeffries et al., 2014). However, determining whether these organisms have the adaptive capacity to respond to HIREC, and if so by which mechanisms, remains an ongoing challenge (Beever et al., 2016; Funk et al., 2019).

Since environmentally-induced phenotypes may have a greater potential to result in adaptive responses than mutations (Moczeck et al., 2011; West-Eberhard, 2005), quantifying the degree of developmental plasticity to HIREC is increasingly seen as critical to determine whether organisms possess the adaptive capacity (Beever et al., 2016; Funk et al., 2019) necessary to respond to environmental change (Desmond, 2018). In response to elevated environmental stress, developing offspring of ectothermic fish could respond plastically in at least two ways. First, there is increasing evidence that environmental stressors experienced by the mother (i.e., such as impacts on maternal physiology induced by direct and indirect effects of climate change) can be transmitted to offspring via the egg in the form of elevated stress (glucocorticoid) hormones (Meylan et al., 2012; Taylor et al., 2016), inducing developmental plasticity that may benefit or even hinder the offspring in that same stressful environment (Capelle et al., 2016; Nettle & Bateson, 2015; Sopinka et al., 2016). Second, it is known that the development of eggs in warmer water temperatures induces a suite of developmental responses in fish (Murray & McPhail, 2009; Paaijmans et al., 2013; Zuo et al., 2012). Moreover, while plasticity in phenotypic traits such as body size, growth, behaviour, etc. are increasingly being examined within this more integrative, multi-stressor (i.e., pre- and post-natal stress) framework,

fewer studies have examined the underlying mechanisms (e.g., gene transcription) by which these interactions produce variation in offspring phenotypes. Given the pleiotropic nature of the genes that underscore environmentally-initiated phenotypes (e.g., Chesler et al., 2005; Kim et al., 2016), and the advancements in high throughput genomics (Connon et al., 2018; Houde et al., 2019; Lowe et al., 2017) a focus on transcriptional mechanisms that quantify the degree to which an organism can respond to an environmental stressor within their current genotypic constraints seems overdue. Through approaches such as quantitative real time polymerase chain reactions (qRT-PCR), researchers can target specific genes of interest to use as biomarkers to identify the degree to which organisms alter their gene expression in response to environmental conditions (Akbarzadeh et al., 2018; Olsvik et al., 2013). The ability to quantify variation in transcriptional profiles for genes across multiple functional groups (e.g., growth, metabolism, immune function, stress response, etc.) at multiple time periods through development is what makes qRT-PCR an indispensable tool when performing a 'Time-Series' approach for identifying how and why environmentally stressful conditions alter early, and transient, responses to environmental stressors (e.g., chronically elevated temperatures, prenatal stress, etc.; Aubin-Horth & Renn, 2009). The goal of this type of mechanistic approach is to examine the underlying early developmental mechanisms which respond to differential prenatal stress signals to ultimately alter an individual's response to an overall decline in environmental quality. As such, examining variation in gene expression in response to interacting stressors that developing offspring experience may help to determine the role that prenatal stress signals play during embryonic development: perhaps a larger role than previously appreciated in modifying future (possibly adaptive) responses to chronically elevated thermal regimes that offspring will face under climate change.

Using an experimental approach in a semelparous Pacific salmonid - Chinook salmon (*Oncorhynchus tshawytscha),* our goal in the present study was to examine stage-dependent (i.e., eyed-egg, alevin, and fry) developmental plasticity in gene transcription in response to the altered environmental conditions resulting from exposure to i) maternal stress signals (i.e., prenatal exposure to elevated egg glucocorticoids - GCs) and ii) elevated water temperature. Using the evolutionary framework of the Environmental-Matching Hypothesis (Sheriff & Love, 2013) our aim was to identify the capacity for each individual environmental treatment (i.e., prenatal and chronic thermal stress) to modulate cellular processes in these aquatic ectotherms. Specifically, within the construct of this framework we are able to examine whether exposure to a maternally-derived stress signal (elevated prenatal GCs) induces predictive adaptive responses (Gluckman et al., 2005) in offspring to better match developmental phenotypes to expected future environmental stressors (i.e., elevated water temperature). Examining the mechanisms underlying this potential adaptive capacity of Pacific salmon to current and future climatic changes using this predictive framework is relevant for multiple reasons. From an applied (conservation) perspective, Pacific salmon are a relevant study model since wild native populations in North America are experiencing severe population declines, many of which have been attributed to the direct and indirect effects of increasing water temperatures (Crossin et al., 2008; Crozier et al., 2010; Isaak et al., 2012). From a mechanistic perspective, Pacific salmonids are ideal study models for testing the effects of maternal- and temperature-stressors during development. This is because Pacific salmon are ectothermic and therefore highly susceptible to the effects of elevated water temperatures (McCullough et al., 2009) coupled with the fact that environmental stressors experienced by female Pacific salmon during migration can still increase circulating plasma GCs beyond that resulting from the stress of migration itself (Carruth et al.,

2000; Cook et al., 2014) and egg GCs (Mingist et al., 2007). Finally, maternal spawning grounds and the offspring's early developmental environment overlap spatially (i.e., a maternal stress signal has the potential to act as an honest indicator of the quality of the offspring's future environment; Love et al. 2013). To simulate exposure of eggs to elevated maternal stress during migration, we experimentally increased egg cortisol (the primary GC in bony fish; Sopinka et al., 2017) within a biologically relevant range (Auperin & Geslin, 2008; Sopinka et al., 2015; Capelle et al 2017). We then divided equal proportions of cortisol- and control-exposed eggs and reared them under temperature regimes that followed natural seasonal fluctuations of the natal river, but were maintained at either current ambient water temperatures or at elevated temperatures (i.e., +3°C above current) a level chosen based on predictions from climate change models for the next century (van Vliet et al., 2013). By taking advantage of advances in qRT-PCR technology, specifically nanofluidic taqman Openarray™ chip, we aimed to quantify how exposure to prenatal cortisol and development in elevated water temperatures altered fish transcriptional profiles through early development using gene transcription at selected known-function genes (Evans, 2015; Wellband & Heath, 2017). Based on previous research in salmonids examining developmental responses to environmental stressors, we chose a set of 26 genes spread across four key biological processes (i.e., growth, metabolism, stress response, immune function) to examine these transcriptional responses. We expected elevated temperature exposure to accelerate cellular and metabolic functions, since metabolism increases exponentially with increasing temperature in fish (Schulte, 2015), which often drives offspring to reach developmental stages earlier, and at reduced body size for early life history stages since more resources are being utilized for the response to elevated thermal conditions (Zuo et al., 2012). We also expected that genes associated with innate stress responses to increase in

expression (e.g., chaperonins, peroxidases, superoxide dismutase; Liu et al., 2018) to aid/rescue cellular activity due to elevated temperatures and the possible free radical accumulation associated with this stressor. We expected that immune gene expression would be reduced at elevated temperatures given that chronically elevated temperatures often impair immune function (Dittmar et al., 2014). As a result of this impairment, we also expected overall survival under elevated water temperatures to be lower (Daufresne et al., 2009; Whitney et al., 2013). However, based on predictions of the Environmental-Matching Hypothesis, we expected preparatory prenatal (egg GC) signalling to aid in mitigating possible negative effects associated with development in elevated thermal environments. Under elevated developmental temperatures, we would expect exposure to elevated egg GCs to result in a positive 'rescuing effect' of transcriptional responses in key biological processes (i.e., growth, metabolism, stress, and immune response) compared to fish raised under elevated water temperatures that did not receive the predictive prenatal stress signal, ultimately leading to increased survival. Overall then, within the context of this experimental design we aimed to answer two main questions; [1] do chronically elevated thermal conditions significantly alter gene transcription of important biological processes during early development, [2] does a prenatal stress signal, predictive of future stressful conditions, have the capacity to modulate gene transcription within those same biological processes to help mitigate some of the negative aspects of those deteriorating thermal environmental conditions?, and [3] does a prenatal stress signal have the capacity to rescue offspring from the negative effects of elevated rearing temperatures and reduce mortality for any early developmental stages? The importance of developing a foundational understanding of how chronic thermal stress modulates gene transcription profiles, and whether a prenatal stress signal may mitigate these responses, is an important step in evaluating how predicted future

environmental conditions will affect the underlying cellular processes during sensitive developmental stages in freshwater fishes.

Methods

Study Population

Intensive stocking of Chinook salmon in Lake Ontario began in 1967 by Ontario's Ministry of Natural Resources and Forestry to naturalize the species to the ecosystem to increase biodiversity, manage predator-prey interactions, and for recreational fishing (OMNRF, 2014). We focused on the Credit River breeding population (43°34'40.0"N 79°42'06.3"W), a tributary of Lake Ontario, during the fall (October-November) when mature fish migrate upstream to spawn. This population is relevant for this study since global climate change is one of the main challenges that aquatic species in the Laurentian Great Lakes will face (Collingsworth et al., 2017) and is predicted to worsen based on future global climate models (Austin & Colman, 2007; Wigley & Raper, 2001). Therefore, it is important to evaluate how this population responds to chronic and acute temperature increases, with the goal of eventually determining the effects of temperature stressors on the naturalized Great Lakes Chinook salmon as well as for Pacific salmon populations in their native ranges. We collected eggs and milt from eight females and eight males on October 17th, 2017. Gametes were expressed by applying pressure to the abdomen, the gametes were then placed in individual plastic bags and brought back on ice to the Great Lakes Institute for Environmental Research (GLIER) in Windsor, ON, for fertilization, cortisol exposure, and incubation. All work was approved by, and completed under, the University of Windsor Animal Use Project Proposal (AUPP: #15-15).

Egg Fertilization and Cortisol Treatments

Each female's eggs were separated into eight containers (90g or ~300 eggs per container) for fertilization, for an approximate total of 2400 eggs per female. Milt from the eight males was pooled in equal volumes to give each male similar likelihood of fertilizing each egg. We then added ~0.5mL of the pooled milt to each egg container and gently mixed the eggs. To activate the sperm, 60mL of the collected river water was added to each container (Lehnert et al., 2018). After a two minute incubation period, 2mL of 400mg/L concentration of cortisol *(*H4001, Sigma-Aldrich Canada Co*)* dissolved in 90% ethanol (HPLC grade, Sigma-Aldrich Canada Co) was added to half of the containers, along with 740mL of Credit River water, to generate a final cortisol concentration of 1000ng/L (Capelle et al., 2016; Sopinka et al., 2014). The other half of the containers were exposed to a vehicle only solution (ethanol and river water) to act as a control treatment. All the containers were exposed to their respective treatment solutions for two hours. The incubation times and concentrations were based on literature that produced environmentally relevant egg cortisol concentrations (Auperin & Geslin, 2008; Burton et al., 2011; Capelle et al., 2016; Sopinka et al., 2016). After the two-hour incubation period, the eggs were removed from the solutions and rinsed with dechlorinated water. A subset of three eggs were removed from each female prior to fertilization, as well as after undergoing either the cortisol or control treatments, to examine the effectiveness of the treatments compared with each female's background egg cortisol concentration.

Temperature Treatment

To determine the effects of elevated temperatures on offspring development, we developed two identical incubation systems with two different temperature regimes. One egg incubation stack

was maintained at the river temperatures the offspring would have naturally experience, based on monthly records collected from stations close to the spawning grounds of the Credit River by the Provincial Water Quality Monitoring Network (2010-2014). The second egg incubation stack was consistently maintained at +3°C above the current river temperature incubation stack to mimic future climate projections for the region (van Vliet et al., 2013). Both incubation stacks were adjusted throughout the rearing process to mimic seasonal fluctuations that they would naturally experience in the current river system, always maintaining the +3°C difference (**Figure 2.1**). Each incubation stack was equipped with seven trays which were subdivided into 16 (10.2cm x 7.6cm) cells. From each female, two cortisol, and two control treated groups were randomly assigned to each of the incubation stacks. The ~300 eggs from each cortisol treatment container, for each female, were randomly assigned to three different cells (i.e.,~100 eggs/cell) within their respective incubation stacks (**Figure 2.2**). This design resulted in four distinct treatment groups (control:current; cortisol:current; control:elevated; cortisol:elevated) and provided two replicates per female for each treatment to control for fertilization container effects, and three replicates for positional effects in the incubation trays.

Rearing and Sampling

During egg incubation, developmental progress and mortality were monitored every third day through visual inspection. Any eggs that had died between checks were removed, placed in labelled containers (with tray and cell ID), and stored in Stockard's solution (5% formaldehyde, 4% glacial acetic acid, 6% glycerin, 85% water) to quantify successful fertilization and at what stage a death occurred up to eyed stage (see below for stage descriptions). Once individuals reached the eyed stage, stage at death was visually evident. Total mortality (i.e., number of

offspring that died between fertilization and fry sampling) was recorded across development to be used later in survival analysis.

To determine the effects of incubation temperature and prenatal cortisol exposure on gene transcription in juvenile Chinook salmon, we focused on three important developmental stages during their early life: eyed-egg (304/317 ATU elevated and current, respectively), alevin (537/541 ATU elevated and current, respectively), and fry (868/848 ATU elevated and current, respectively). When offspring had reached these developmental stages, we collected three individuals from each cell in the incubation stack and placed them in a tube filled with 10mL of a high salt preservative (700g ammonium sulfate, 935mL ddH_2O, 40g sodium citrate, 25mL 0.5M EDTA, adjusted to pH of 5.2 using 1M sulfuric acid, filtered using 0.45µm membrane filters) per gram of sample. We stored tubes at room temperature for 24 hrs to allow the solution to permeate the tissue, and then moved the samples to -20°C to preserve their mRNA for future transcriptional analyses. Eyed-eggs were perforated to allow the high salt solution to interact with the tissue, and the alevins had their yolk-sacs removed to increase the solution:tissue ratio and to reduce the presence of proteins during future extraction. Since ectotherms develop at faster rates under elevated temperatures due to increased metabolic and cellular activity (Alderdice & Velsen, 1978; Zuo et al, 2012), we used accumulated thermal units (ATUs) to identify when the current temperature group had reached the same developmental stage as the elevated temperature group (Neuheimer & Taggart, 2007). We monitored the daily temperatures that offspring experienced using temperature probes (accurate to ±0.2°C) to identify how many ATUs were required for the elevated temperature group to reach the desired developmental stage. Once the current temperature group matched them in ATUs, they were removed and preserved (hish salt buffer) for future transcriptional analysis.

Cortisol Assays

We used enzyme-linked immunosorbent assays (ELISA; Cayman Cortisol kits; Capelle et al., 2016) to measure egg cortisol levels to ensure that the cortisol treatment successfully increased egg cortisol levels to mimic an environmentally relevant maternal stress signal. For each female, we measured cortisol in eggs sampled both prior to fertilization and after receiving the cortisol or control treatment (N=24). Eggs (n=3) from each treatment group within each female were weighed, homogenized in 1.2mL of assay buffer, and cortisol was extracted using 3mL of diethyl ether (Capelle et al., 2016). Samples were then agitated by vortexing for 30 seconds, centrifuged for 5 min at 3500 rpm and allowed to rest for 30 min, after which they were flash frozen at -80°C for 30 min. The supernatant was decanted, and the remaining sediment was left overnight to allow the rest of the liquid to evaporate. Samples were then reconstituted in 1.2mL of assay buffer and stored at -80°C for future analysis. Samples were run in triplicate at a 1:50 dilution and the cortisol concentration was measured at a wavelength of 412nm. Intra and inter-plate coefficients of variation (CV) were 5.0% and 22.5%, respectively.

Primer and Probe Development

The direct and indirect effects of elevated water temperature play a key role in cellular processes for aquatic ectotherms, and have been linked to important effects on fish growth, metabolism, immune function, and stress response (Forster et al., 2012; Gillooly et al., 2001; Smith et al., 2013; Yang et al., 2015). Due to temperature having such broad effects, we included candidate genes from four key physiological processes for transcriptional profiling. Most of our Taqman© primers and probes used were from Toews et al., (2019). However, a number of primer/probe combinations were developed for this study using cDNA sequences from the National Center for

Biotechnology Information (NCBI) using Chinook salmon transcript sequence or transcript sequences from species within the same genus (*Oncorhynchus*) or family (*Salmonidae*). Primer and probe sequences were designed using Primer Express 3.0 software and optimized for amplicon length, melting temperature, and minimal dimerization. Primers were tested for successful amplification using a gradient PCR to identify that primers functioned at target annealing temperatures (i.e., ≥61°C) for qPCR, and generated the proper amplicon length, using gel electrophoresis. See **Appendix A1** for full list of candidate gene primer and probe sequences.

mRNA Extraction and cDNA Synthesis

A total of 417 fish samples (140 eyed-egg, 138 alevin, and 139 fry) were included in the gene transcription analysis. RNA extraction from whole body samples (average mass; eyed=10mg, alevin=38mg, fry=347mg) was performed using TRIzol™ Reagent (Ambion) following manufacturing protocols (Invitrogen) with slight modifications. First, while eyed and alevin samples followed the protocol exactly, fry were too large to homogenize in a single 2.0mL graduated tube (UltiDent Scientific) with 1mm silica beads using an unequal centrifugation bead breaker (Biospec Products). To work around this, each fry sample was dissected into approximately three equal proportions, placed in three separate tubes, underwent homogenization in TRIzol (following manufactures protocol), mixed into a single 15 mL Falcon tube, and then 750µL of the mixed solution was added to a new 2.0mL tube to complete the rest of the protocol. Secondly, modification to storage technique included RNA samples being stored in the 75% ethanol solution, rather than immediately resuspending in water, due to the extended period that RNA could be stored at higher temperatures (-20°C instead of -80°C) while still preventing degradation (Amisten, 2016). Prior to qRT-PCR analysis, RNA samples were resuspended in water and RNA concentration was measured by spectrophotometry on a NanoVue

spectrophotometer (General Electric Company); average RNA concentration was 945ng/µL. We synthesized cDNA from 250ng total RNA for each sample using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) using half reactions of the manufacturers protocol. All cDNA samples were stored at -20°C until qRT-PCR analysis.

Quantitative Real Time - PCR

Taqman® OpenArray® chips from Applied Biosystems were used to quantify variation in gene transcription using the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Each chip consisted of 48 subarrays, consisting of the 56 wells that were loaded with a solution consisting of 1.2µL cDNA solution, 1.3µL ddH_2O, and 2.5µL Taqman® OpenArray® Real-Time PCR Master Mix (Applied Biosystems) using the QuantStudio 12K Flex Accufill System (Applied Biosystems) to reduce intra-assay variation. The 56 wells were preloaded with primers and probes, as described above (**Appendix A**), to quantify gene transcription at all target genes within each sample. Each chip could therefore process 48 samples with each of the 28 candidate genes run in duplicate to give greater confidence in accuracy of Cq values. A total of nine chips were run to account for all 417 cDNA samples.

General Statistical Approaches

All statistical models were graphed for visual inspection of homogeneity (residual vs. fitted graphs), and normality (residual quantile-quantile plots). All datasets that did not meet these assumptions underwent transformation and were then reassessed. All statistical analyses were completed using R software, version 3.6.0 (R Core Team, 2019).

Survival Analysis

Mortality data collected throughout development were converted to a binary dataset using the buildBinary function in the *fullfact* package (Houde & Pitcher, 2016). The package assigned each deceased individual with a value of 0 and all living individuals with a value of 1. To analyze the effects of prenatal stress and rearing temperatures on survival during early development, we ran General Linear Mixed Models (GLMM) using a binomial distribution with temperature regime, cortisol treatment, and developmental stage as fixed effects, examining all of them individually and in three two-way interactions. The model also included female ID, cortisol treatment nested within female ID, and cell-position nested within tray as random effects. To test the fixed effects, we used likelihood ratio tests (LRT) by fitting full models for survival with maximum likelihood (ML) estimation to determine significance of the interactions by comparing the full models, with the interactions, to one that did not contain the interaction (Zuur et al., 2009). If no significant interactions were found ($p>0.05$), we removed the interactions from the model and we sequentially tested the fixed effects again using LRT with ML estimation. All significant interactions ($p\leq0.05$) remained within the model and underwent restricted maximum likelihood estimation (REML), and pairwise analysis within each developmental stage using False Discovery Rates (FDR) in the *emmeans* package (Pike, 2011).

Egg Cortisol Analysis

Using the results from the cortisol assay, we analyzed differences in egg cortisol concentrations due to our cortisol treatments (i.e., cortisol-dosed and control-dosed). The data underwent a Box-Cox power transformation (Osborne, 2010), using the *boxcox* function in the *MASS* package ($\lambda=0$, Venables & Ripley, 2003), to normalize the dataset. The effect of the cortisol treatments on

post-fertilized eggs were analyzed using a Linear Model (LM) with the cortisol treatment groups as fixed effects.

Treatment Analysis of Candidate Gene Transcription

Prior to all gene transcription analysis, the target genes were standardized based on relative primer efficiencies using LinRegPCR software, to determine relative individual gene Cq (Tuomi et al., 2010). All target gene expression levels underwent a Box-Cox power transformation to normalize the data (Osborne, 2010) for Linear Mixed Models (LMM), to ensure that data was as normal as possible within individual candidate genes. All target gene expression underwent the same transformation ($\lambda=0$) for Multivariate Analysis of Variance (MANOVA) and Principle Coordinate Analysis (PCoA), performed using *vegan* package (Oksanen et al., 2019), to ensure variance was due to similarities in response and not variation due to differing transformations. Sample sizes differed across genes due to mortality, or due to samples being removed if they exceeded the acceptable maximum for Cq (i.e., >30 cycles) since the stochasticity of the assay can generate detectable thresholds for a single copy of cDNA for the gene, within 33 nL of solution, above 30 cycles (Wellband et al., 2018).

To analyze the effects of our combined environmental stressors on gene transcription associated with our biological processes of interest (i.e., growth, metabolism, immune, and stress), we took a two-stage approach. First, we examined the effect of rearing temperature on gene expression within developmental stages within the control group (i.e., eggs did not receive cortisol exposure prior to incubation). We then examined the effects of cortisol at specific developmental stages within temperature treatments separately. This approach to our analyses allowed us to first establish whether elevated rearing temperature alone induces changes in our

candidate gene transcription. We then addressed the potential for exposure to prenatal cortisol could alter the established transcriptional responses within each of the two temperature regimes.

Taking a hierarchical approach to evaluating the effects of elevated rearing temperatures and prenatal cortisol, separate multivariate analysis of variance (MANOVA) were performed for complete gene profiles for each developmental stage. To test for temperature effects, all non-cortisol (control) individuals, across both current and elevated rearing temperatures, were subdivided by developmental stage. To test for cortisol effects, all developmental stages were divided into two separate datasets based on rearing temperatures to evaluate cortisol's capacity to modulate gene transcription under current ambient and predicted future thermal environments. All candidate genes that had sufficient numbers of samples were then loaded into the MANOVA as dependent variables to be compared for correlational effects of our independent variable (i.e., temperature treatment or prenatal cortisol). Female, and Tray nested within Female were not significant independent variables, they were originally included to account for possible residual effects and were therefore removed from the final models to provide more power to examine temperature effects. Any developmental stages that showed significant correlational responses to temperature or prenatal cortisol then underwent MANOVA for all functional gene groups (i.e., growth, metabolism, immune, and stress) to identify if one or more functional gene groups were being significantly more affected by the independent variable than others. Any functional gene groups significantly affected by temperature or cortisol then underwent individual candidate gene analysis, using linear mixed modelling (LMM), to identify if any genes transcriptional phenotypes were being significantly modulated by temperature or cortisol. Each LMM had either temperature or cortisol, depending on which treatment was significantly altering correlational responses at the functional gene group level, as a fixed effect. To account for residual effects due

to possible maternal differences, incubation positional biases, and treatment effects, the random effects were all nested within female ID and included tray ID, cortisol treatment replicate nested within cortisol treatments, and cell replicate. Random effects found to account for no residual variance were kept in the model to reduce the risk of falsely removing a variance component that is approaching zero, but not equal to it (Matuschek, et al., 2017).

Principal coordinate analysis (PCoA) was used in congruence with the MANOVA analyses for both the complete gene set and the functional gene groups, to visualize significant relationships identified in the MANOVA. Bray-Curtis dissimilarity was used to quantify the compositional dissimilarities between each of our candidate genes that were used in each MANOVA.

Results

Egg Cortisol Treatment

Cortisol treated eggs (n_{cort}=8) had significantly higher cortisol concentrations compared to the non-cortisol (control; $n_{control}$=8) treatment (LM: t = -6.7, p<0.001, cortisol: 46.6±12.8 ng/g, control: 8.1±4.4 ng/g). Cortisol levels in the dosed group are comparable to literature examining relevant environmental maternal stress signals in Pacific salmonids (Capelle et al., 2016; Sopinka et al., 2016, 2017).

Survival Analysis

Survival analyses indicated there was no significant interaction effect between cortisol and temperature treatments (LRT: χ^2 = 0.0038, p = 0.95; **Figure 2.3**), or between cortisol and developmental stage (χ^2 = 0.82, p = 0.84; **Figure 2.3**). However, there was a significant

interaction between temperature and developmental stage on survival (χ^2 = 9.8, p = 0.0074; **Figure 2.3**). After reviewing the summary of the model, there were significant differences in survival between the three developmental stages (p<0.001 all stages). After performing a False Discovery Rate (FDR) post-hoc analysis on the temperature effects within each developmental stage, there was a significant difference in survival due to temperature at the eyed stage (q=0.015). There was a significant decrease in survival under elevated thermal conditions when compared to the current thermal environment's offspring would experience at the eyed developmental stage.

Prenatal Cortisol and Temperature Effects on Candidate Gene Transcription

Examination of complete gene sets for our three developmental stages of interest (i.e., eyed, alevin, and fry) identified significant differences in candidate gene transcriptional responses at the eyed and alevin stages to the elevated water temperature treatment (**Table 2.1**). Visual inspection of the ordination map (**Figure 2.4**) indicates that most genes have a similar response with metabolic genes (COI and INSIG1) and stress gene (hsp70) being the most dissimilar at the eyed stage. At the eyed stage, both metabolic and stress functional gene groups were significantly affected by the elevated temperature treatment (**Table 2.2**). Visual inspection of the ordination maps for the metabolic (**Figure 2.5**) and the stress (**Figure 2.6**) functional groups would indicate that transcriptional response for COI, HK1, and INSIG1 were the most dissimilar compared to the other genes in the metabolic functional group while hsp90a and GPx1 were the most dissimilar in the stress functional group. However, individual gene transcriptional analysis for the candidate genes loaded into the metabolic and stress (**Table 2.3**) functional groups at the eyed-egg stage indicate the elevated temperatures do not significantly alter any individual gene transcriptional phenotypes. Similarly, the alevin developmental stage exhibited significant

effects of the temperature treatment on the complete gene set profile (**Table 2.1**) with MYO1A, INSIG1, and hsp70 clustering as the most dissimilar from the other genes (**Figure 2.7**). Gene transcriptional profiles for the metabolic functional gene group were significantly affected by the temperature treatment (**Table 2.2**), with COI, HK1, and INSIG1 being the most dissimilar in response for all candidate genes in this gene group (**Figure 2.8**). Again, no significant individual gene transcriptional responses were observed for this developmental stage for any metabolic candidate genes examined (**Table 2.3**).

There were no significant effects of prenatal cortisol on gene transcriptional profiles for complete gene set analysis at any developmental stage, across any temperature treatment (**Figure 2.4**). Because of this, no further analysis was necessary to evaluate hierarchical effects of cortisol throughout development.

Discussion

Our study of the impacts of early-life stressors on the transcriptional responses of developing Chinook salmon was an experimental approach to testing the Environmental Matching Hypothesis (Sheriff & Love. 2013), and it had three primary goals. First, to examine whether exposure to elevated water temperatures (compared to current conditions) modified the transcriptional phenotypes of offspring at three sensitive developmental stages. Second, to determine whether a prenatal stress signal had the capacity to further modify the transcriptional phenotypes of offspring already responding to the chronic thermal challenge. Finally, whether there were any fitness benefits to receiving a thermal or hormonal signal of future life stressors (i.e., decreased mortality rates). We identified effects of chronically elevated temperatures on gene transcriptional profiles for candidate genes within certain functional gene groups at the

eyed and alevin developmental stages, but no significant effects on individual gene transcriptional phenotypes. This would seem to support previous literature which has shown that elevated temperatures during development have the capacity to modulate functional processes evaluated in this thesis (see Introduction), but these significant modifications appear to be due to coordinated changes within biological functional groups and not due to individual gene responses. However, exposure to prenatal cortisol did not generate any significant effects on gene transcriptional profiles during early development under current ambient or predicted future thermal environments, suggesting prenatal stress signals may not be a potent modifier of the biological functions examined in this experiment, under either thermal regime. Finally, in terms of survival, while elevated temperatures significantly increased mortality at the eyed-stage, prenatal cortisol did not rescue offspring mortality under chronically elevated thermal rearing conditions, indicating prenatal cortisol does not confer some adaptive effect on fitness in response to our thermal stressor. Our results for transcriptional plasticity in response to early-life stressors shed light on the capacity of Chinook salmon (and possibly other temperate-water fish species) to respond to climate change.

Prenatal Cortisol Does Not Rescue Survival Under Chronically Elevated Thermal Conditions

Based on previous research, we expected to see a significant negative effect of the temperature treatment on early survival across development stages, with exposure to elevated prenatal cortisol potentially buffering these effects. We found a significant difference in mortality rates across the development stages, but only within the eyed stage did we find a significant difference in survival due to the temperature treatment. Eyed-eggs are extremely sensitive to environmental perturbations (Myrick & Cech, 2004; Pankhurst & King, 2010) which may account for why we observed the highest mortality rates occurring at this stage in response to our temperature

treatment. As for the significant differences in mortality across the developmental stages, hatching is energetically costly (Del Rio et al., 2019) and elevated temperatures affect rates of biological processes in ectothermic organisms, such as aerobic metabolism (Schulte, 2015) which may have resulted in the significant difference in survival between the eyed and alevin developmental stages. Temperatures would normally be lower during these periods and therefore cellular metabolic demand may have been too much to overcome while growing at these accelerated rates, ultimately resulting in greater mortality. The difference in mortality rates we detected between the alevin and fry stages may be due to the 'critical period hypothesis' (Hjort, 1914) where a disproportionate degree of mortality occurs during early developmental stages due to accelerated physiological changes, compared to later life history stages, associated with functions driving the offspring from endogenous to exogenous feeding. Interestingly, early survival in this study did not appear to be affected by the exogenous prenatal cortisol treatment, or the interaction between the cortisol and temperature treatments. There has been considerable research that has identified the negative effects of temperature on early survival in larval fish (Campos et al., 2019; Murray & McPhail, 2009; Tomalty et al., 2015). In Chinook salmon in particular, Warriner et al. (2020) recently showed significant negative impacts of temperature on early survival. This correlates well with our results from the eyed stage mortality rates being higher under elevated temperature conditions. Our results do not support prenatal stress signal acting as a potential mediator to improve offspring survival rates under elevated rearing temperatures. Interpreting the impacts of elevated prenatal cortisol on survival is also complex because prenatal stress signals have been shown to both positively and negatively impact early mortality rates across multiple taxa (Capelle et al., 2016; Love et al., 2005; Love & Williams, 2008; Mccormick, 1998; Meylan et al., 2005). In Chinook salmon in particular, exposure to

similar doses of prenatal cortisol has been shown to improve survival (Capelle et al., 2016) as well as negatively impact survival when paired with elevated incubation temperatures (Warriner et al., 2020). Our observed lack of any impact of prenatal cortisol could be due to a number of factors, including that the prenatal cortisol signal's ability to modify the offspring's response to elevated temperatures might be overwhelmed by the effects of elevated temperature.

Predicted Increases in Water Temperature Significantly Modulate Gene Transcriptional Profiles

Based on previous research across a diversity of fish species we predicted that being reared under chronic hyperthermic conditions would significantly impact gene transcriptional profiles across multiple functional gene groups in a diversity of ways (see Introduction). We did indeed identify overall gene transcriptional profiles had significantly different responses to elevated temperature for the developmental stages eyed-egg and alevin. Within these developmental stages, the candidate genes used as biomarkers for alterations to the functional gene groups metabolism (both eyed and alevin) and stress (eyed only) had significantly different overall gene transcriptional profiles. However, when examining the candidate genes at the individual level for variation in transcriptional phenotypes we found no significant effect of our temperature treatment on gene transcription, at either developmental stage. This indicates that elevated water temperatures have the capacity to alter overall gene transcription for the functional gene groups identified, but not to a significant level for any individual gene. Therefore, chronically elevated thermal rearing temperatures may be acting as a priming signal for these early developmental stages, altering overall gene transcription to cope with the correct intensity of response that is not reflected by a singular gene, but by multiple small changes across functional groups. These group differences may be an example of an adaptive response across functional gene groups since offspring sampled at these stages would have survived the initial high mortality in response

to elevated temperatures at the eyed-egg stage, and may be exhibiting greater thermal competency. Fish also have the capacity to acclimate to their thermal environments (Beaman et al., 2016; Donelson et al., 2011; Hutchison & Maness, 1979) which may explain why the fry developmental stage did not show significant alterations to their gene transcription profiles. Once they had achieved that stage in their development, the chronic increase in temperature would be a normal environmental condition and possibly no longer a perturbation as it was at earlier stages.

Prenatal Cortisol Does Not Modulate Early Gene Transcriptional Profiles

We exposed eggs to an exogenous cortisol treatment designed to mimic a natural prenatal stress signal that offspring might receive from a mother experiencing stressful migratory or breeding conditions (see Introduction). Under the current ambient and predicted future environmental thermal regimes, Chinook salmon offspring exhibited no significant differences in gene transcriptional profiles at any developmental stage in response to prenatal cortisol exposure. However, previous work has identified significant alterations to fish early life phenotypes in response to prenatal cortisol (Capelle et al., 2016; Mccormick, 2006; Nettle & Bateson, 2015; Sopinka et al., 2016). Therefore, we cannot discard the Environmental-Matching Hypothesis (Sheriff & Love, 2013), but possibly infer that the maternal stress signal was not appropriate to alter gene transcriptional profiles in response to current ambient conditions and predicted future thermal conditions Pacific salmonids might experience in their habitat ranges.

What Could This Mean for Pacific Salmonids?

Many important fisheries are being negatively impacted by elevated water temperatures (Free et al., 2019) and wild fish populations are in a significant decline on a global scale (Dulvy et al., 2006). Salmonids, including Chinook salmon, are among the wild populations that have seen

their numbers dwindle to the point of some populations being listed as endangered or threatened (Albaugh et al., 2011; Crozier, 2016). Early developmental stages are exposed to fluctuating thermal conditions due to the greater daily and seasonal variation in temperature in river systems relative to larger bodies of water such as lakes or oceans (Van Vliet et al., 2011; Webb & Nobilis, 2007). Elevated environmental variation of any kind has a significant impact on offspring developmental plasticity (Carter, 2005; Melack et al., 1997; Whitney et al., 2013), and maternal stress signals have been shown to act as preparatory cues to offspring concerning their future environmental conditions that alter developmental plasticity (Bateson et al., 2014; Sheriff & Love, 2013). Thus, it is important to evaluate to what degree predicted future climactic events and maternal stress signals may interact to alter cellular responses in juvenile Chinook salmon. In our study, we examined the effects of a maternal stress signal (i.e., egg cortisol) and elevated water temperatures on gene transcription, which is an underlying mechanism for the developmentally plastic traits observed in response to an environmental stressor (Wellband & Heath, 2017). Overall, the maternal stress signal did not have any significant effects on gene transcriptional profiles, but chronically elevated thermal conditions did. This could be an indicator that native and naturalized populations of Pacific salmonids are either unable to react with any more plasticity in their gene transcription, even when the maternal stress signal would indicate a need, or that prenatal cortisol is not the appropriate signal necessary to elicit alterations in plasticity under current ambient or future elevated water conditions. However, while we did not find wide-spread evidence that a maternal stress signal alters offspring gene transcription, indicative of environmental matching under chronically elevated thermal regimes for these developmental stages, we cannot preclude the possibility that later life history stages might have significantly different gene transcriptional responses to extreme environmental conditions (ex.

acute thermal fluctuations) when reared under these conditions. We identified significant alterations in functional gene transcriptional profiles for eyed-eggs and alevins in response to the elevated thermal conditions. While individual gene transcriptional analysis of the candidate genes in these functional gene groups indicated no significant effects due to the elevated temperatures, the overall gene transcription profile was affected. These alterations may be indicative of possible predictive-adaptive responses to important functional systems due to the environmental stressor. Analysis of whether these differences in functional gene transcription persist into later life history stages need to be evaluated and if these modulations to gene transcription are indeed possible examples of adaptive cellular response. Finally, we found no evidence that maternal stress signals dampen the effects of elevated temperatures, as our egg cortisol treatment did not enhance survival. Therefore, exposing fertilized eggs to prenatal cortisol would not be an advised technique to enhance fitness of offspring in response to chronic hyperthermic conditions.

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Tables

Table 2.1 - Complete Gene Profile Analysis of Temperature Effects

All candidate genes with significant numbers of samples expressing the gene at detectable levels (>70% total sample size) were loaded into a MANOVA as dependent variables to be compared in their response to the independent variable, temperature treatment. Eyed-egg and alevin were significant enough in differences for their complete gene sets to warrant further analysis at the functional gene group level. ***p<0.001, **p<0.01, *p<0.05, •p<0.1.

Fixed Effect	MANOVA Results	Eyed	Alevin	Fry
Temperature	Pillai trace	0.68	0.72	0.72
	F-value	2.20	2.56	1.75
	DF and Error DF	(1,18)	(1,18)	(1,15)
	p-value	**0.053 •**	**0.027 ***	0.13

Table 2.2 - Functional Gene Group Profile for Temperature Affected Developmental Stages

All candidate genes used in the complete gene profile analysis were loaded into their respective functional gene groups analyzed using a MANOVA to identify which gene groups were significantly different in candidate gene transcription due to temperature treatment. Metabolism (both eyed and alevin) and stress (eyed) were significantly different in their responses to the temperature treatment, indicating the need to evaluate individual gene transcriptional phenotypes for those gene groups. ***p<0.001, **p<0.01, *p<0.05, •p<0.1.

Developmental Stage	MANOVA Results	Growth	Metabolism	Immune	Stress
Eyed	Pillai trace	0.045	0.41	0.013	0.36
	F-value	0.91	3.43	0.39	2.87
	DF and Error DF	(1,39)	(1,34)	(1,59)	(1,31)
	p-value	0.41	**0.0070 ****	0.68	**0.024 ***

Alevin	Pillai trace	0.068	0.37	0.017	0.23
	F-value	0.98	3.04	0.50	1.69
	DF and Error DF	(1,40)	(1,42)	(1,57)	(1,33)
	p-value	0.41	**0.011 ***	0.61	0.16

Table 2.3 - Temperature Affected Functional Gene Group Individual Candidate Gene Analysis

Each candidate genes response to the temperature treatment for the functional gene groups significantly affected by the temperature treatment. Results are based on a Linear Mixed Model with temperature treatment as the fixed effect. To account for residual effects due to possible maternal differences, incubation positional biases, and treatment effects, the random effects were all nested within female ID and included tray ID, cortisol treatment replicate nested within cortisol treatments, and cell replicate. The independent variable was Cq values, normalized using BoxCox transformations, of each gene. All p-values are raw values from the ANOVA of the LMMs. ***p<0.001, **p<0.01, *p<0.05, •p<0.1.

Developmental Stage	Functional Gene Group	Candidate Gene	F-value	p-value
Eyed	Metabolism	COI	$F_{(1,18.7)} = 0.24$	0.63
		CPT1	$F_{(1,12.0)} = 0.00$	0.98
		CYP1A	$F_{(1,8.74)} = 0.24$	0.63
		ELOVL7	$F_{(1,40.3)} = 0.31$	0.58
		FAS	$F_{(1,10.5)} = 0.076$	0.79
		HK1	$F_{(1,9.25)} = 0.028$	0.87
		INSIG1	$F_{(1,57.7)} = 0.080$	0.78
Eyed	Stress	GPx1	$F_{(1,5.26)} = 0.061$	0.81
		GR-2	$F_{(1,5.56)} = 0.11$	0.75
		hsp70	$F_{(1,8.23)} = 0.25$	0.63
		hsp90a	$F_{(1,9.27)} = 0.20$	0.67
		metA	$F_{(1,10.1)} = 0.66$	0.44
		TrxR3a	$F_{(1,9.91)} = 0.32$	0.58
Alevin	Metabolism	COI	$F_{(1,7.47)} = 2.50$	0.15
		CPT1	$F_{(1,34.3)} = 0.11$	0.74
		CYP1A	$F_{(1,3.95)} = 2.51$	0.19
		ELOVL7	$F_{(1,37.8)} = 1.17$	0.29
		FAS	$F_{(1,8.70)} = 1.34$	0.28
		HK1	$F_{(1,6.67)} = 0.17$	0.69
		INSIG1	$F_{(1,9.20)} = 0.019$	0.78

Table 2.4 - Complete Gene Profile Analysis of Cortisol Effects

All candidate genes with significant numbers of samples expressing the gene at detectable levels (>70% total sample size) were loaded into a MANOVA as dependent variables to be compared in their response to the independent variable, cortisol treatment. No developmental stages, across any of the temperature regimes were significantly different in response to the cortisol treatment. Therefore, no further analysis was necessary. ***p<0.001, **p<0.01, *p<0.05, •p<0.1.

Fixed Effect	MANOVA Results	Eyed (Current)	Alevin (Current)	Fry (Current)	Eyed (Elevated)	Alevin (Elevated)	Fry (Elevated)
Cortisol	Pillai trace	0.26	0.60	0.69	0.45	0.60	0.64
	F-value	0.29	1.60	1.79	0.63	0.94	1.11
	DF & Err DF	(1,14)	(1,21)	(1,17)	(1,13)	(1,12)	(1,14)
	p-value	0.99	0.15	0.11	0.81	0.56	0.43

Figures

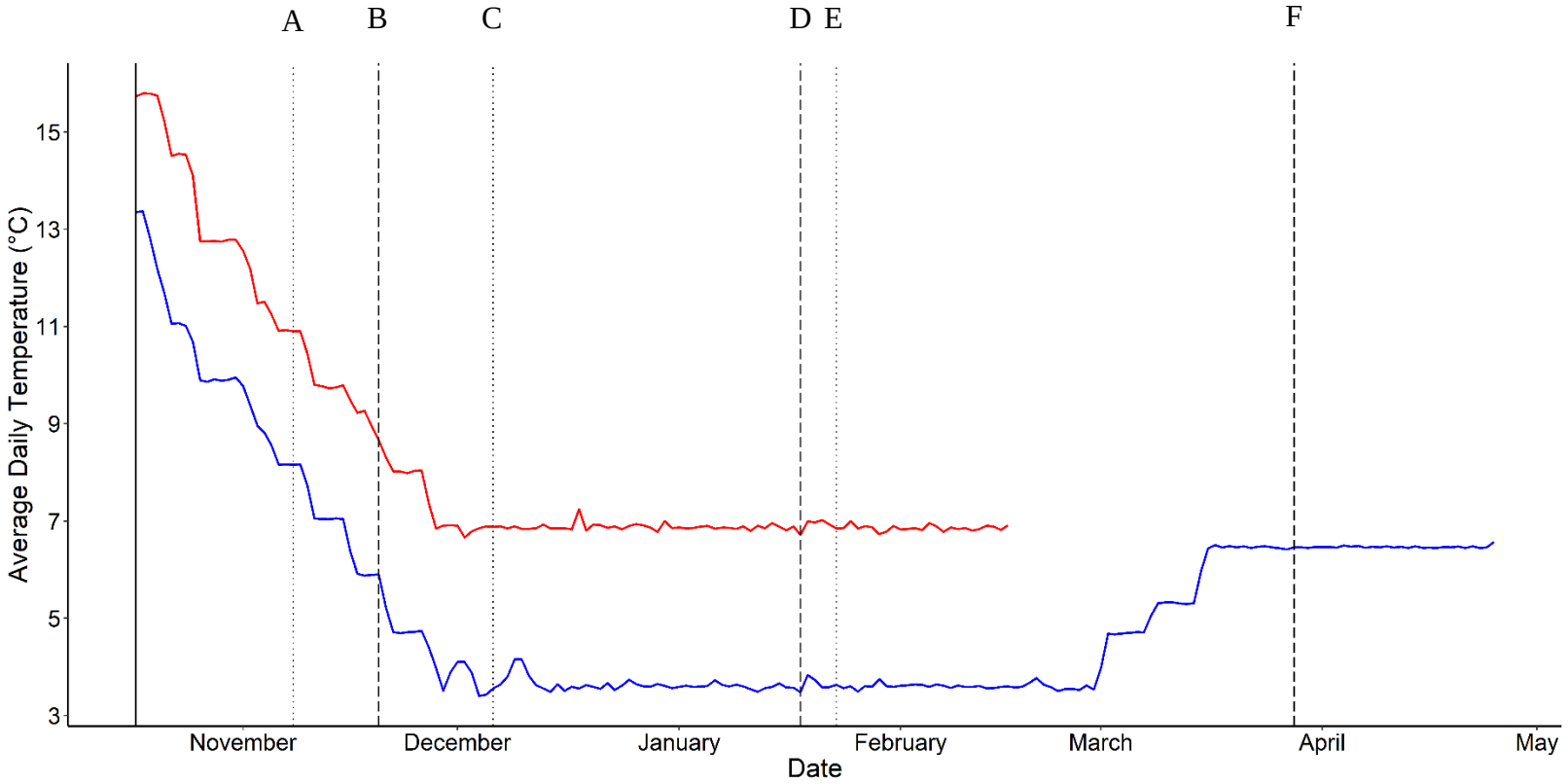

Figure 2.1 -The temperature regimes that the offspring experienced during development in the incubation stacks to mimic current and predicted future thermal conditions while following natural seasonal fluctuations. The bottom, blue line indicates the temperature regime for the current river condition stack while the top, red line indicates the temperature regime for the elevated incubation stack while. The letters indicate the time periods at which each of the developmental stages were sampled across both temperature regimes. [A] Elevated Eyed-Egg, [B], Current Eyed-Egg, [C] Elevated Alevin, [D] Current Alevin, [E] Elevated Fry, and [F] Current Fry

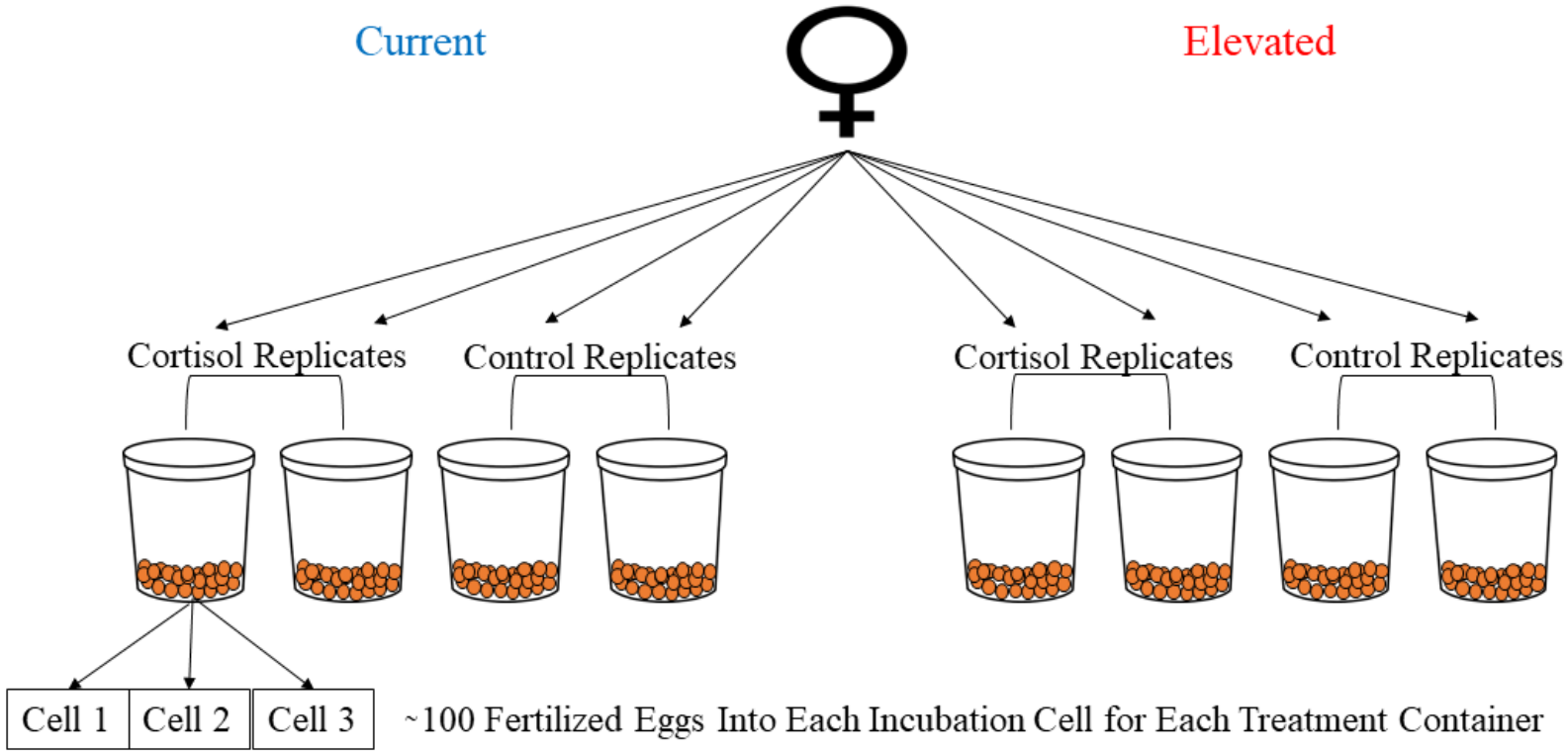

Figure 2.2 - Experimental design for egg cortisol manipulation and incubation. Each females eggs were equally dispersed across eight containers to receive either an environmentally relevant exogenous cortisol dosage, mimicking a maternal stress signal, or a control dosage, containing no cortisol. Each cortisol treatment was run in duplicate to provide replicates of treatment effects across the two temperature treatments (i.e., elevated or current). From each container,~300 eggs were randomly distributed to three cells within the incubation stacks to give a total of~100 eggs/cell to control for positional biases within the incubation stack during development.

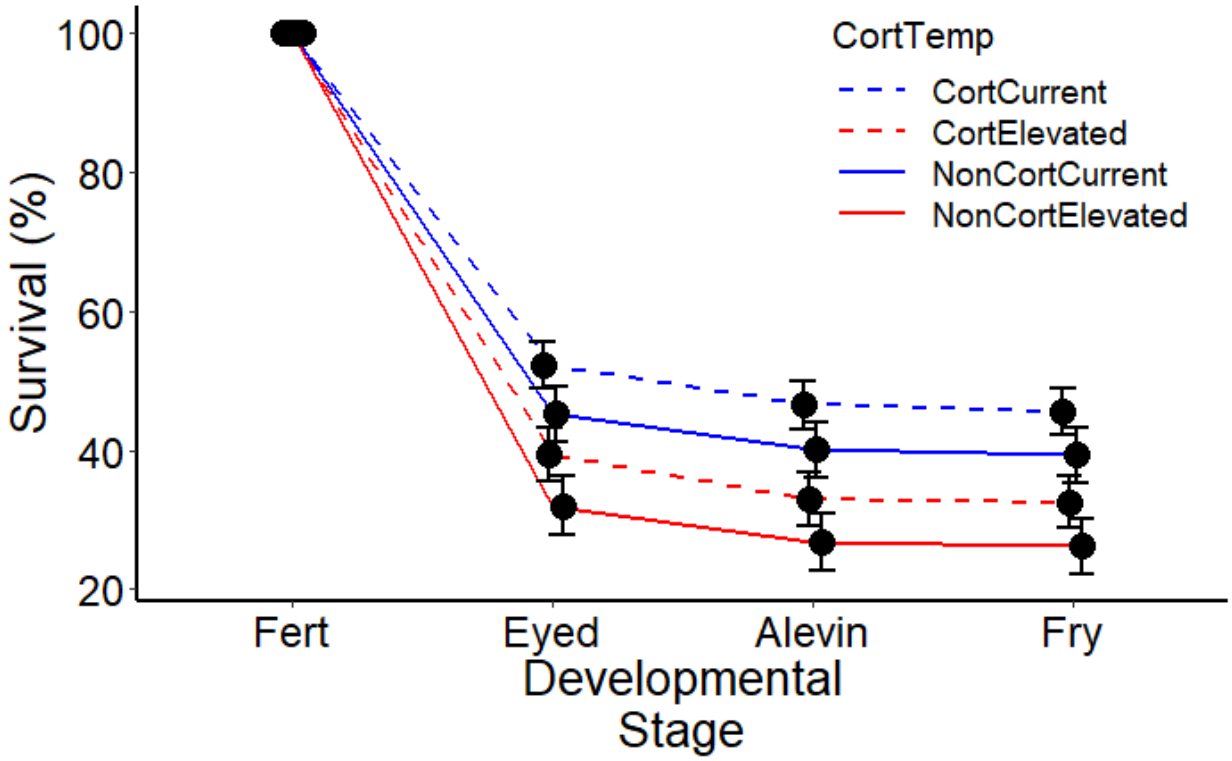

Figure 2.3 - Cumulative survival across developmental stages (eyed, alevin, and fry). There was no significant difference in survival across any of the developmental stages or temperature treatments due to the cortisol treatment. There was a significant interaction of temperature x developmental stage ($\chi^2 = 9.8$, $p = 0.0074$) with the temperature treatment evaluated within each developmental stage using False Discovery Rate (FDR) significantly reducing survival within the eyed stage ($q = 0.015$). No other developmental stages showed significant differences in survival due to temperature.

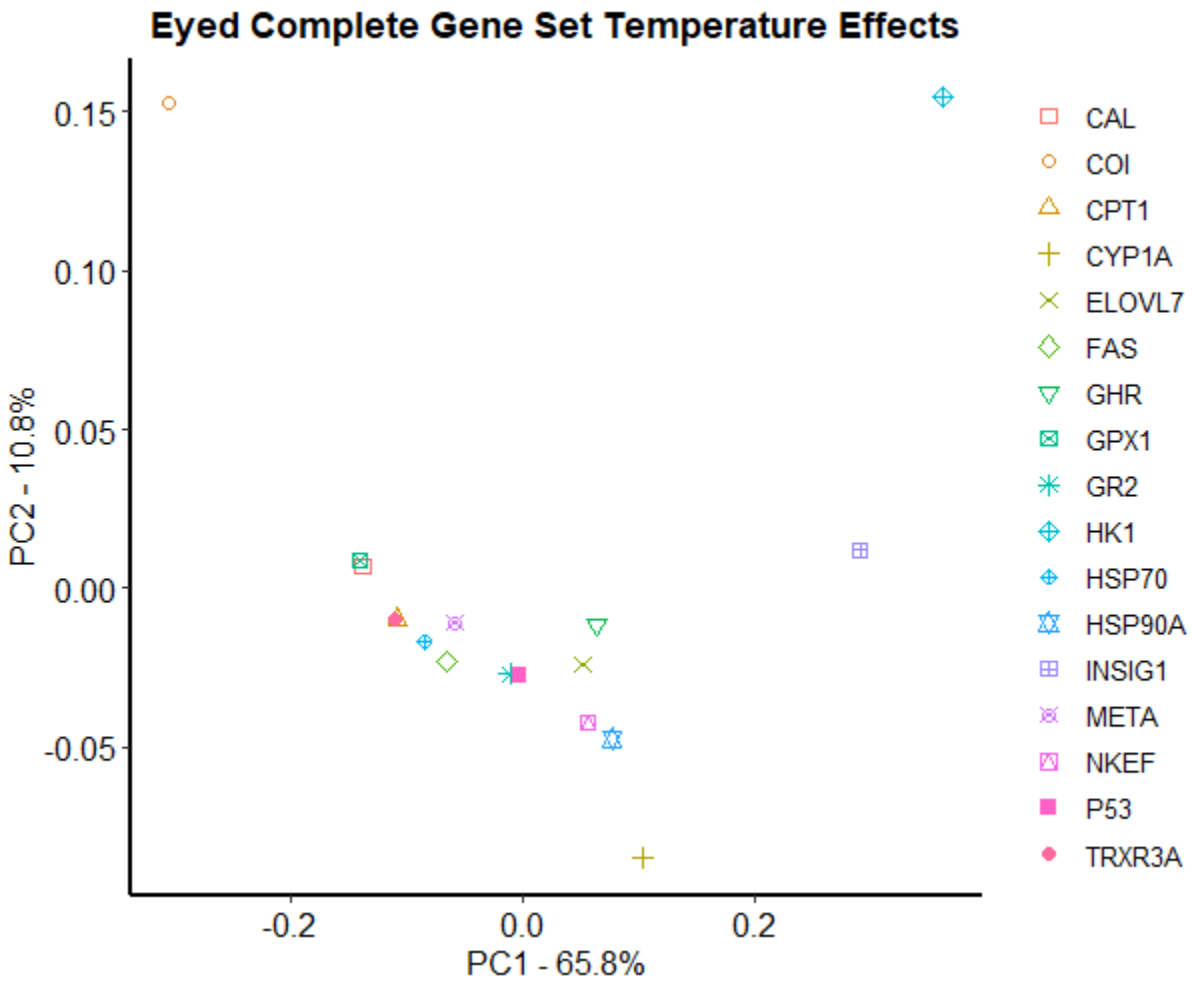

Figure 2.4 - PCoA ordination map depicting complete gene set analysis for the eyed-egg developmental stage. Ordination map indicates dissimilarities between candidate gene transcriptional profiles in response to the elevated water temperature treatment. The greater the distance between two vector points, the greater the difference in response to the temperature treatment. The greatest dissimilarities would be between COI, INSIG1, and HK1.

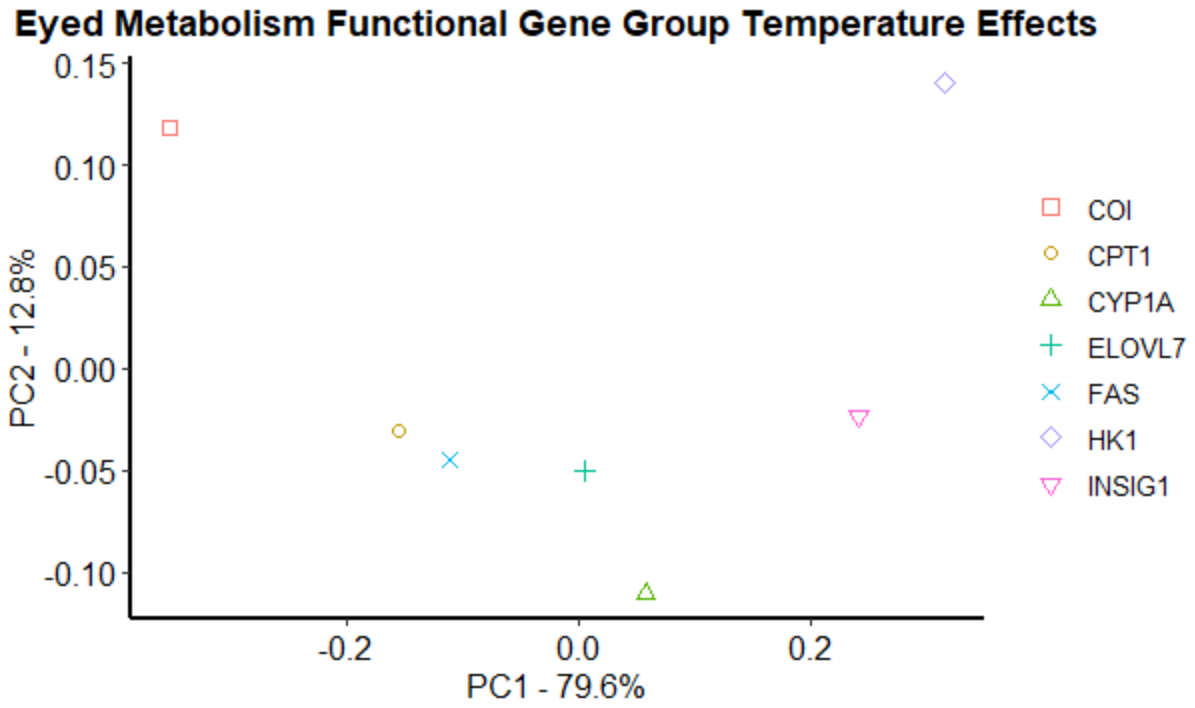

Figure 2.5 - PCoA ordination map depicting candidate gene transcriptional variation in the metabolic functional gene group for the eyed-egg developmental stage. Ordination map indicates dissimilarities between candidate gene transcriptional profiles in response to the elevated water temperature treatment. The greater the distance between two vector points, the greater the difference in response to the temperature treatment. The greatest dissimilarities would be between COI, INSIG1, and HK1.

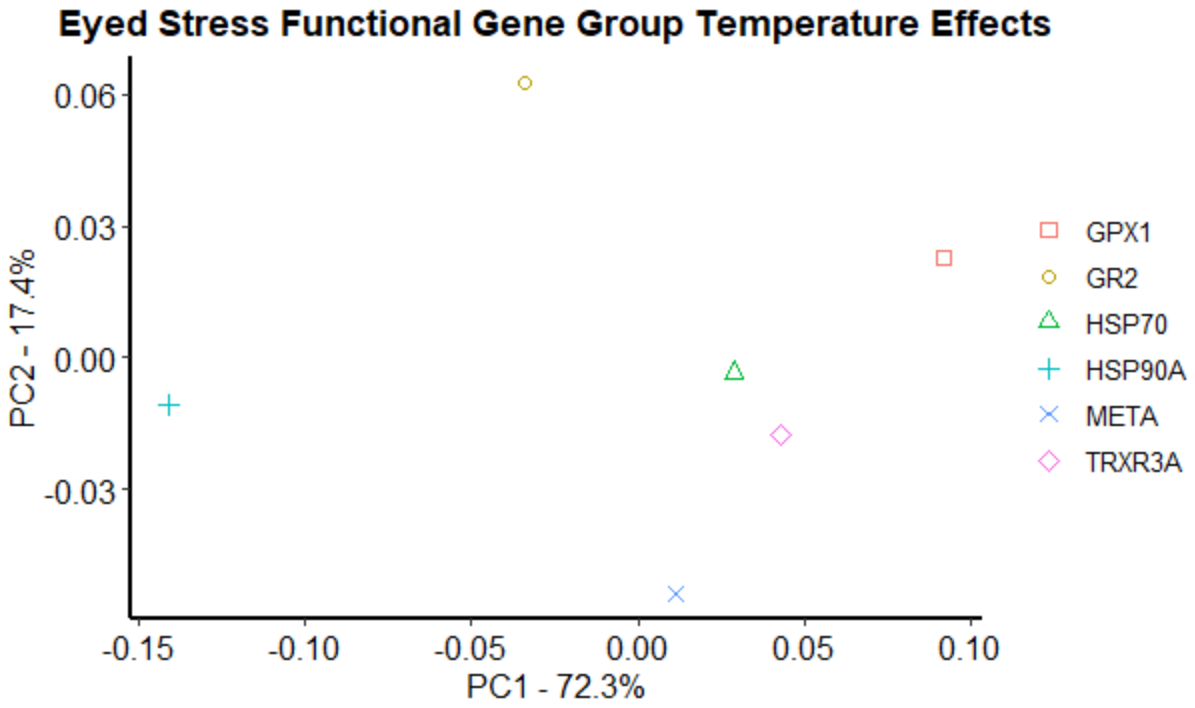

Figure 2.6 - PCoA ordination map depicting candidate gene transcriptional variation in the stress functional gene group for the eyed-egg developmental stage. Ordination map indicates dissimilarities between candidate gene transcriptional profiles in response to the elevated water temperature treatment. The greater the distance between two vector points, the greater the difference in response to the temperature treatment. The greatest dissimilarities would be between hsp90a and GPx1.

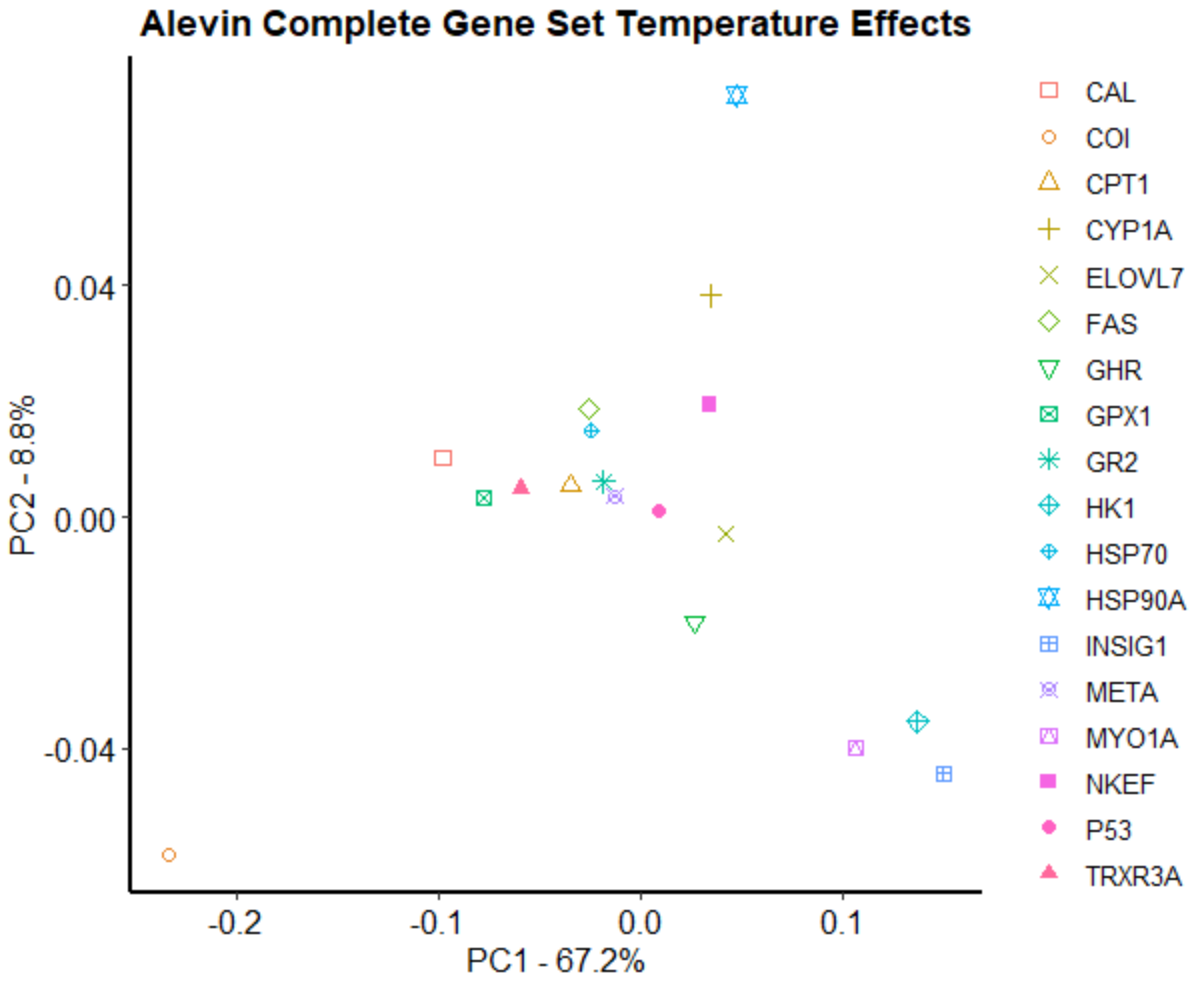

Figure 2.7 - PCoA ordination map depicting complete gene set analysis for the alevin developmental stage. Ordination map indicates dissimilarities between candidate gene transcriptional profiles in response to the elevated water temperature treatment. The greater the distance between two vector points, the greater the difference in response to the temperature treatment. The greatest dissimilarities would be between MYO1A, HK1, and hsp70 which cluster as outliers.

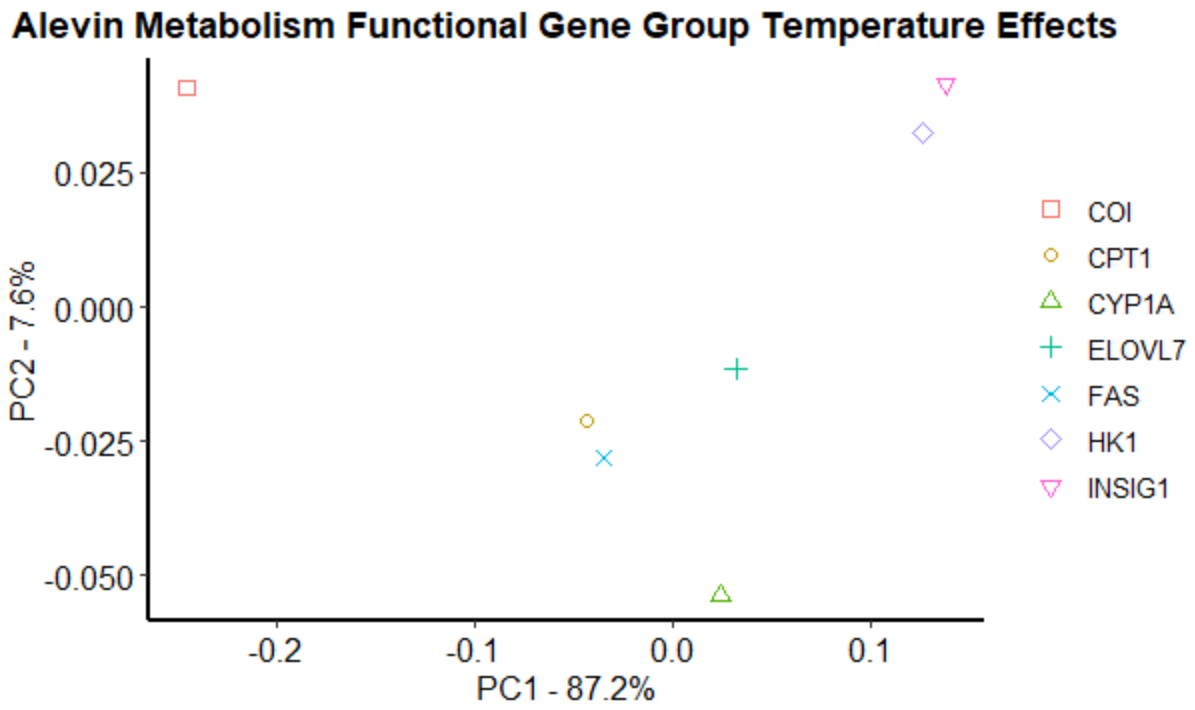

Figure 2.8 - PCoA ordination map depicting candidate gene transcriptional variation in the metabolic functional gene group for the alevin developmental stage. Ordination map indicates dissimilarities between candidate gene transcriptional profiles in response to the elevated water temperature treatment. The greater the distance between two vector points, the greater the difference in response to the temperature treatment. The greatest dissimilarities would be between COI, INSIG1, and HK1.

Chapter 3 – EXPOSURE TO ELEVATED DEVELOPMENTAL TEMPERATURES, BUT NOT PRENATAL CORTISOL, ADAPTIVELY MODULATES TRANSCRIPTIONAL RESPONSES TO A FUTURE THERMAL STRESSOR

Introduction

Human Induced Rapid Environmental Change (HIREC) is causing organisms to respond at their biological limits to novel combinations of stressors (Cloern et al., 2016; Heino et al., 2015; Nolan et al., 2018). In aquatic environments, global surface water temperatures are continuing to increase (Wigley & Raper, 2001; NOAA, 2019) that have collectively resulted in decreased ice coverage (Collingsworth et al., 2017; Karl & Trenberth, 2003), shifts in biodiversity ranges (Pörtner et al., 2008; Vinagre et al., 2015), alterations in dissolved oxygen content (Cloern et al., 2016; Mahaffey et al., 2020), and increased frequency of extreme weather events (Walsh et al., 2016; Zhang et al., 2019). For aquatic ectotherms like the majority of fish, elevated water temperatures can directly alter biochemical reaction rates as well as physiological responses (Campos et al., 2019; van Vliet et al., 2013). Altered responses to both the direct and indirect effects of chronically elevated water temperature can be further exacerbated by increasingly frequent acute temperature fluctuations that push physiological balance outside of species' thermal ranges (Jeffries et al., 2014). Unfortunately, due to a lower volume of water, freshwater systems have a lower capacity to buffer these acute fluctuations compared to large marine environments, meaning these events may occur more even frequently than expected (Austin & Colman, 2007; Kintisch, 2015; Zhang et al., 2016). If mortality is not a direct result of these acute fluctuations, organisms may respond by altering their behaviour or physiology in an attempt to mitigate the effects of the stressor and prolong survival during the perturbation (Crossin et al., 2008; Dengiz Balta et al., 2017; Dickens & Romero, 2016; Mahaffey et al.,

2020). Thermal conditions during early rearing can play a role in modifying developmental trajectories of offspring and their later responses to acute thermal fluctuations (Konecki et al., 1995; Scott & Johnston, 2012; Vinagre et al., 2015), often resulting in higher upper thermal tolerances, especially for smaller individuals (Messmer et al., 2017; Peck et al., 2009). Global climate change models predict an increase in average annual surface water temperatures for lakes and rivers over the next century (Muñoz et al., 2015; van Vliet et al., 2011, 2013), changes which may be integral for acclimating offspring developing in those environments for acute thermal stressors later in life. Furthermore, additional early (i.e., maternal effects) signals about the future environmental conditions they will experience may be passed from mother to offspring when their environments overlap spatially (Breuner, 2008).

The transfer of environmental information from mothers to offspring via maternally derived hormones (e.g., glucocorticoids) has been widely studied given their demonstrated capacity to alter progeny phenotypic plasticity in response to expected future stressors (Meylan et al., 2012; Monk et al., 2013; Sheriff & Love, 2013). In oviparous species, prenatal stress signals are often associated with offspring phenotypes of reduced size and mass at key life history stages (Burton et al., 2011; Hayward & Wingfield, 2004; Love et al., 2005; Sopinka et al., 2017), alterations to behaviour (Love & Williams, 2008; Sopinka et al., 2015), as well as physiological functions such as metabolic activity (Sloman, 2010), immune function (Li & Leatherland, 2012), and stress responses (McNeil et al., 2016; Van Kesteren et al., 2019). Some of these altered phenotypes have been found to result in higher survival within stressful environments impacted by HIREC (Brown et al., 2014; Capelle et al., 2016; Eriksen et al., 2011). As such, the role of hormonal prenatal stress signals as potential adaptive modulators of offspring phenotype has gained more attention recently, with the prediction that stress- signal

induced offspring phenotypes should have higher performance when offspring face a similarly stressful future rearing environment (i.e., Environmental Matching Hypothesis; Sheriff & Love, 2013). Identifying whether exposure to prenatal stress generates adaptive responses to acute thermal stressors (as predicted under the environmental match hypothesis) within the background framework of early development under is important for testing whether environmentally-induced phenotypes have the capacity to respond to expected changes into the future (Moczeck et al., 2011; West-Eberhard, 2005). Gene transcriptional variation is an increasingly targeted trait for determining how phenotypic response to both acute and chronic environmental stressors in a diversity of organisms (Balenger et al., 2015; Houde et al., 2019; Shen et al., 2019). Since the transcriptional phenotype of offspring can indicate modifications to cellular activity associated with the underlying mechanism of gene expression (Aubin-Horth & Renn, 2009; Connon et al., 2018; Komoroske et al., 2015), we can use changes in gene transcription to quantify the degree to which offspring are altering their responses to cope with these environmental stressors and to what capacity, if any, early rearing conditions may alter future responses to extreme environmental fluctuations.

Using a multi-level experimental approach, we examined whether two early environmental signals - exposure to pre-natal glucocorticoids and elevated temperatures during development – adaptively modified future responses to an acute thermal challenge in juvenile Chinook salmon (*Oncorhynchus tshwytscha*). Within the framework of the Environmental-Matching Hypothesis (Sheriff and Love 2013), we specifically predicted that both prenatal exposure to elevated cortisol and elevated rearing temperatures would adaptively alter the transcriptional responses of fish exposed to an acute thermal challenge later in life. Moreover, individuals exposed to the prenatal cortisol signal and then raised in a chronically stressful

thermal environment (i.e., expecting and then raised in a stressful post-natal environment) should show the most optimized (i.e., be best prepared) transcriptional responses to a future acute thermal challenge. We chose to conduct our work in a semelparous Pacific salmonid species given that: [1] adult females have the capacity to continue to increase cortisol release during migration in response to increasingly stressful conditions (Carruth et al., 2000; Cook et al., 2014); [2] females are oviparous which allows them to expose developing eggs to exogenous cortisol during migration (Mingist et al., 2007; Moore & Johnston, 2008; Sopinka et al., 2016); [3] adult females and offspring overlap spatially (i.e., offspring develop in the same streams/rivers that mother must travel and spawn within), enabling prenatal stress signals to be an honest signal of future environmental quality (Love et al., 2013); and [4] salmonids are ectothermic making them highly susceptible to elevated water conditions since they have little to no physiological ability to regulate body temperature (Heino et al., 2015; Wood & McDonald, 1997). Semelparity also imparts a further constraint on these fish since maternal signals should be both honest and beneficial given that mothers have only one opportunity in their lives to maximize their fitness (Kuijper & Hoyle, 2015). We first exposed eggs to an exogenous cortisol solution that resulted in elevated, but environmentally relevant egg cortisol concentrations (Auperin & Geslin, 2008; Capelle et al., 2016; Sopinka et al., 2015). Eggs were then divided across two rearing regimes based on normal seasonal changes in the natal rearing environment; one mimicking current water temperatures offspring would naturally experience, and the other mimicking predicted climate change conditions of +3°C above current river water temperatures (van Vliet et al., 2013). At the parr developmental stage (normally reached in the wild by late summer for Fall migratory life history Chinook salmon), offspring were exposed to either a multiday (and unexpected) acute thermal stressor of +9°C (1°C/hr for 9 hr) that peaked at 27°C,

or maintained at a constant 18°C (see Warriner et al., *subm.*). To evaluate cellular changes in response to the acute thermal stressor, we quantified gene transcriptional phenotypes using candidate genes shown to respond to environmental stressors across four broad biological processes (i.e., growth, metabolism, immune function, and stress response; Evans, 2015; Toews et al., 2019). Acute thermal stressors in many aquatic ectotherms significantly reduce growth rate, decrease metabolism as temperatures approach maximum thermal limits (Gillooly et al., 2001; Schulte, 2015) and can have immunosuppressive effects (Chadwick & McCormick, 2017; Dittmar et al., 2014; Lim et al., 2017; Whitney et al., 2016), thus we expected reductions in growth, immune function, and increases metabolic gene transcription levels. Nonetheless, the Environmental Matching Hypothesis predicts that if exposure to chronically elevated water temperatures adaptively prepare ectothermic offspring for future acute thermal stressors, we would expect these fish to perform better than to fish facing the same acute thermal stressor, but raised under current water conditions. Moreover, the Environmental Matching Hypothesis further predicts that individuals receiving a pre-natal cortisol signal (predictive of low environmental quality) and then reared under chronically elevated water conditions should fair the best of all groups when faced with the acute thermal challenge. We expected 'adaptive' transcriptional responses to take the form of higher relative levels in growth, immune, and metabolic genes, and lower relative levels in stress response genes. Overall, the result would be stress-induced predictive adaptive developmental plasticity that provides individuals exposed to early-life stressors with a greater capacity to function at higher thermal limits in the future.

Methods

Egg Fertilization and Cortisol Treatments

On October 4th, 2016, we collected 15 adult female and 9 adult male Chinook salmon from the Credit River (43°34'40.0"N 79°42'06.3"W) in the Laurentian Great Lakes population in Ontario, Canada. We removed gametes by applying pressure to the fish's abdomens and transported these on ice in coolers. Once at the University of Windsor, ~300 eggs from each female were added to a container (n_{eggs}≈2400 per female, 8 containers per female). We mixed the milt across sires to ensure each male a similar likelihood of fertilizing each egg and added ~0.5mL of the mixed milt to each container. We added 60mL of river water to activate the sperm for a two minute fertilization incubation (Lehnert et al., 2018). Immediately following fertilization, half of the containers received 740mL of a cortisol solution (*H4001, Sigma-Aldrich Canada Co*) dissolved in 90% ethanol (HPLC grade, Sigma-Aldrich Canada Co) and river water to make a final cortisol concentration of 1000ng/L. The remaining containers received a vehicle only solution (ethanol and river water) to act as a control group. We specifically chose the cortisol solution incubation times (2 hours) and exogenous cortisol concentrations based on literature that produced environmentally relevant elevations in egg cortisol concentrations that mimic a maternal stress signal (Auperin & Geslin, 2008; Burton et al., 2011; Capelle et al., 2016; Sopinka et al., 2016). After a two-hour incubation, we removed all eggs from the treatment solutions and eggs were rinsed using dechlorinated water. Details of the effects of the cortisol treatment on egg cortisol concentrations can be found in Warriner et al. (2020).

Temperature Rearing Conditions

Egg incubation stacks were developed to mimic either the natural temperature regimes offspring currently experience in the river system based on monthly records collected from monitoring stations close to the Credit River (Provincial Water Quality Monitoring Network (2010-2014) to act as a control, or consistently maintained at +3°C above the current river temperature incubation stack to mimic future climate projections for the region (van Vliet et al., 2013). The ~300 eggs per female from each cortisol treatment container were then equally and randomly distributed to two incubation cells within each of the two incubation stacks, with ~150 eggs within each replicate, and two replicates per treatment group giving a total of 16 cells, per female, across both stacks. This design resulted in four distinct treatment groups (e.g., prenatal treatment: temperature treatment) applied to each female's eggs (control:current; cortisol:current; control:elevated; cortisol:elevated; **Figure 3.1**).

We transferred the elevated and current fry on December 23rd, 2016 and February 16th, 2017, respectively, to 10L perforated buckets that housed 100 offspring combined from replicate cortisol treatment containers and replicate incubation cells. Buckets within temperature treatments were housed within five separate 320L recirculating-system housing tanks kept as separate systems to maintain the +3°C difference between the two systems, giving a total of 10 tanks. We raised the developing offspring under red light conditions that followed a 12:12 hour light and dark cycle, they were fed 3-4 times a day ad libitum, and tanks received water changes daily to maintain water conditions. All work was approved by, and completed under, the University of Windsor Animal Use Project Proposal (AUPP: #15-15).

Acute Temperature Trials

When the offspring had reached the parr developmental stage (Trial #1 = 2611ATU, Trial #2 = 2665ATU, Trial#3 = 2637ATU, Trial#4 = 2694ATU), they were subjected to one of two acute thermal spike procedures designed to evaluate transcriptional responses to multiple acute thermal stressors approaching the offspring's critical thermal maximum (CT_{Max}; outlined in Warriner et al. (2020)). Half the fish in all four of the treatment group combinations underwent an experiment which consisted of a +9°C temperature spike once per day over three consecutive days to evaluate transcriptional response to multiple acute thermal stressors approaching the offspring's critical thermal maximum (CT_{Max}) (previously evaluated by Warriner et al. 2019). The other half of the fish went through identical handling procedures, but without the temperature spikes, In fish, the individual is determined to have reached their CT_{Max} when they lose equilibrium under steadily increasing water temperatures and would die (Konecki et al., 1995; Lutterschmidt & Hutchison, 1997). To conduct these acute stressor experiments, two identical experimental arenas were developed that consisted of four 10L perforated buckets placed approximately equidistance apart in a 320L recirculating housing system: an immersion circulating heater to increase the temperature, a pump to uniformly circulate the heated water, a drop-in chiller to make the temperature increases gradual and maintain desired temperature, and a steel temperature probe to monitor temperature increases (**Figure 3.2**). The trials took place from May 25th – June 2nd and July 12th – 20th for the elevated and current treatments, respectively ($n_{replicate\ trials}$=4; **Figure 3.3**). On the night prior to the beginning of each trial, we transferred 32 individuals from their housing tanks and placed them in the perforated buckets with mesh covers (n=4 per bucket) to allow them to acclimate to their new environment. Within each arena, two buckets contained individuals from each of the cortisol and control treatment groups. Both arenas

began at 18°C, but one was maintained at this constant temperature to act as a control to account for any possible effects of transfer stress. The other arena experienced an increase of 0.5°C every 30 minutes for 9 hours and was then reduced at the same rate for the next 9 hours. This treatment was repeated once each day of the trial, for three consecutive days. On the fourth day, the fish were euthanized using clove oil and their blood was collected in 10µL heparinized microcapillary tubes using tail ablation method within 5 minutes. Fish were then weighed and stored in high salt solution to preserve RNA in a ratio of 10mL of salt solution per gram of sample tissue.

Primer and Probe Development

To analyze how a prenatal stress signal and chronically elevated rearing temperatures impact offspring responses to acute thermal stressors, we quantified transcriptional variation in growth, metabolism, immunity and stress- related genes of offspring. We used primers and probes from Toews et al. (2019), which were previously optimized in Chinook salmon, due to the study examining environmental stress on Chinook salmon gene transcriptional profiles. We also developed new primers to evaluate specific tissue responses (i.e., hepatic and skeletal muscle) to acute heat stress. We collected transcript sequences used to generate primers and probes from the National Center for Biotechnology Information (NCBI), and all originated from Chinook salmon transcript sequence or from species within the same genus (*Oncorhynchus*) or family (*Salmonidae*). Using Primer Express 3.0 software, we identified the specific amplicon length, melting temperatures, and possible dimerization (in order to reduce these occurrences) of the chosen primer and probe sequences. Primers were tested for proper annealing temperatures (i.e., ≥61°C) using gradient PCR to ensure that they would amplify during qPCR thermocycling. We confirmed the proper amplicon length from the gradient PCR using gel electrophoresis with

Chinook salmon cDNA. See **Appendix A2** for complete sequences of primers and probes as well as for amplicon lengths for each candidate gene.

mRNA Extraction and cDNA Synthesis

We chose to evaluate how a prenatal stress signal and chronically elevated rearing temperatures might modify the transcriptional response to an acute thermal environmental stressor using two tissues: liver and muscle. Liver is an important tissue to evaluate since hepatocyte gene transcription can be modified through cortisol binding to extracellular glucocorticoid receptors, and hepatic tissue plays a critical role in coping with an environmental stressor in fish due to the ability to regulate gluconeogenesis to regain homeostasis (Aluru & Vijayan, 2007; Evans et al., 2011). We chose skeletal muscle because it has been shown to respond differently to thermal stress in fish when reared under differing thermal conditions (Jesus et al., 2016). We excised whole livers (0.032±0.0091g; n_{liver}=117) and sections of muscle approximately equidistance above and below the lateral line (0.035±0.0052g; n_{muscle}=117) which then underwent RNA extraction. Total RNA was extracted using TRIzol™ Reagent (Ambion) following manufactures protocols (Invitrogen) with one slight modification: Rather than immediately resuspending the RNA in water, the RNA was washed with 75% ethanol, poured off, and 750 µL of new 75% ethanol was added to each tube to store the RNA at -20°C for up to a maximum of 12 months (Amisten, 2016). Prior to qRT-PCR analysis, RNA samples were resuspended in water put into solution and concentration determined by spectrophotometry on a NanoVue spectrophotometer (General Electric Company); average RNA concentrations were 1336ng/µL and 378ng/µL for liver and muscle, respectively. We then synthesized cDNA from 250ng total RNA for each sample using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) using half

reactions but following the manufacturer's protocol. All cDNA samples were stored at -20°C until qRT-PCR analysis.

Quantitative Real Time - PCR

Taqman® OpenArray® chips from Applied Biosystems were used to quantify variation in gene transcription using the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Each chip consisted of 48 subarrays, with of 56 through-holes per subarray, which were loaded with a solution of 1.2µL of sample cDNA, 1.3µL of ddH_2O, and 2.5µL Taqman® OpenArray® Real-Time PCR Master Mix (Applied Biosystems). The solution was loaded using the QuantStudio 12K Flex Accufill System (Applied Biosystems) to reduce intra-assay variation. Each through-hole came preloaded with forward primer, reverse primer, and Taqman probe for a single candidate gene. This allowed for 48 samples to be run on a single chip, with each of the 28 candidate genes run in duplicate to provide greater confidence in Cq values. In total, three chips were run for the 234 combined liver and muscle samples.

General Statistical Approaches

All statistical models were graphed for visual inspection of both homogeneity (residual vs. fitted graphs) and normality (residual quantile-quantile plots). All datasets that did not meet these assumptions underwent transformation and were then reassessed. All statistical analyses were completed using R software, version 3.6.0 (R Core Team, 2019).

Treatment Analysis on Candidate Gene Transcription

Gene transcription data were standardized for relative primer efficiency using LinRegPCR software, to determine relative primer candidate gene Cq values and then underwent

standardization using endogenously corrected relative target gene transcription levels (ΔCq; Tuomi et al., 2010). All target gene transcription levels underwent Box-Cox power transformation to normalize the data (Osborne, 2010) to perform Linear Mixed Modelling (LMM). To compare transcription variation across genes in response to our treatments, candidate genes underwent the same log transformation for all Multivariate Analysis of Variance (MANOVA) and Principle Coordinate Analyses (PCoA). All samples with Cq values >30 were removed from the datasets to offset stochasticity in the assay (Wellband et al., 2018).

Cortisol by Acute Temperature Effects – Gene Transcriptional Profile Analysis

Prior to analysis of the main and interaction effects of cortisol and acute thermal stress on individual candidate gene transcriptional phenotypes, we separated the transcription data for all candidate genes into sub-datasets based on rearing temperature and tissue analyzed. This allowed us to analyze the specific effects cortisol and rearing temperature in response to an acute thermal stressor in liver and muscle tissues while avoiding the need to examine, and interpret, a 4-way interaction between a prenatal stress signal, temperature rearing condition, acute thermal stressor, and tissue type. This setup also allowed for qualitative comparisons across rearing temperature treatments for which functional gene groups and individual candidate genes appear to have variations in response, if any

Multivariate Analysis of Variance (MANOVA) was performed on the complete gene set lists for each rearing temperature:tissue combination to identify if the prenatal cortisol, temperature spike, or combination of both treatments resulted in significant differences in transcriptional profiles. All candidate genes with sufficient numbers of individuals expressing the gene (>70% of total samples; 20 candidate genes in liver, 17 in muscle) at relevant levels (<30

Cq) were loaded into the MANOVA as dependent variables. The independent variables for the model included cortisol treatment, spike treatment, the interaction of cortisol and spike, as well as trial number and bucket ID to control for variation in date tested and rearing conditions, respectively. Any temperature:tissue combinations significantly affected by either cortisol, spike, or cortisol:spike were then analyzed at the functional gene group level. Candidate genes were loaded into a MANOVA as dependent variables within their respective functional gene groups, with the same independent variables as in the complete gene set analysis. Broad, transcriptional responses of functional systems indicates, at the cellular level, whether any systems of an organism are exhibiting possible adaptive, compensatory, or detrimental responses to environmental stressors (Connon et al., 2018; Williams et al., 2016). Any functional gene groups significantly affected by cortisol, spike, or cortisol:spike were then analyzed at the individual gene level to identify to what extent the treatments altered each candidate gene and if there were any qualitative differences across rearing temperatures that may be an indication of a predictive adaptive response. Linear Mixed Models (LMM) were performed on each individual gene. Fixed effects for the LMM included cortisol treatment, spike treatment, and the interaction of the two. Random effects in the models were trial number and bucket ID, again to control for residual effects of sampling period and rearing conditions, respectively. Random effects found to account for no residual variance were kept in the model to reduce the risk of falsely removing a variance component that is approaching zero, but not equal to it (Matuschek et al., 2017). Qualitative comparisons across rearing temperatures were performed through visualizing differences in graphical representations and means of the relative transcriptional responses of the individual genes in responses to the spike treatment.

Principle Coordinate Analysis (PCoA) was performed in parallel with the MANOVA at each level to visualize the differences, and similarities in response for the complete gene set and the functional gene groups to our treatments.

Results

Neither prenatal cortisol, nor its interaction with the spike treatment, had a significant effect on the complete gene set (**Table 3.1**) or any of the functional gene group (**Table 3.2**) transcriptional profiles in either liver or muscle, or in either rearing temperature (current or elevated).

The spike treatment significantly affected all complete gene set transcriptional profiles for all temperature:tissue combinations (**Table 3.1**). Visual inspection of the PCoA ordination maps for each temperature:tissue combination provided a graphical representation of which candidate genes were exhibiting similar, or dissimilar variation in gene transcriptional responses for all candidate genes analyzed in the MANOVAs indicating the shared responses to the significant treatment effects in the MANOVAs (**Figures 3.4-3.7**). The spike treatment also significantly affected all functional gene transcriptional profiles, with the exception of the metabolic functional group for the Elevated:Muscle group (**Table 3.2**). Therefore, all functional gene groups, excluding the Elevated:Muscle metabolic functional gene group, underwent individual gene transcriptional analysis. Again, visual inspection of the PCoA ordination maps for each functional gene group indicated similarities in response for candidate genes loaded into each functional gene group (**Figures 3.8-3.11**). Since the immune function gene group for both current and elevated temperatures in muscle tissue had only two candidate genes, ordination maps could not be completed for those. Multiple candidate genes within each functional group were significantly affected by the spike treatment across both rearing temperatures or for only a

singular rearing temperature for both tissues evaluated. Overall, growth, immune, and metabolic candidate genes observed down-regulations in gene transcription while stress candidate genes observed up-regulation in gene transcription in response to the acute thermal stressor. See **Tables 3.3 and 3.4** for all individual gene transcriptional analysis results for each temperature:tissue combination for all functional gene groups significantly affected by the spike treatment. Graphs visualize intensity and directionality of change due to the acute temperature treatment on individual gene transcriptional variation (**Figures 3.12-3.19**). Larger values indicate increases in gene transcription. Qualitative analysis of mean transcriptional responses for individual candidate genes across functional groups allowed us to make comparative assumptions across rearing conditions if relative gene transcription, due to the spike treatment, showed similar responses to our predictions. A summary of this analysis can be found in **Table 3.5**.

Discussion

Working within the evolutionary framework of the Environmental-Matching Hypothesis (Sheriff & Love, 2013) we aimed to identify whether previous exposure to a prenatal cortisol signal and elevated water temperatures during development could modulate gene transcription phenotypes for a diverse group of candidate genes representative of key functional systems (i.e., growth, metabolism, immune function, and stress response) in response to an acute thermal stressor later in life. In line with predictions of the EMH and predictive adaptive responses (see Introduction), exposure to elevated temperatures during early development appeared to upregulate and downregulate a number of possibly adaptive gene pathways across functional systems in both muscle and liver tissue when individuals were faced with an unpredictable acute thermal stressor later in life. Contrary to our predictions, a prenatal stress signal showed no capacity to alter gene transcriptional profiles for our candidate genes across all functional gene groups in response to

the acute thermal stressor, regardless of initial rearing temperature or tissue examined (i.e., liver and muscle). Here we explore why we believe prenatal stress signals had no capacity in inducing transcriptional plasticity, and how adaptive temperature-induced plasticity might benefit Chinook salmon and other Pacific salmonids under current and predicted future elevated conditions when facing unexpected temperature spikes approaching their CT_{max}.

Prenatal Exposure to Elevated Cortisol Does Not Alter Gene Transcriptional Profiles

We predicted that prenatal stress would result in predictive adaptive responses in gene expression to allow offspring to cope better in the long-term under the context of a chronic stressful environment (i.e., developing under elevated water temperatures; i.e., Environmental-Matching Hypothesis; Sheriff and Love, 2013). Indeed, exposure to prenatal cortisol has been linked to environmentally induced phenotypes in previous literature across a diversity of taxa (Meylan et al., 2012; Sheriff & Love, 2013; Sopinka et al., 2014). The complete lack of an impact of exposure to prenatal cortisol on gene transcription at the parr life history stage is therefore surprising and suggests that although prenatal cortisol has been shown to alter early developmental transcriptional responses in some studies (Best et al., 2017; Li et al., 2010), either these effects are not present in our study system (see Chapter 2), or possible early effects may not always persist into later developmental stages. Our results at this later parr developmental stage are also consistent with those from Chapter 2 where we did not identify any effects of exposure to exogenous prenatal cortisol during early embryonic development. Given the lack of response at earlier stages then, it is perhaps not surprising that the prenatal cortisol signal did not generate any transcriptional response later in life (Capelle et al., 2016; Van Kesteren et al., 2019). These results are in contradiction with previous studies showing that maternal stress signals alter offspring behavioural and physiological phenotypes, as well as gene transcription

(Best et al., 2017; Kleppe et al., 2013) potentially in an adaptive sense in preparation for future stressful conditions (Love et al. 2013; Sheriff and Love, 2013). However, in combination with recent studies suggesting a lack of a rescue effect for elevated egg cortisol on survival in salmonids under elevated temperatures (Eriksen et al., 2006; Warriner et al., 2020), our results suggest that elevated egg cortisol does not act as strong priming signals within the context of future hyperthermic environments, at least for salmonids. An alternative conclusion for our results is that the strength of our cortisol exposure did not match the degree to which the eventual thermal stressor was manifested, despite our cortisol elevation being both biologically relevant and based on previous studies. Nonetheless, it may be valuable to investigate relative gene transcription responses under elevated thermal conditions in response to higher cortisol doses.

Acute and Chronic Thermal Conditions Alter Gene Transcription

Acute thermal conditions approaching the upper thermal limits in our study species (see Warriner et al., *in prep)* had a significant impact on gene transcriptional profiles for all temperature:tissue combinations at both the complete gene set and at the functional gene groups levels, excluding the stress gene group for the Elevated:Muscle group. These broad functional differences in gene transcription can be interpreted as possible adaptive, compensatory, or even detrimental responses across functional systems. Interpreting differences in individual gene transcriptional phenotypes within our functional groups not only indicates which processes were being altered to the greatest degree within the perspective of a given functional gene group, but they can also be used to qualitatively indicate whether any PAR occur as a result of variation in thermal rearing conditions. Differences across rearing temperatures for relative gene transcription in function genes thought to respond adaptively under thermal stress would support

the concept that development under elevated thermal conditions induces higher upper thermal tolerances later in life (Messmer et al., 2017; Peck et al., 2009).

Examination of individual candidate gene transcription in the liver (**see Table 3.3**) and muscle (see **Table 3.4)** across the current and elevated rearing temperatures indicated some similarities in genes responses to the acute thermal spike, while others were only affected by the thermal spike for a single rearing environment. Candidate genes that showed qualitative differences then evaluated for mean transcriptional responses to possibly indicate predictive adaptive responses that were a result of the elevated rearing conditions that would denote enhanced thermal tolerance (**Table 3.5**). We predicted that growth gene transcription would vary across rearing conditions, with elevated temperature rearing conditions producing higher relative gene transcription in response the acute thermal stressor compared to current ambient conditions. This response would indicate an enhanced capacity to maintain normal functional processes under acutely stressful conditions, which would be a possible indication of higher upper thermal tolerance as a response to elevated rearing conditions. Within the liver we observed that the temperature spike treatment significantly altered gene transcription compared to the benign conditions in the current temperature conditions but not for individuals reared under elevated thermal conditions. Specifically, early growth response protein 1 (EGR-1) observed a significant down-regulation in gene transcription in response to the spike treatment and growth hormone receptor (GH-R) observed a significant up-regulation, both important genes whose products regulate cell proliferation. However, evaluating average mean transcription levels across rearing temperatures, in response to the acute spike treatment, it would appear that liver is very similar in response to the temperature spike, possibly indicating that rearing under elevated temperatures did not alter transcriptional growth response when experiencing an acute thermal stressor. This

result suggests that rearing under elevated temperatures did not enhance liver thermal tolerances since cellular activity remained similar. Additionally, muscle tissue observed a significant up-regulation in GH-R and a tumor suppressor protein 53 (p53), both regulators of cellular proliferation, and a down-regulation myostatin 1A (MYO1A), an antagonist of muscle growth, showing quantitative changes across rearing temperatures in response to the thermal spike. Qualitatively, the relative gene transcription was similar in response to the spike treatment as well, further suggesting that elevated temperatures during rearing did not alter thermal tolerances for muscle tissue. However, growth is an energetically costly function (Williams et al., 2016), and growth transcriptional responses are often reduced under acutely stressful conditions (Nakano et al., 2013). Mechanistically, it could be an example of possible allostatic load directly reducing biological functions, in this case growth activity, for both liver and muscle tissue to prolong an energetic response to a chronic stress and made worse when exposed to an acute stressor (McEwen & Wingfield, 2003; Schreck & Tort, 2016). As such, the transcriptional differences that do not align with our early predictions of possible rescued growth due to elevated rearing conditions may be a normal response under any circumstance to reduce energy output for continued survival. Similarly, we predicted that immune gene transcription should vary across rearing conditions, with elevated temperature conditions producing upregulated gene transcription in response to the acute thermal stressor. Again, this mechanistic ability to maintain 'normal' functioning at the cellular level would be a strong indicator for higher thermal tolerance. For both liver and muscle tissue, immune function was significantly altered by the temperature spike treatment for all candidate gene transcription, except for calmodulin (CAL), an important gene for inflammation and apoptosis, in muscle under current temperature conditions. Qualitatively, immune function responses in liver were often slightly more down-regulated in

relative gene transcription in response to the acute thermal stressor for offspring reared under chronically elevated thermal conditions, which would indicate that elevated rearing conditions might have negatively enhanced the immunosuppressive effects of the acute thermal stressor. Previous literature has identified that in salmonids, elevated temperatures during rearing has the capacity to increase adaptive immunity while reducing innate immunity (Alcorn et al., 2002). Examination of the candidate genes significantly affected by the temperature spike treatment in each tissue would indicate a reduction in innate immune activity, specifically complement component 3 and 5 (C3 and C5) in the liver and natural killer enhancing factor (NKEF) in muscle. Together, these results indicate that elevated rearing temperatures do not result in higher thermal tolerance to a significant enough level for offspring to produce a stronger innate immune response. Again, this is a possible indication of the mechanism of allostatic load reducing energy consumption by shunting resources away from energetically costly biological functions to prolong survival under acute thermally stressful conditions. We predicted a decrease in stress response gene transcription under elevated thermal rearing conditions. Both liver and muscle showed a significant difference due to the temperature spike treatment for many of the candidate genes in this functional group, all exhibiting an up-regulation in gene transcription due to the spike treatment. However, qualitative differences in stress candidate genes across rearing conditions in response to the temperature spike treatment indicate that there was not as significant an up-regulation in candidate gene transcription in offspring reared under elevated rearing conditions for both liver and muscle. Reduction in relative transcription for these genes supports the concept of higher thermal tolerance due to elevated rearing temperatures. Metabolic activity was predicted to down-regulate transcription, but to be higher in tissues under elevated thermal conditions, due to temperatures approaching an ectothermic organism's maximum

thermal limit often resulting in an inversion of metabolic activity, reducing metabolic reactions (Gillooly et al., 2001; Schulte, 2015). If offspring reared under elevated thermal conditions did not down-regulate gene transcription for candidate genes evaluating metabolic activity to the same degree as the offspring reared under current ambient conditions, this would be an indicator of higher thermal tolerance. Muscle tissue did not significantly respond to the acute thermal stressor for any of the candidate genes in the metabolic functional group, indicating no significant down-regulation in gene transcription such as those observed in the offspring reared under current ambient conditions. This lack of response at the functional gene group level would indicate a higher thermal tolerance for these individuals in muscle tissue. However, quantitative analysis of the metabolic genes significantly affected by the temperature spike treatment in liver in fact showed greater transcriptional change for fatty acid synthase (FAS) under elevated conditions, and greater transcriptional change for insulin induced gene 1 (INSIG1) under current temperature conditions. Qualitatively, metabolic gene transcription appears to be lower, or comparable, across both rearing, possibly indicating no adaptive effects to thermal tolerance in liver tissue. Variation in tissue metabolism to elevated temperatures are due to variations in mitochondrial densities, ribosomal concentrations, as well as other differing component processes (Clarke & Fraser, 2004), meaning that identifying higher thermal tolerance in offspring reared under elevated conditions muscle and not liver is possible at the cellular level.

Environmental Signals as Inducers of Predictive Adaptive Responses to Future Thermal Stress

Currently, fisheries are being negatively impacted by the elevated thermal water conditions associated with global climate change (Free et al., 2019) and these conditions are expected to continue to deteriorate based on predicted continued anthropogenic factors (van Vliet et al., 2013). These deteriorating conditions are resulting in many populations of Pacific salmonids

(among other species) facing endangered or threatened status within their natural ranges (Albaugh et al., 2011; Crozier, 2016), while others have been extirpated. While phenotypic (developmental) plasticity is believed to be how most of these populations have recently (or may be able to) adapted to cope with these environmental disturbances (Crozier et al., 2008; Muñoz et al., 2015), those responses can have important repercussions for later life history stages (Bateson et al., 2014; Nettle & Bateson, 2015). Our findings, some supporting predictions of the Environmental Matching Hypothesis (specifically metabolic and stress functional response), suggest that exposure to elevated water temperatures during development induce transcriptional plasticity that can better prepare Chinook salmon offspring for future thermal stressors, but that exposure to elevated prenatal glucocorticoids does little to further modify these adaptive changes. Overall, these mechanistic (i.e., gene transcription) responses suggest promising avenues by which exposure to relevant environmental stressors can better prepare individuals for future increases in environmental stress. Nonetheless, more studies are needed that link these underlying responses to the organismal performance traits that ultimately influence survival, growth, and reproductive potential. Without these studies, ultimately assigning an 'adaptive' label to plastic changes in underlying mechanisms is not possible, but it is a first, and promising, step in predicting how ectothermic fish will adapt to future increases in thermal stress.

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Tables

Table 3.1 - Complete Gene Set Profile Analysis for all Temperature:Tissue Combinations

All candidate genes with significant numbers of samples expressing the gene at detectable levels (>70% total sample size) were loaded into a MANOVA as dependent variables to be compared in their response to the independent variables, cortisol treatment, spike treatment, and the interaction of the two. All temperature:tissue combinations were significantly affected by the spike treatment. ***p<0.001, **p<0.01, *p<0.05, •p<0.1.

Treatment Effect	MANOVA Results	Current-Liver	Elevated-Liver	Current-Muscle	Elevated-Muscle
Cortisol	Pillai trace	0.56	0.54	0.57	0.62
	F-value	1.26	1.31	1.10	0.68
	DF & Err DF	(1,20)	(1,22)	(1,14)	(1,7)
	p-value	0.30	0.27	0.43	0.76
Spike	Pillai trace	0.92	0.88	0.99	0.98
	F-value	11.7	8.14	129.9	26.4
	DF & Err DF	(1,20)	(1,22)	(1,14)	(1,7)
	p-value	**<0.001 *** **	**<0.001 *** **	**<0.001 *** **	**<0.001 *** **
Cort:Spike	Pillai trace	0.44	0.39	0.57	0.76
	F-value	0.78	0.69	1.08	1.34
	DF & Err DF	(1,20)	(1,22)	(1,14)	(1,7)
	p-value	0.71	0.79	0.45	0.36

Table 3.2 – Functional Gene Group Profile for All Temperature:Tissue Combinations

All candidate genes used in the complete gene profile analysis were loaded into their respective functional gene groups analyzed using a MANOVA to identify which gene groups were significantly different in candidate gene transcription due to the temperature spike treatment. All functional groups were significantly affected by the spike treatment, except for the metabolic functional gene group for the Elevated-Muscle samples. ***p<0.001, **p<0.01, *p<0.05, •p<0.1.

Temp:Tissue	Treatment	MANOVA Results	Growth	Metabolism	Immune	Stress
Current:Liver	Spike	Pillai trace	0.70	0.40	0.54	0.30
		F-value	17.8	5.21	13.8	3.43
		DF & Err DF	(1,39)	(1.47)	(1,48)	(1,40)
		p-value	**<0.001**	**<0.001**	**<0.001**	**0.011**
Elevated:Liver	Spike	Pillai trace	0.54	0.36	0.45	0.27
		F-value	9.18	4.21	9.02	3.26
		DF & Err DF	(1,39)	(1,45)	(1,45)	(1,43)
		p-value	**<0.001**	**0.0019**	**<0.001**	**0.014**
Current:Muscle	Spike	Pillai trace	0.88	0.45	0.60	0.97
		F-value	97.3	4.05	35.4	226.3
		DF & Err DF	(1,40)	(1,30)	(1,48)	(1,43)
		p-value	**<0.001**	**0.0043**	**<0.001**	**<0.001**
Elevated:Muscle	Spike	Pillai trace	0.74	0.24	0.78	0.91
		F-value	33.4	1.24	76.4	57.3
		DF & Err DF	(1,35)	(1,23)	(1,42)	(1,36)
		p-value	**<0.001**	0.32	**<0.001**	**<0.001**

Table 3.3 - Liver Individual Gene Transcriptional Analysis

Each candidate gene was analyzed for significant effects of the temperature spike treatment, due to the significant effects the treatment had on functional gene group transcriptional profiles. Results are based on a Linear Mixed Modelling (LMM). To account for residual effects due to possible variation in sampling dates and incubation position, trial number and bucket ID were included as random effects. The dependent variable was relative target gene transcription (ΔCq), normalized using BoxCox transformations, of each gene. All p-values are raw values from the ANOVA of the LMMs. ***p<0.001, **p<0.01, *p<0.05, •p<0.1.

Tissue	Functional Gene Group	Candidate Gene	Treatment	LMM Results	Current	Elevated
Liver	Growth	EGR-1	Spike	F-value	$F_{(1,49.9)}$=9.89	$F_{(1,45.1)}$=0.31
				p-value	**0.0028 ****	0.58
		GH-R	Spike	F-value	$F_{(1,55.1)}$=4.42	$F_{(1,47.9)}$=1.90
				p-value	**0.040 ***	0.17
		IGF-1	Spike	F-value	$F_{(1,50.0)}$=1.53	$F_{(1,43.9)}$=2.49
				p-value	0.22	0.12
		IGFBP2b	Spike	F-value	$F_{(1,56.0)}$=0.84	$F_{(1,43.6)}$=2.32
				p-value	0.84	0.14
		p53	Spike	F-value	$F_{(1,44.7)}$=81.0	$F_{(1,51.0)}$=25.9
				p-value	**<0.001 *****	**<0.001 *****
	Metabolism	COI	Spike	F-value	$F_{(1,57.0)}$=1.39	$F_{(1,48.9)}$=2.97
				p-value	0.24	0.091 •
		CPT1	Spike	F-value	$F_{(1,57.0)}$=2.99	$F_{(1,53.1)}$=2.48
				p-value	0.089 •	0.12
		CTSL	Spike	F-value	$F_{(1,57.0)}$=3.15	$F_{(1,53.9)}$=0.21
				p-value	0.081 •	0.65
		CYP1A	Spike	F-value	$F_{(1,58.0)}$=0.04	$F_{(1,54.0)}$=0.47
				p-value	0.85	0.49
		FAS	Spike	F-value	$F_{(1,56.0)}$=1.00	$F_{(1,47.4)}$=4.64
				p-value	0.32	**0.036 ***
		INISG1	Spike	F-value	$F_{(1,55.1)}$=5.52	$F_{(1,47.7)}$=0.04
				p-value	**0.022 ***	0.84
	Immune	C3	Spike	F-value	$F_{(1,51.3)}$=47.1	$F_{(1,53.0)}$=24.3
				p-value	**<0.001 *****	**<0.001 *****
		C5	Spike	F-value	$F_{(1,50.9)}$=17.9	$F_{(1,53.0)}$=25.9
				p-value	**<0.001 *****	**<0.001 *****
		CAL	Spike	F-value	$F_{(1,54.0)}$=3.23	$F_{(1,48.5)}$=2.74
				p-value	0.078 •	0.10
		NKEF	Spike	F-value	$F_{(1,50.6)}$=0.13	$F_{(1,53.0)}$=0.26
				p-value	0.72	0.61
		GPx1	Spike	F-value	$F_{(1,43.5)}$=3.62	$F_{(1,45.3)}$=11.6
				p-value	0.064 •	**0.0014 ****
		GR-2	Spike	F-value	$F_{(1,55.0)}$=2.70	$F_{(1,53.0)}$=0.004

	Stress			p-value	0.11	0.95
		hsp70	Spike	F-value	$F_{(1,50.9)}$=3.55	$F_{(1,49.4)}$=1.25
				p-value	0.065 •	0.27
		metA	Spike	F-value	$F_{(1,56.0)}$=7.44	$F_{(1,54.0)}$=0.40
				p-value	**0.0085 ****	0.53
		TrxR3a	Spike	F-value	$F_{(1,56.0)}$=3.51	$F_{(1,53.1)}$=5.75
				p-value	0.066 •	**0.020 ***

Table 3.4 - Muscle Individual Gene Transcriptional Analysis

Each candidate gene was analyzed for significant effects of the temperature spike treatment, due to the significant effects the treatment had on functional gene group transcriptional profiles. Results are based on a Linear Mixed Modelling (LMM). To account for residual effects due to possible variation in sampling dates and incubation position, trial number and bucket ID were included as random effects. The dependent variable was relative target gene transcription (ΔCq), normalized using BoxCox transformations, of each gene. All p-values are raw values from the ANOVA of the LMMs. ***p<0.001, **p<0.01, *p<0.05, •p<0.1.

Tissue	Functional Gene Group	Candidate Gene	Treatment	LMM Results	Current	Elevated
Muscle	Growth	GH-R	Spike	F-value	$F_{(1,54.0)}$=32.8	$F_{(1,49.0)}$=13.1
				p-value	**<0.001 *** **	**<0.001 *** **
		MYO1A	Spike	F-value	$F_{(1,41.0)}$=164.2	$F_{(1,39.8)}$=39.0
				p-value	**<0.001 *** **	**<0.001 *** **
		p53	Spike	F-value	$F_{(1,53.0)}$=60.9	$F_{(1,47.0)}$=94.9
				p-value	**<0.001 *** **	**<0.001 *** **
	Metabolism	COI	Spike	F-value	$F_{(1,57.0)}$=5.50	NA
				p-value	**0.023 ***	
		CPT1	Spike	F-value	$F_{(1,53.0)}$=0.034	
				p-value	0.86	
		CTSL	Spike	F-value	$F_{(1,56.0)}$=4.19	
				p-value	**0.045 ***	
		CYP1A	Spike	F-value	$F_{(1,46.6)}$=1.68	
				p-value	0.20	
		FAS	Spike	F-value	$F_{(1,40.4)}$=6.06	
				p-value	**0.018 ***	
		INSIG1	Spike	F-value	$F_{(1,43)}$=0.070	
				p-value	0.79	
	Immune	CAL	Spike	F-value	$F_{(1,47.2)}$=0.59	$F_{(1,50.1)}$=11.8
				p-value	0.45	**0.0012***
		NKEF	Spike	F-value	$F_{(1,52.0)}$=43.7	$F_{(1,46.1)}$=96.4
				p-value	**<0.001 *** **	**<0.001 *** **
	Stress	GPx1	Spike	F-value	$F_{(1,51.0)}$=70.8	$F_{(1,39.7)}$=31.4
				p-value	**<0.001 *** **	**<0.001 *** **
		GR-2	Spike	F-value	$F_{(1,46.5)}$=1.78	$F_{(1,42.4)}$=4.61
				p-value	0.19	**0.038***
		hsp70	Spike	F-value	$F_{(1,55.1)}$=225.1	$F_{(1,44.4)}$=51.0
				p-value	**<0.001 *** **	**<0.001 *** **
		hsp90a	Spike	F-value	$F_{(1,58.0)}$=554.3	$F_{(1,45.2)}$=331.3
				p-value	**<0.001 *** **	**<0.001 *** **
		metA	Spike	F-value	$F_{(1,53.0)}$=0.095	$F_{(1,46.6)}$=0.21
				p-value	0.76	0.65
		TrxR3a	Spike	F-value	$F_{(1,54.0)}$=3.71	$F_{(1,50.1)}$=11.6

				p-value	0.060 •	**0.0013 ****

Table 3.5 - Qualitative Analysis of Means for Candidate Genes Significantly Affected by the Temperature Spike Treatment

Mean gene transcriptional values of liver and muscle tissues based on offspring rearing temperatures. Bolded text indicates comparisons across means that align with predicted transcriptional responses we expected for each functional group. Overall alignment with predictions across functional gene groups are either correct (✓), incorrect (X), or neutral (-). The metabolism functional group was not included for comparison due to this functional group not being significantly affected by the spike treatment in the elevated reared offspring. See Discussion for explanation as to why this is a possible indicator of higher thermal tolerance.

Tissue	Functional Gene Group	Candidate Gene	Current Mean	Elevated Mean	Predicted Transcriptional Response	Aligns with Predicted Response?
Liver	Growth	EGR-1	0.092±0.09	0.081±0.09	Higher under elevated	X
		GH-R	0.031±0.01	0.028±0.01		
		p53	0.020±0.02	0.017±0.02		
	Immune	C3	1.97±1.0	1.37±1.1	Higher under elevated	X
		C5	1.28±0.8	0.82±0.6		
	Metabolism	FAS	0.22±0.2	0.13±0.1	Higher under elevated	-
		INSIG1	**0.021±0.02**	**0.026±0.02**		
	Stress	GPx1	**0.034±0.03**	**0.030±0.03**	Lower under elevated	✓
		metA	**0.66±0.3**	**0.40±0.3**		
		TrxR3a	**0.21±0.2**	**0.11±0.1**		
Muscle	Growth	GH-R	0.083±0.06	0.052±0.03	Higher under elevated	X
		MYO1A	0.005+0.03	0.012±0.009		
		p53	0.093±0.03	0.083±0.03		
	Immune	CAL	0.37±0.2	0.27±0.2	Higher under elevated	X
		NKEF	0.018±0.01	0.014±0.007		
	Stress	GPx1	**0.082±0.04**	**0.043±0.02**	Lower under elevated	✓
		GR-2	**0.27±0.1**	**0.25±0.1**		
		hsp70	**279.4±339.6**	**19.6±32.4**		
		hsp90a	**14.9±9.8**	**8.03±5.7**		
		TrxR3a	**0.18±0.1**	**0.17±0.1**		

Figures

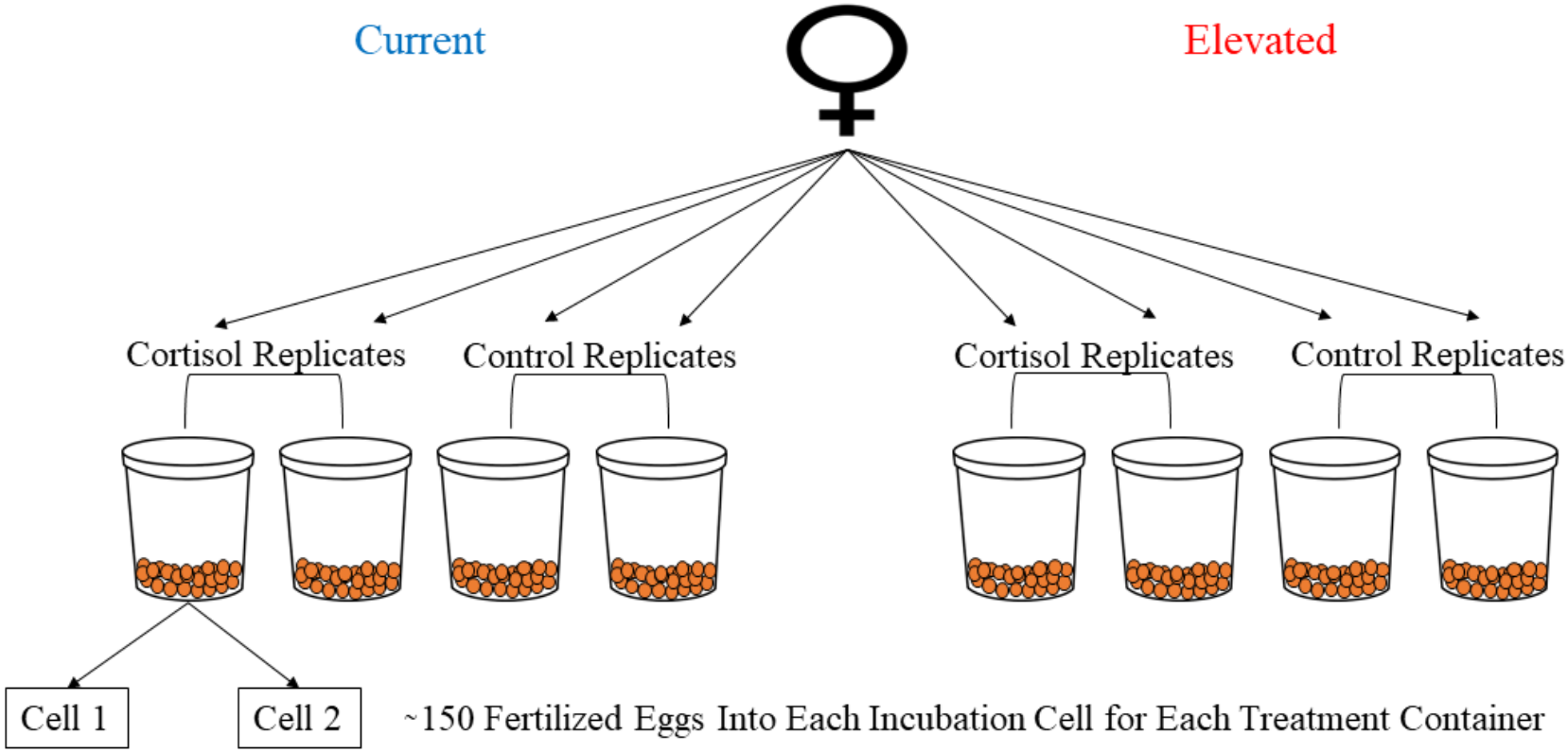


Figure 3.1 - Experimental design for egg cortisol manipulation and incubation. Eggs from each female were equally dispersed across eight containers to receive either an environmentally relevant exogenous cortisol dosage, mimicking a maternal stress signal, or a control dosage, containing no cortisol. Each cortisol treatment was run in duplicate to provide replicates of treatment effects across the two temperature treatments (i.e., elevated or current). From each container, ~300 eggs were randomly distributed to two cells within the incubation stacks to give a total of ~150 eggs/cell to control for positional biases within the incubation stack during development.

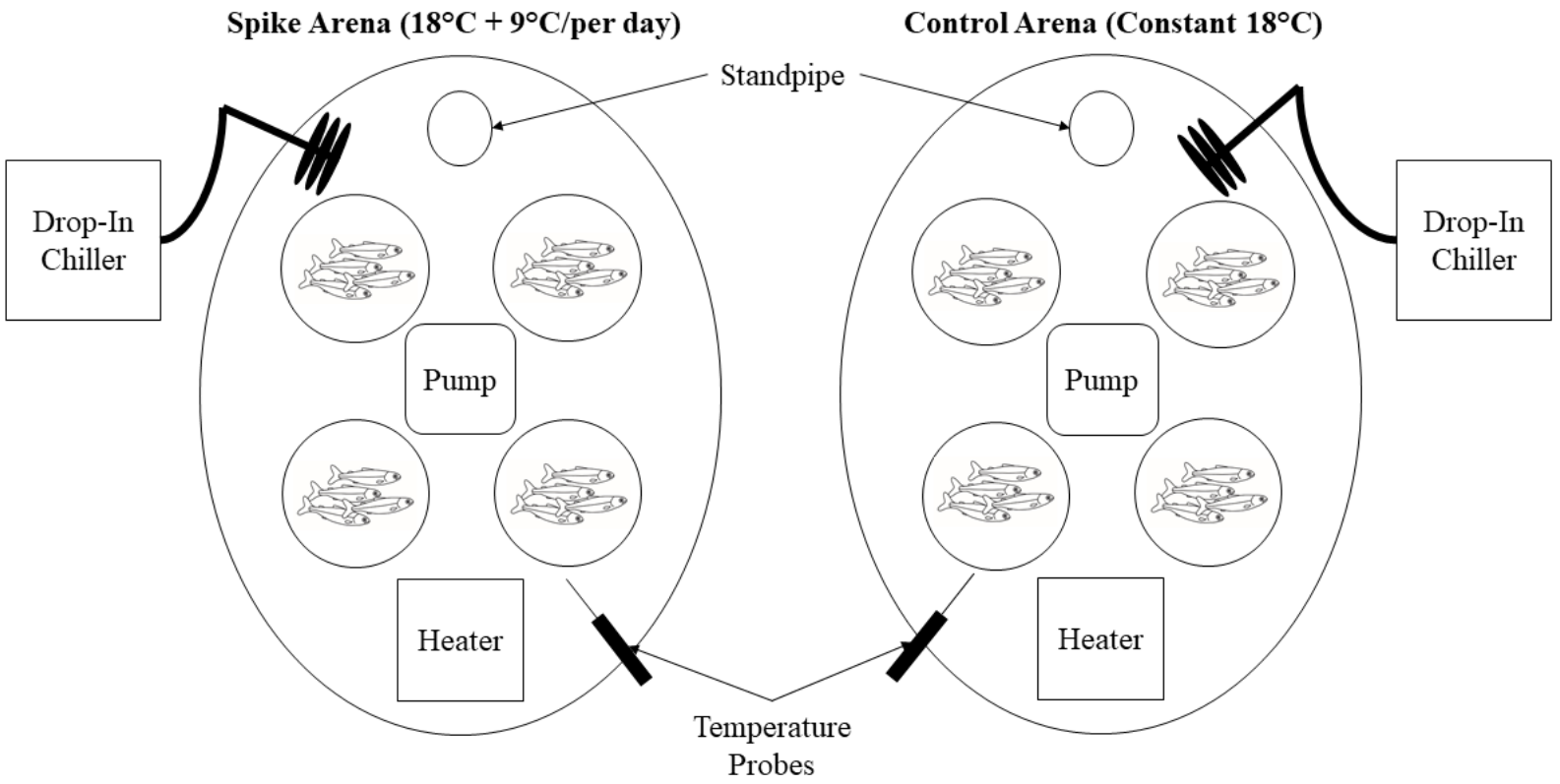


Figure 3.2 - Arena design layout for both spiked and control groups.

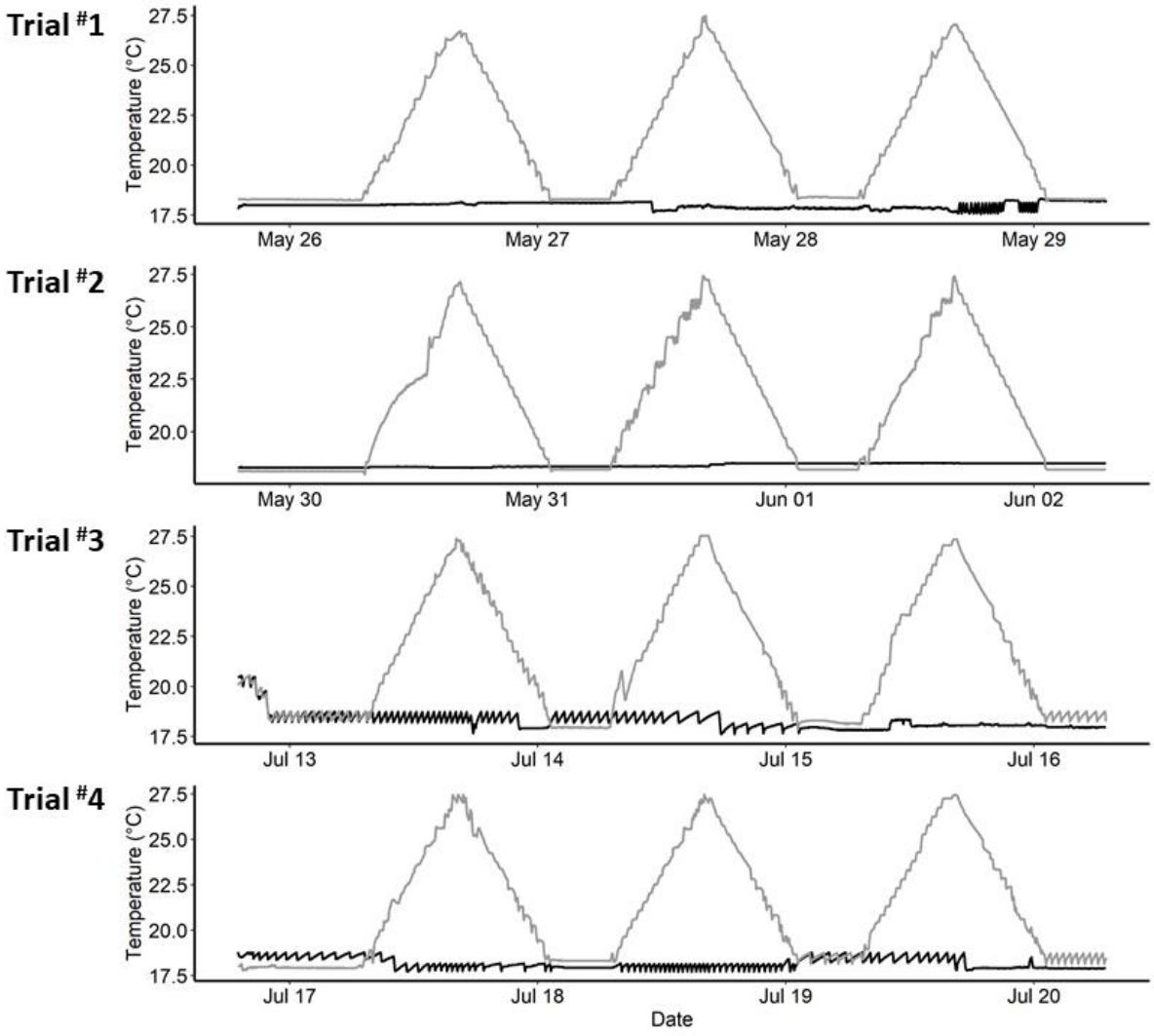


Figure 3.3 - Temperature regime for each trial replicate for both spiked and no-spike groups. Trials #1 and #2 were conducted using the offspring reared in elevated thermal conditions while Trials #3 and #4 used offspring reared in current thermal conditions.

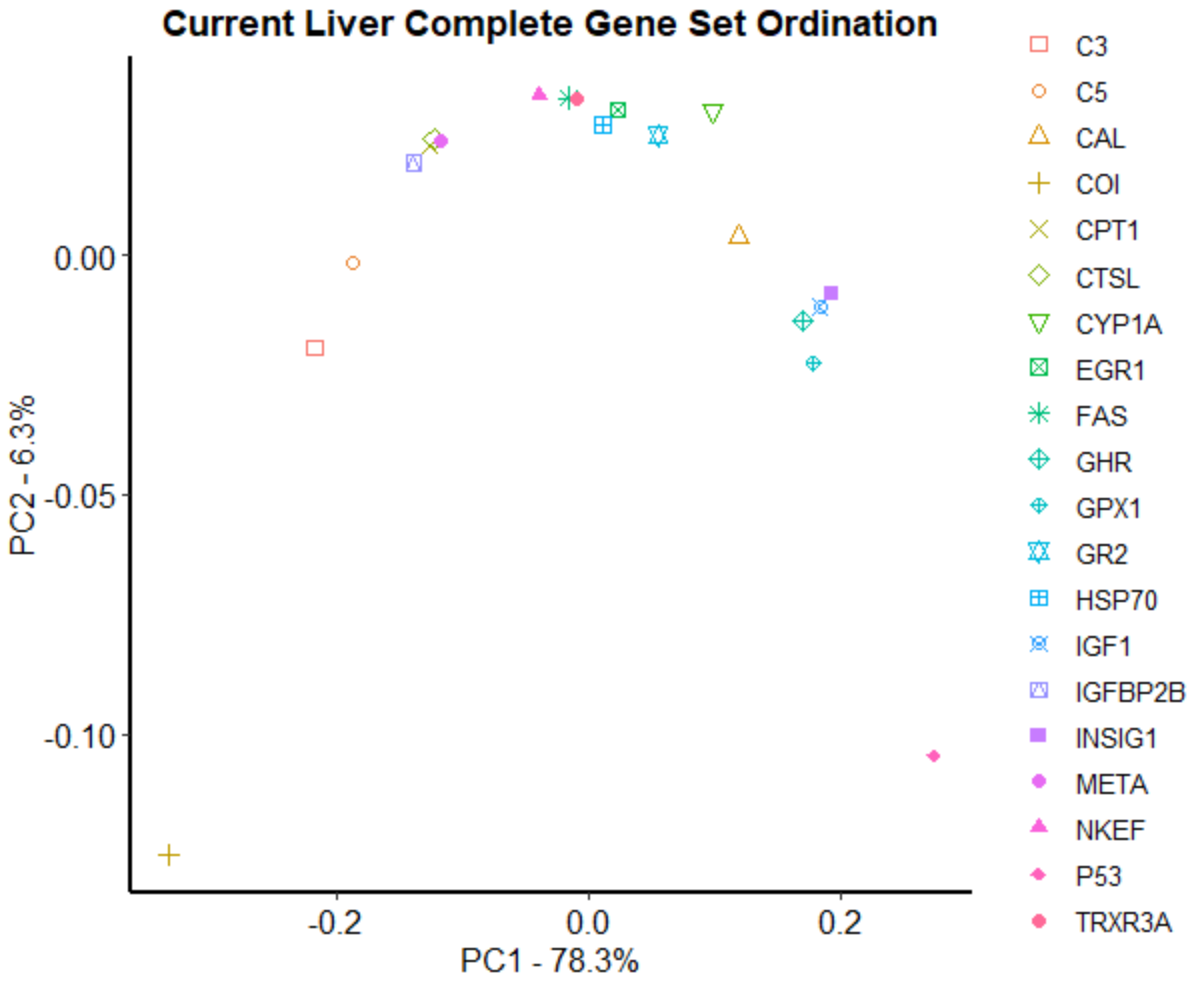


Figure 3.4 - PCoA ordination map for complete gene set profiles of Current-Liver. Candidate genes oriented closer to each other are more similar in response and farther distances indicate increasing dissimilarity. Principle component 1 (PC1) accounts for the largest proportion of the variance (78.3%) and therefore is a better indicator of overall gene transcriptional relationships. Due to the temperature spike treatment being the only fixed effect to significantly alter the complete gene transcriptional profile, the variation is mostly accounted to the spike treatment.

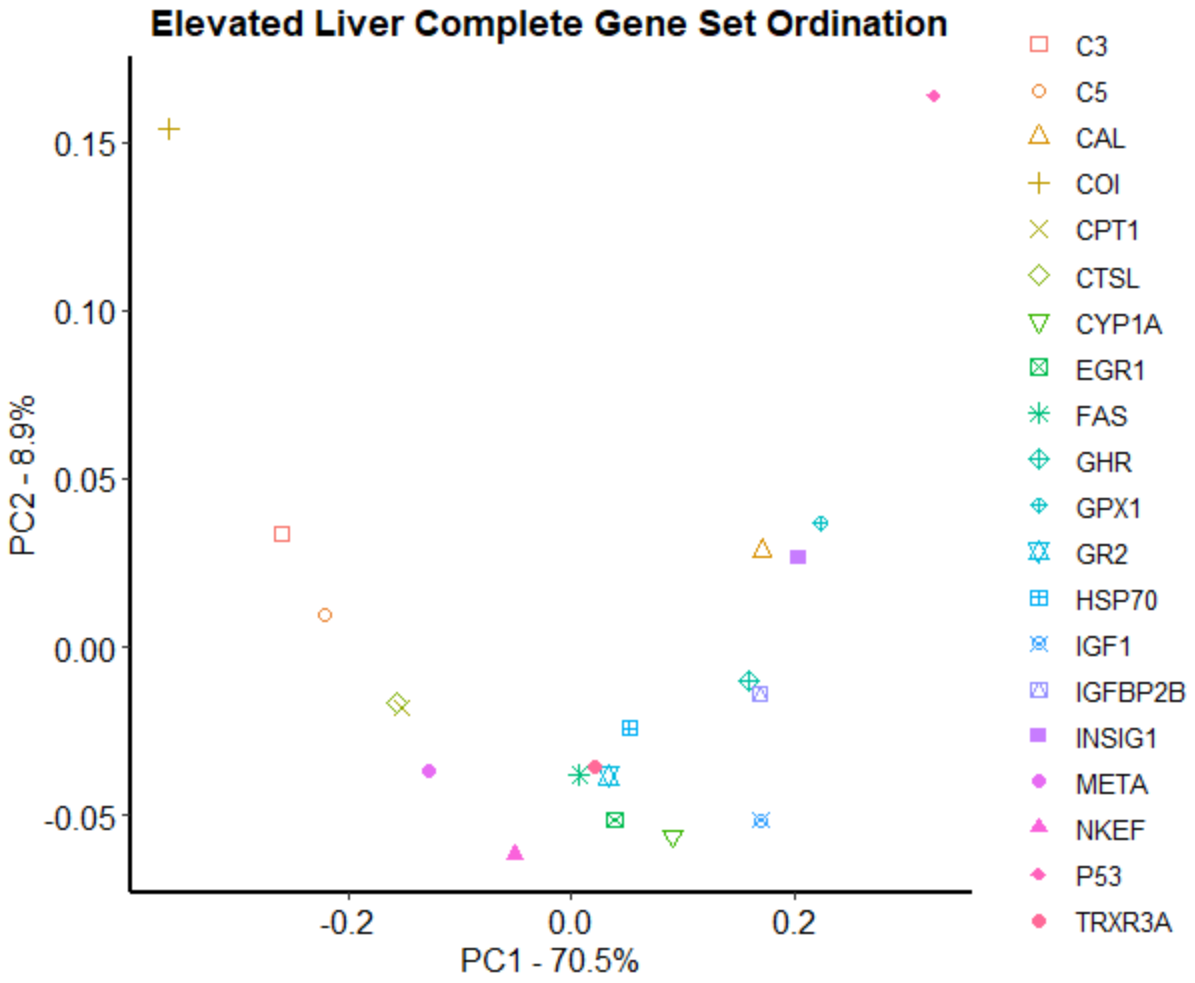


Figure 3.5 - PCoA ordination map for complete gene set profiles of Elevated-Liver. Candidate genes oriented closer to each other are more similar in response and farther distances indicate increasing dissimilarity. Principle component 1 (PC1) accounts for the largest proportion of the variance (70.5%) and therefore is a better indicator of overall gene transcriptional relationships. Due to the temperature spike treatment being the only fixed effect to significantly alter the complete gene transcriptional profile, the variation is mostly accounted to the spike treatment.

Figure 3.6 - PCoA ordination map for complete gene set profiles of Current-Muscle. Candidate genes oriented closer to each other are more similar in response and farther distances indicate increasing dissimilarity. Principle component 1 (PC1) accounts for the largest proportion of the variance (72.4%) and therefore is a better indicator of overall gene transcriptional relationships. Due to the temperature spike treatment being the only fixed effect to significantly alter the complete gene transcriptional profile, the variation is mostly accounted to the spike treatment.

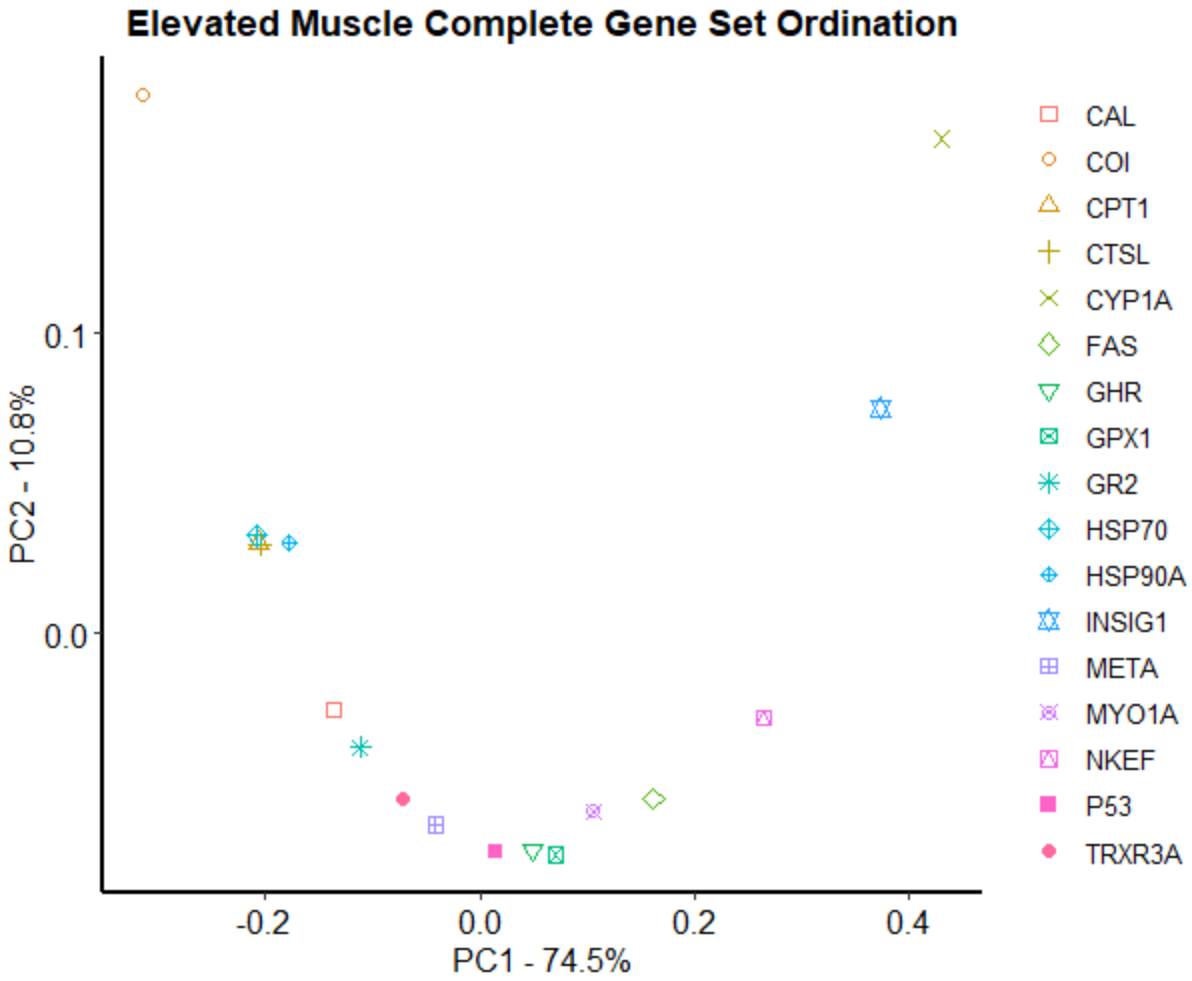


Figure 3.7 - PCoA ordination map for complete gene set profiles of Elevated-Muscle. Candidate genes oriented closer to each other are more similar in response and farther distances indicate increasing dissimilarity. Principle component 1 (PC1) accounts for the largest proportion of the variance (74.5%) and therefore is a better indicator of overall gene transcriptional relationships. Due to the temperature spike treatment being the only fixed effect to significantly alter the complete gene transcriptional profile, the variation is mostly accounted to the spike treatment.

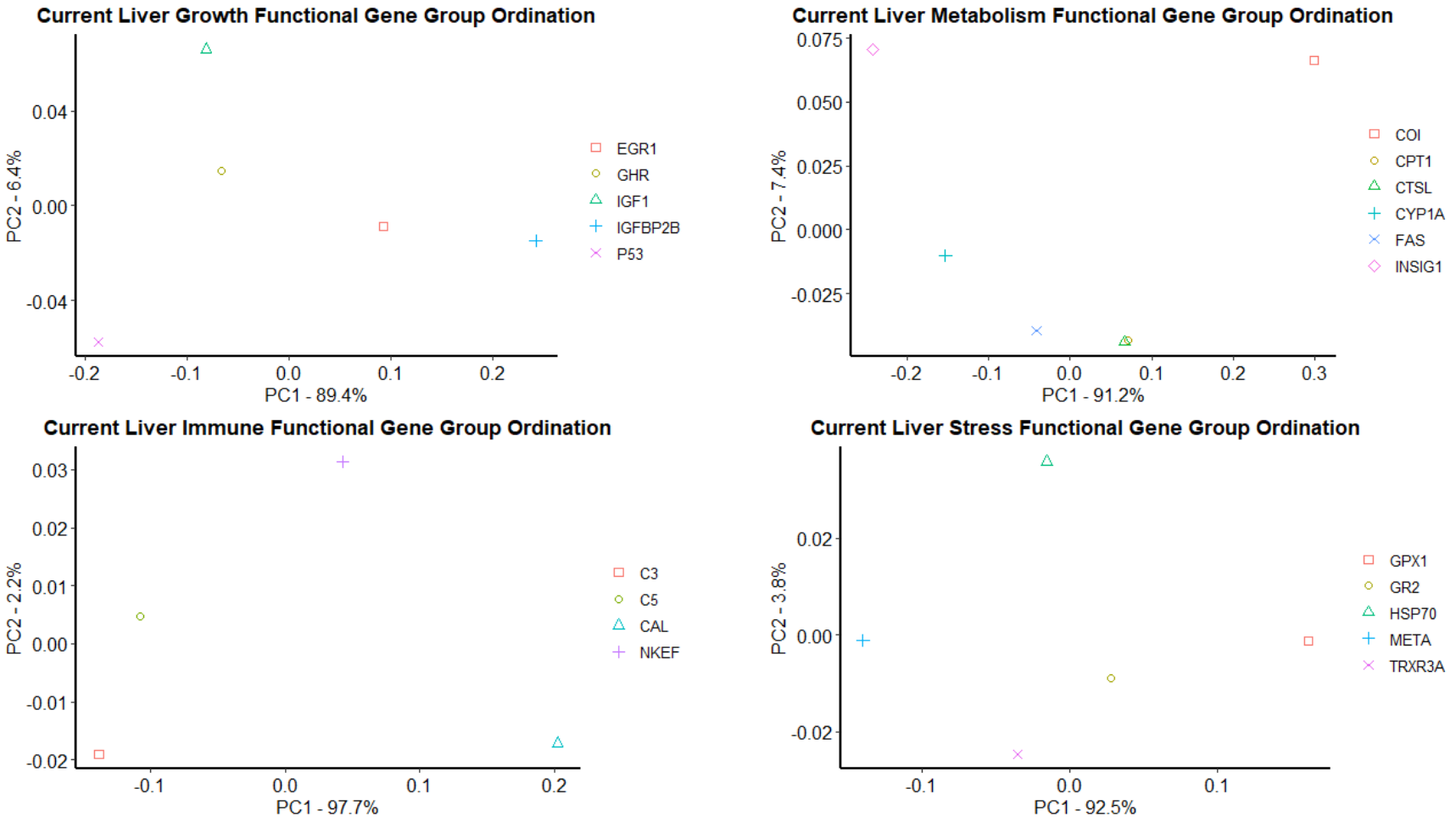


Figure 3.8 - PCoA ordination maps for all functional gene groups significantly affected by the temperature spike treatment for Current-Liver. Candidate genes used in the complete gene set profile analysis were loaded into their respective functional groups. Candidate genes oriented closer to each other are more similar in response and farther distances indicate increasing dissimilarity. Principle component 1 (PC1) accounts for the largest proportion of the variance for all functional gene groups and therefore is a better indicator of overall gene transcriptional relationships in response to the spike treatment.

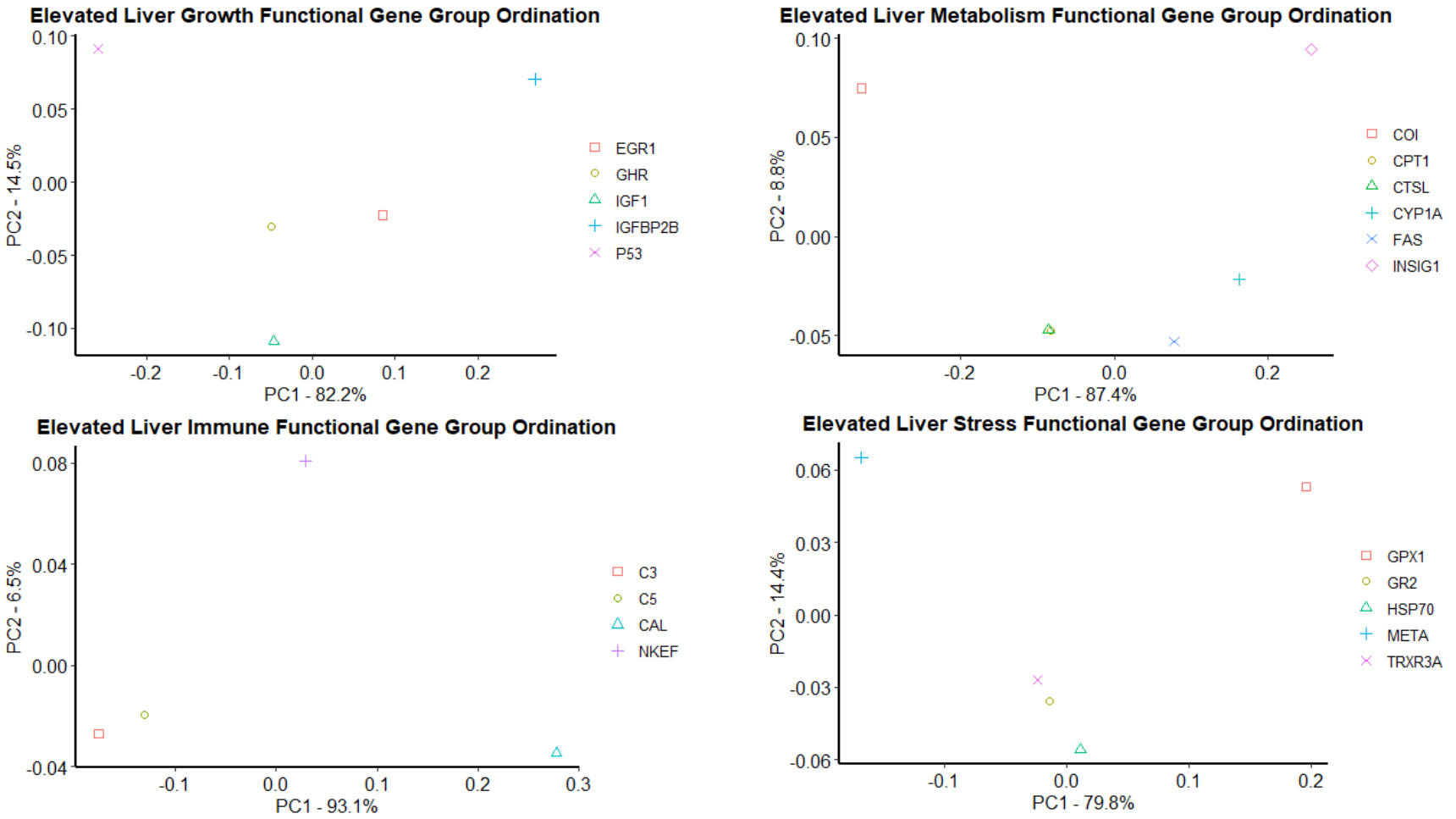


Figure 3.9 - PCoA ordination maps for all functional gene groups significantly affected by the temperature spike treatment for Elevated-Liver. Candidate genes used in the complete gene set profile analysis were loaded into their respective functional groups. Candidate genes oriented closer to each other are more similar in response and farther distances indicate increasing dissimilarity. Principle component 1 (PC1) accounts for the largest proportion of the variance for all functional gene groups and therefore is a better indicator of overall gene transcriptional relationships in response to the spike treatment.

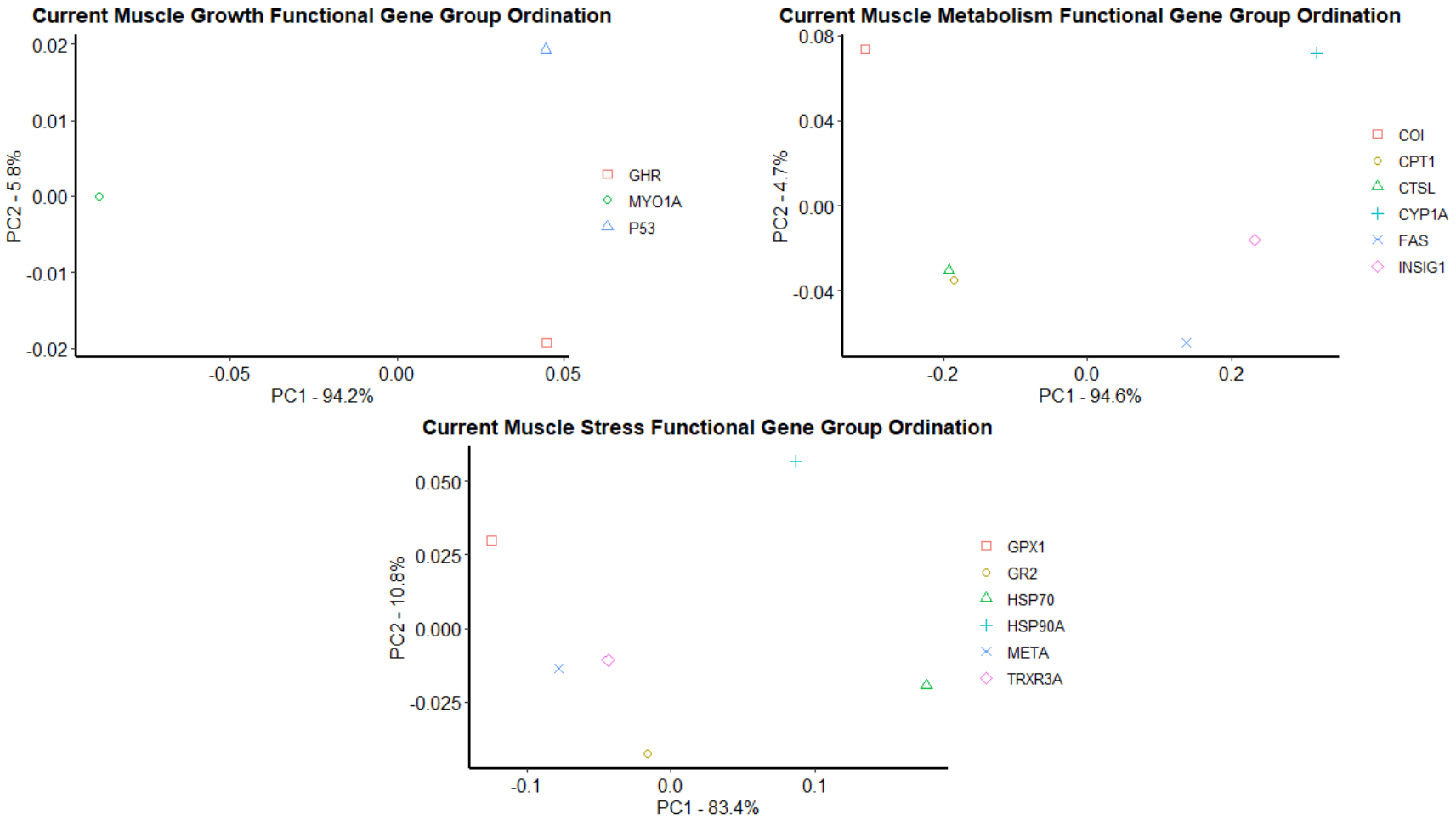


Figure 3.10 - PCoA ordination maps for all functional gene groups significantly affected by the temperature spike treatment for Current-Muscle. Candidate genes used in the complete gene set profile analysis were loaded into their respective functional groups. Candidate genes oriented closer to each other are more similar in response and farther distances indicate increasing dissimilarity. Principle component 1 (PC1) accounts for the largest proportion of the variance for all functional gene groups and therefore is a better indicator of overall gene transcriptional relationships in response to the spike treatment.

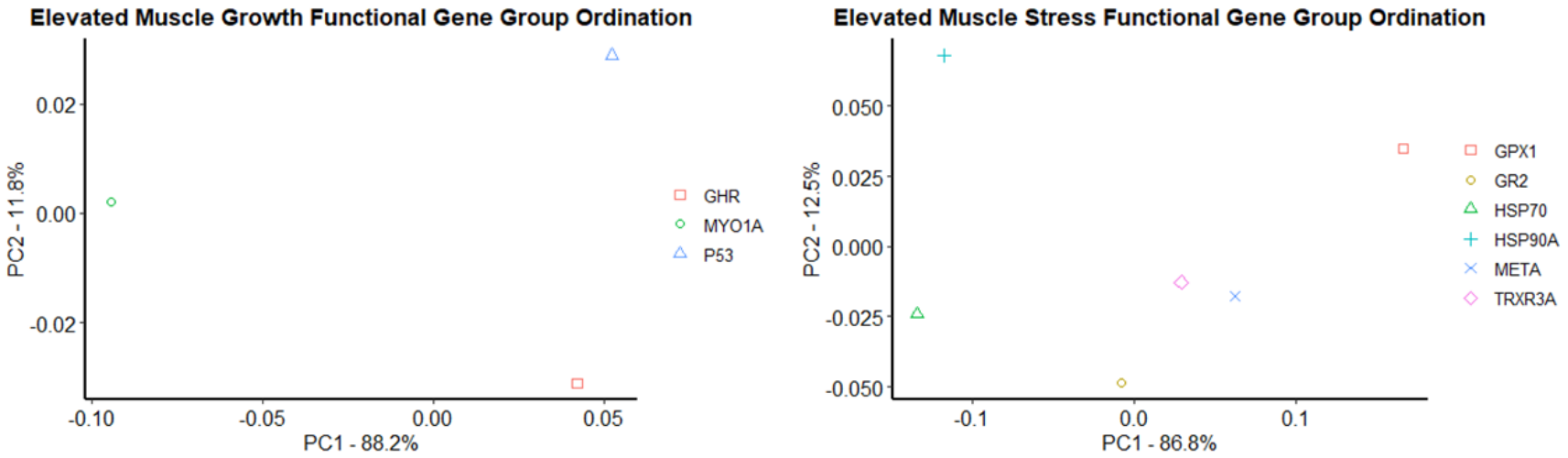
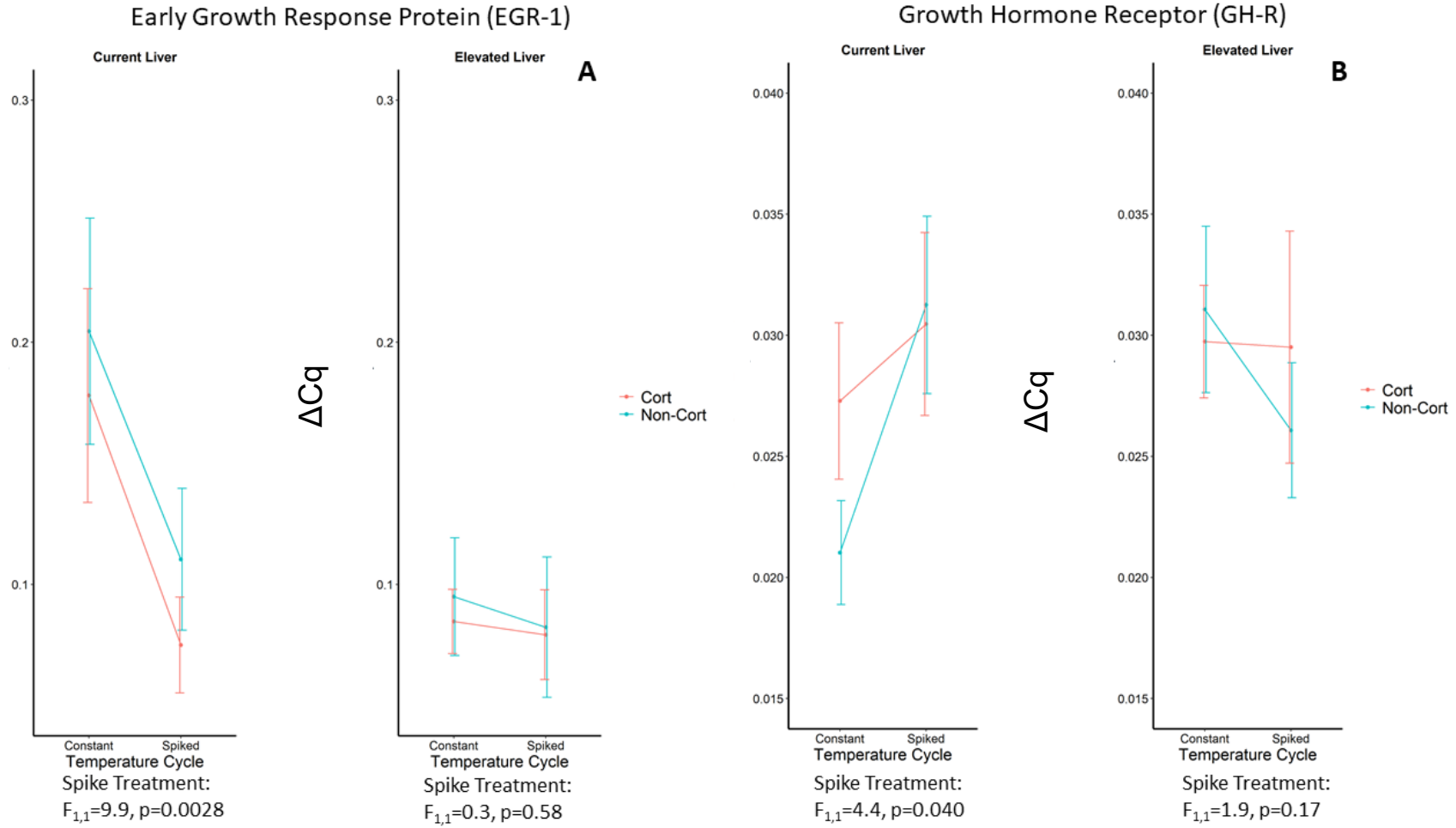


Figure 3.11 - PCoA ordination maps for all functional gene groups significantly affected by the temperature spike treatment for Elevated-Muscle. Candidate genes used in the complete gene set profile analysis were loaded into their respective functional groups. Candidate genes oriented closer to each other are more similar in response and farther distances indicate increasing dissimilarity. Principle component 1 (PC1) accounts for the largest proportion of the variance for all functional gene groups and therefore is a better indicator of overall gene transcriptional relationships in response to the spike treatment.


Early Growth Response Protein (EGR-1)
Growth Hormone Receptor (GH-R)
Current Liver
Elevated Liver
A
B
ΔCq
0.3
0.2
0.1
0.040
0.035
0.030
0.025
0.020
0.015
Constant
Spiked
Temperature Cycle
Cort
Non-Cort
Spike Treatment:
F1,1=9.9, p=0.0028
Spike Treatment:
F1,1=0.3, p=0.58
Spike Treatment:
F1,1=4.4, p=0.040
Spike Treatment:
F1,1=1.9, p=0.17

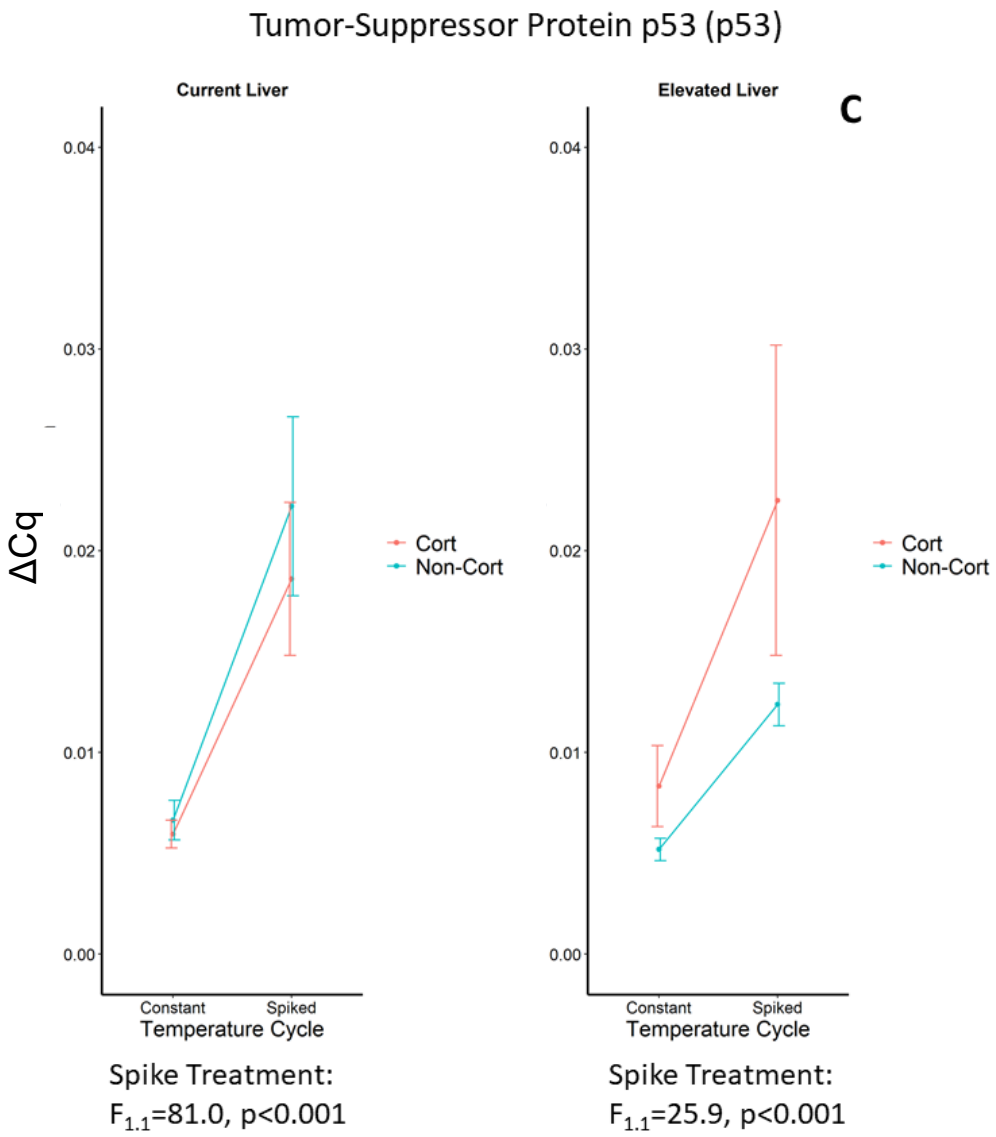


Figure 3.12 A to C – Individual gene transcriptional responses of candidate genes significantly affected by the temperature spike treatment in the growth functional group for the liver tissue across both rearing temperatures. The candidate genes EGR-1 and GH-R were significantly affected by the temperature spike only in the liver of offspring reared under current ambient thermal conditions while p53 was significantly affected by the spike treatment across both rearing conditions. Y-axis is efficiency corrected ΔCq corrected with geometric mean of both endogenous control genes.

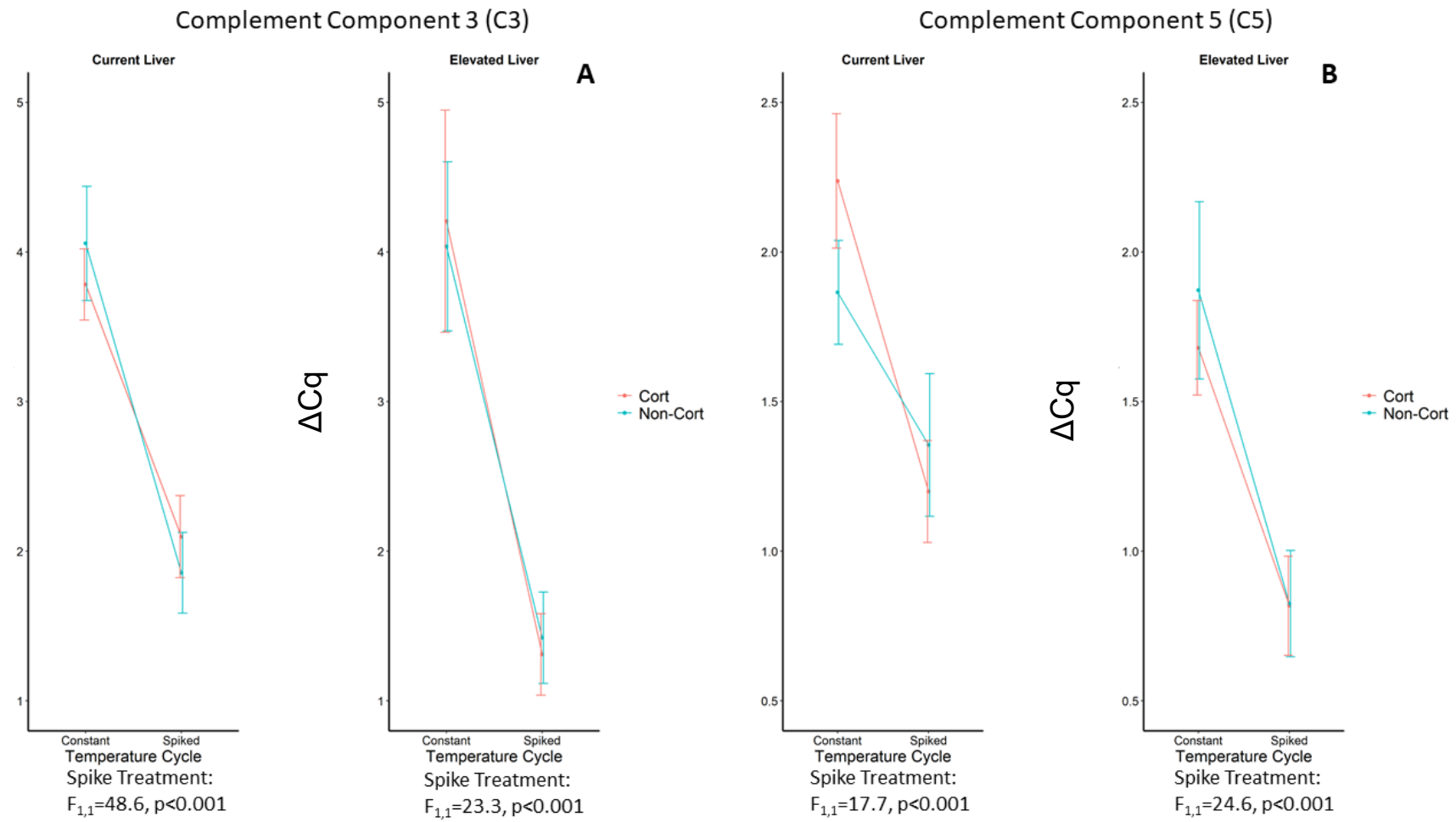


Figure 3.13 A & B – Individual gene transcriptional responses of candidate genes significantly affected by the temperature spike treatment in the immune functional group for the liver tissue across both rearing temperatures. Both candidate gene C3 and C5 exhibited significant decrease in gene transcription in response to the temperature spike treatment. Elevated reared offspring had slightly lower levels of transcription due to the spike treatment which may indicate greater immunosuppressive activity. Y-axis is efficiency corrected ΔCq corrected with geometric mean of both endogenous control genes.

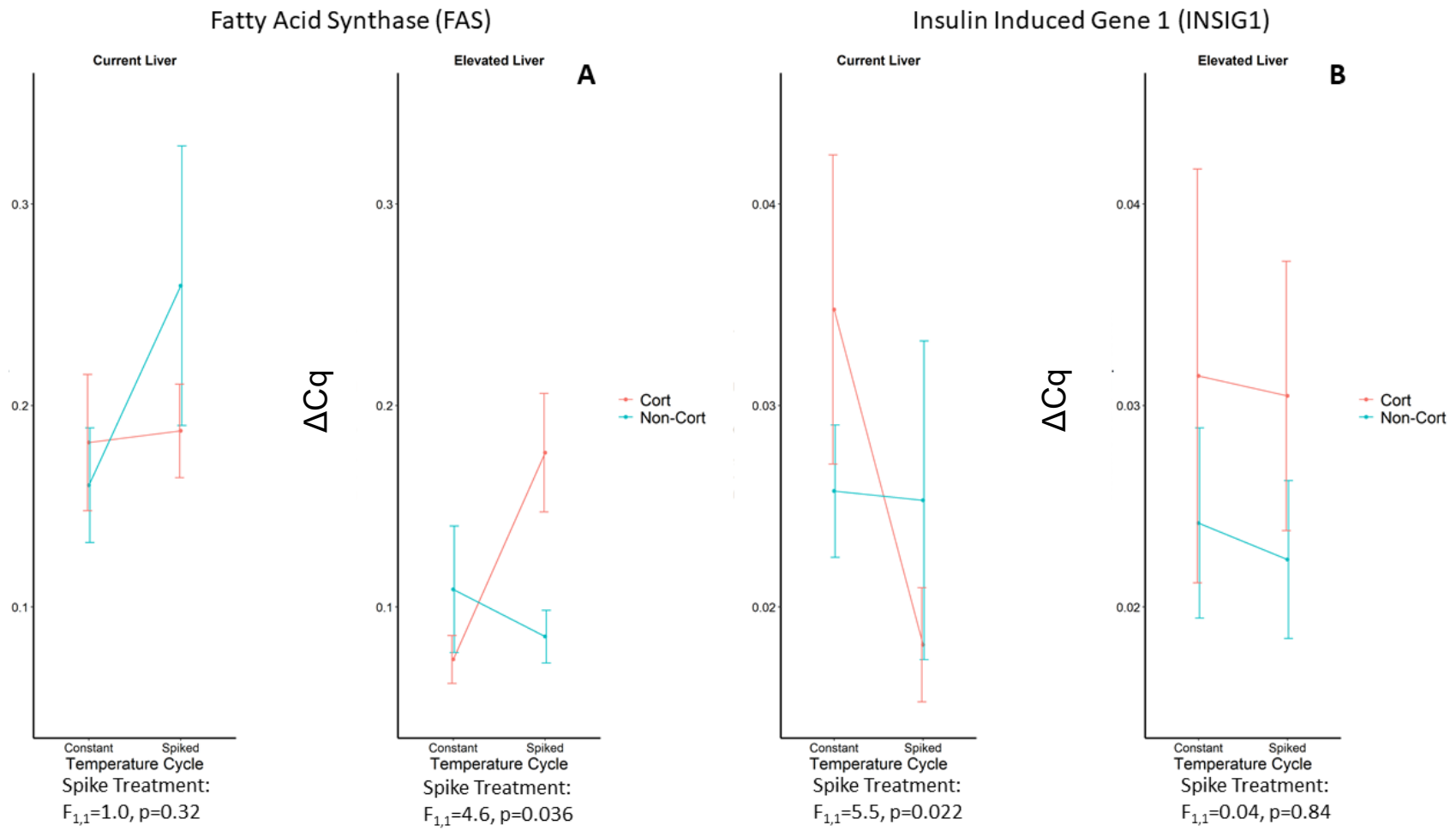
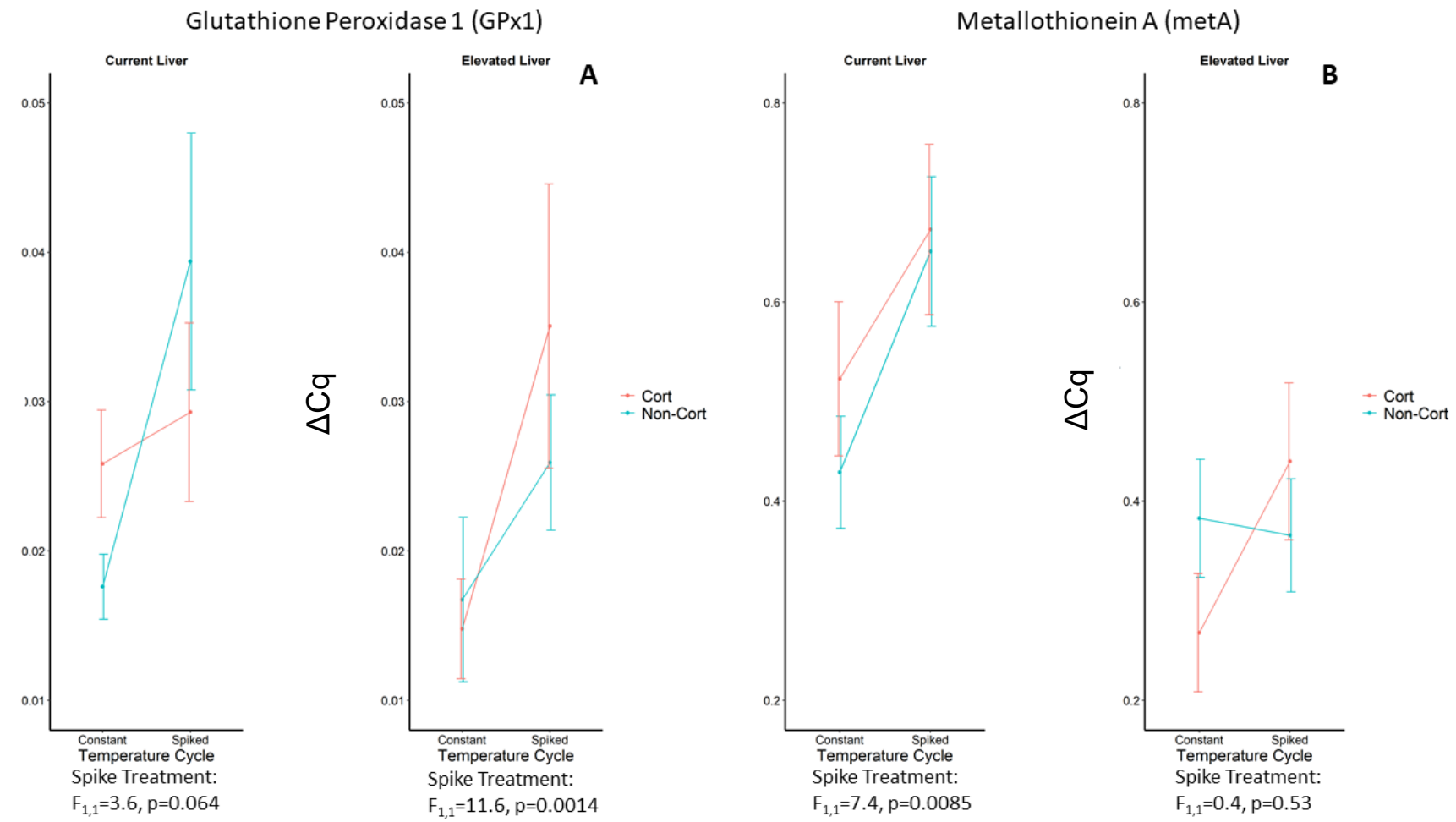


Figure 3.14 A & B – Individual gene transcriptional responses of candidate genes significantly affected by the temperature spike treatment in the metabolism functional group for the liver tissue across both rearing temperatures. The candidate gene FAS was only significantly affected by the temperature spike treatment in the liver of offspring reared under elevated thermal conditions while INSIG1 was only significantly affected by the temperature spike in the offspring reared under current ambient thermal conditions. Y-axis is efficiency corrected ΔCq corrected with geometric mean of both endogenous control genes.


Glutathione Peroxidase 1 (GPx1)
Current Liver
Elevated Liver
A
ΔCq
Cort
Non-Cort
Constant
Spiked
Temperature Cycle
Spike Treatment:
F1,1=3.6, p=0.064
F1,1=11.6, p=0.0014
Metallothionein A (metA)
B
F1,1=7.4, p=0.0085
F1,1=0.4, p=0.53

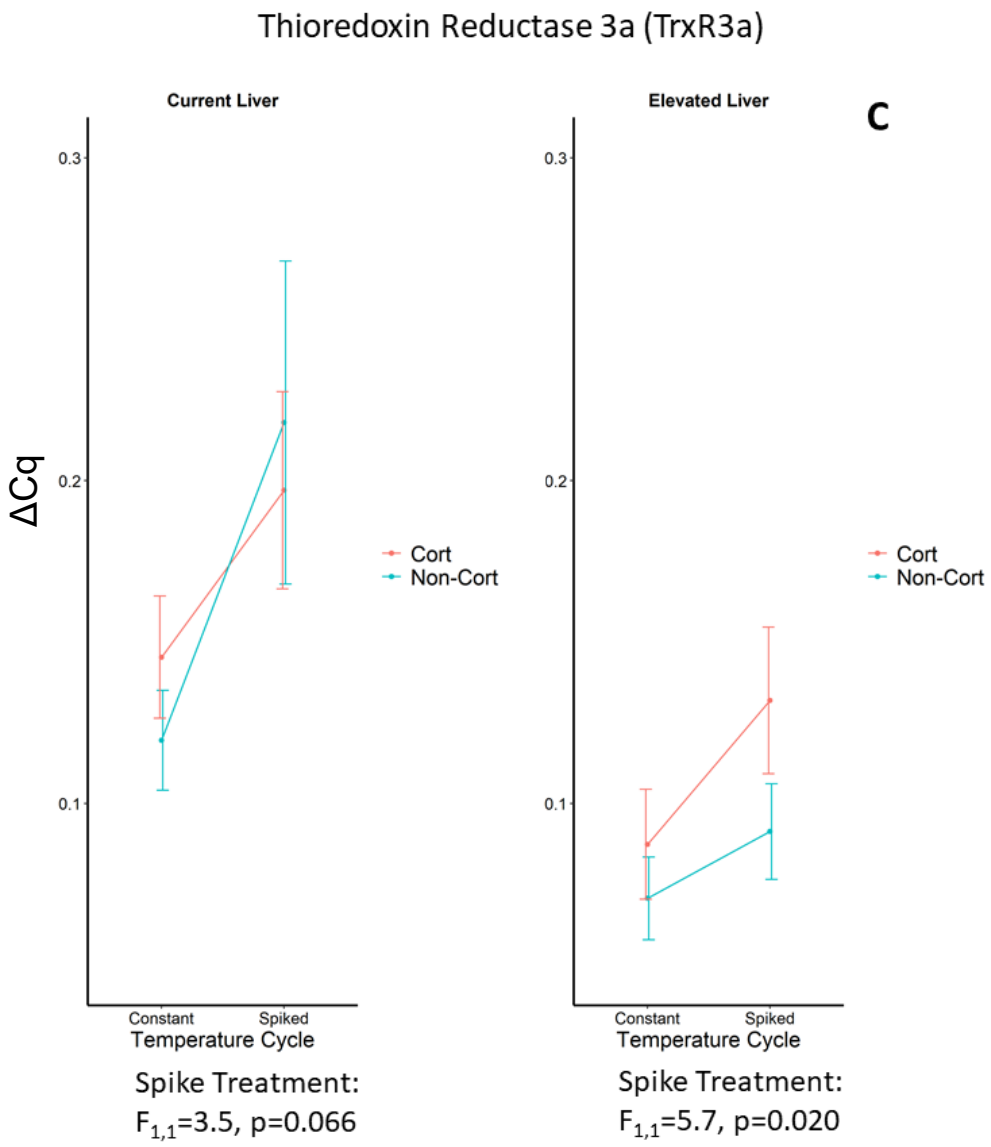
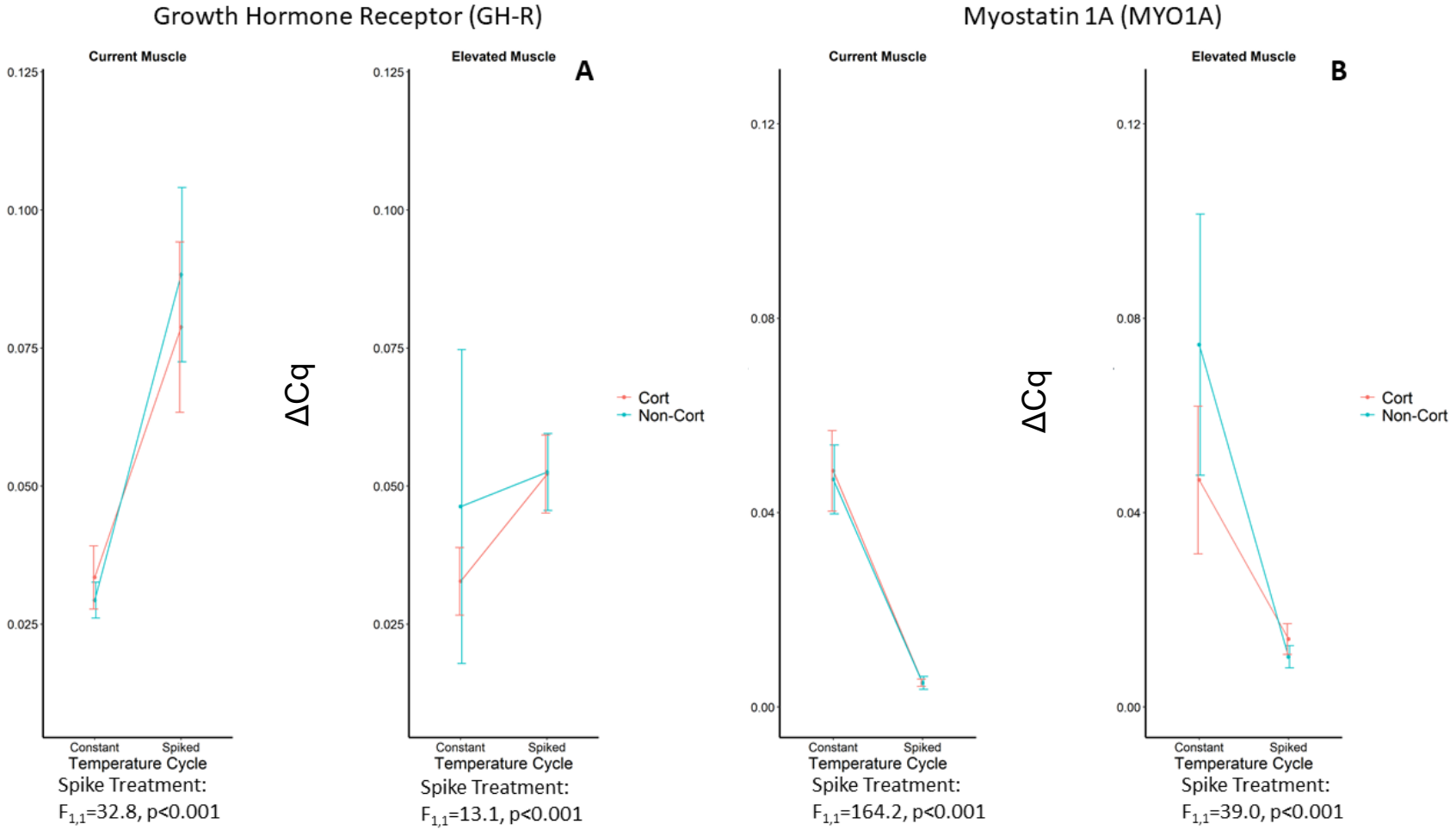


Figure 3.15 A to C – Individual gene transcriptional responses of candidate genes significantly affected by the temperature spike treatment in the stress functional group for the liver tissue across both rearing temperatures. The candidate genes GPx1 and TrxR3a were only significantly affected by the temperature spike treatment in the liver of offspring reared under elevated thermal conditions while metA was only significantly affected by the temperature spike in the offspring reared under current ambient thermal conditions. Y-axis is efficiency corrected ΔCq corrected with geometric mean of both endogenous control genes.


Growth Hormone Receptor (GH-R)
Myostatin 1A (MYO1A)
Current Muscle
Elevated Muscle
A
B
ΔCq
0.125
0.100
0.075
0.050
0.025
0.12
0.08
0.04
0.00
Constant
Spiked
Temperature Cycle
Cort
Non-Cort
Spike Treatment:
F1,1=32.8, p<0.001
F1,1=13.1, p<0.001
F1,1=164.2, p<0.001
F1,1=39.0, p<0.001

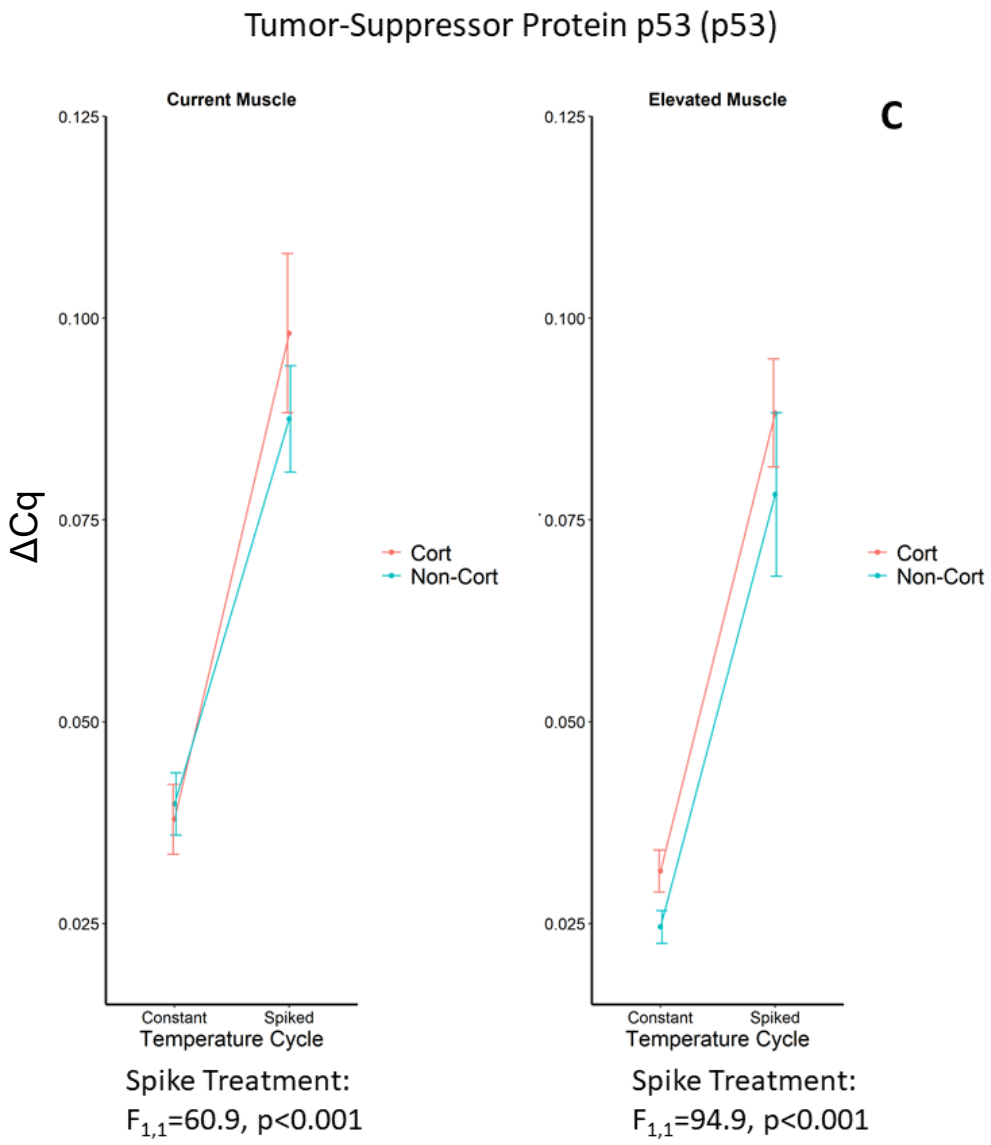


Figure 3.16 A to C – Individual gene transcriptional responses of candidate genes significantly affected by the temperature spike treatment in the growth functional group for the muscle tissue across both rearing temperatures. All candidate genes were significantly affected by the spike treatment across both rearing temperatures, but muscle growth and cellular turnover was slightly lower in the elevated reared offspring which could indicate reduced growth and recovery. Y-axis is efficiency corrected ΔCq corrected with geometric mean of both endogenous control genes.

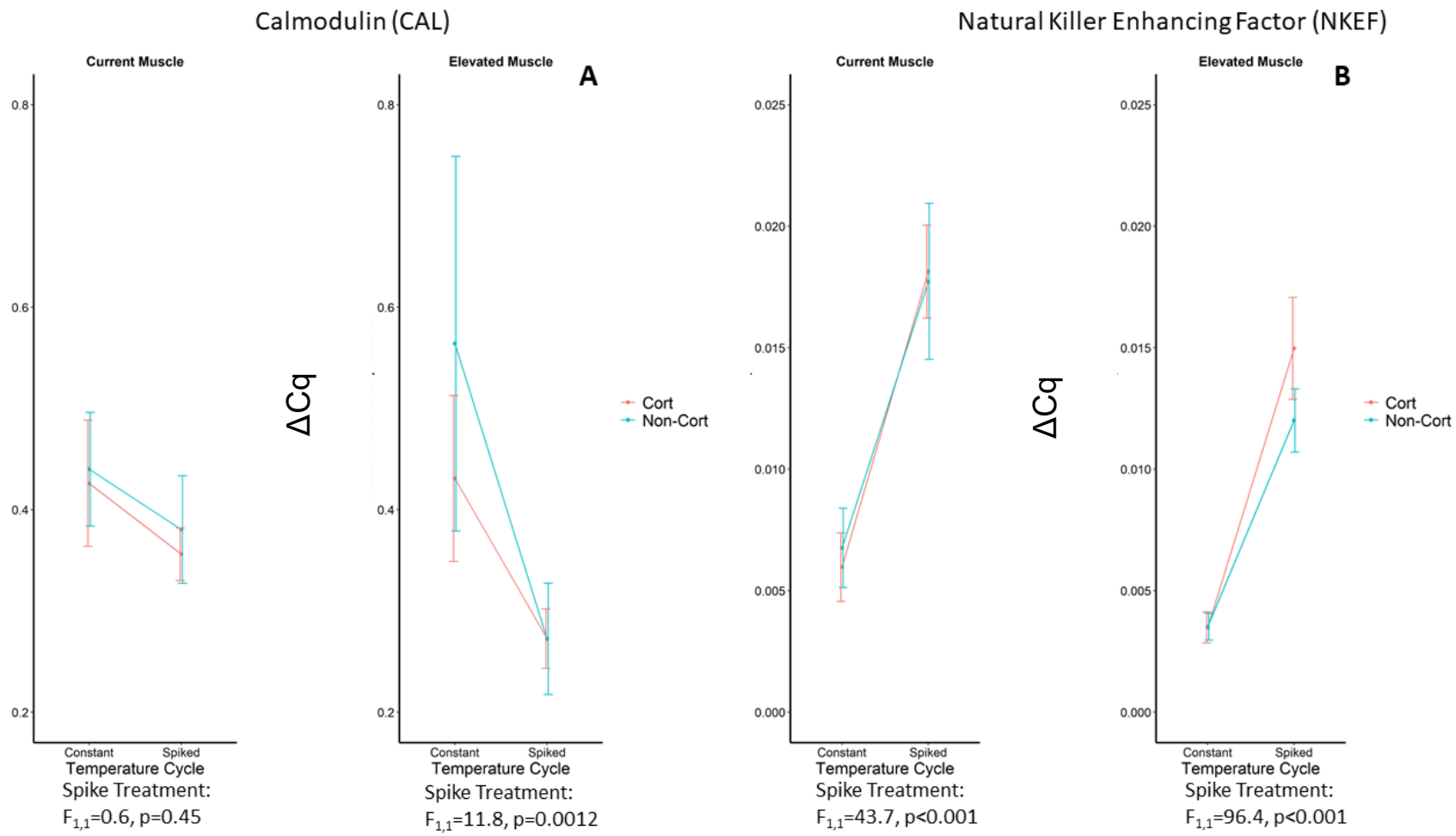
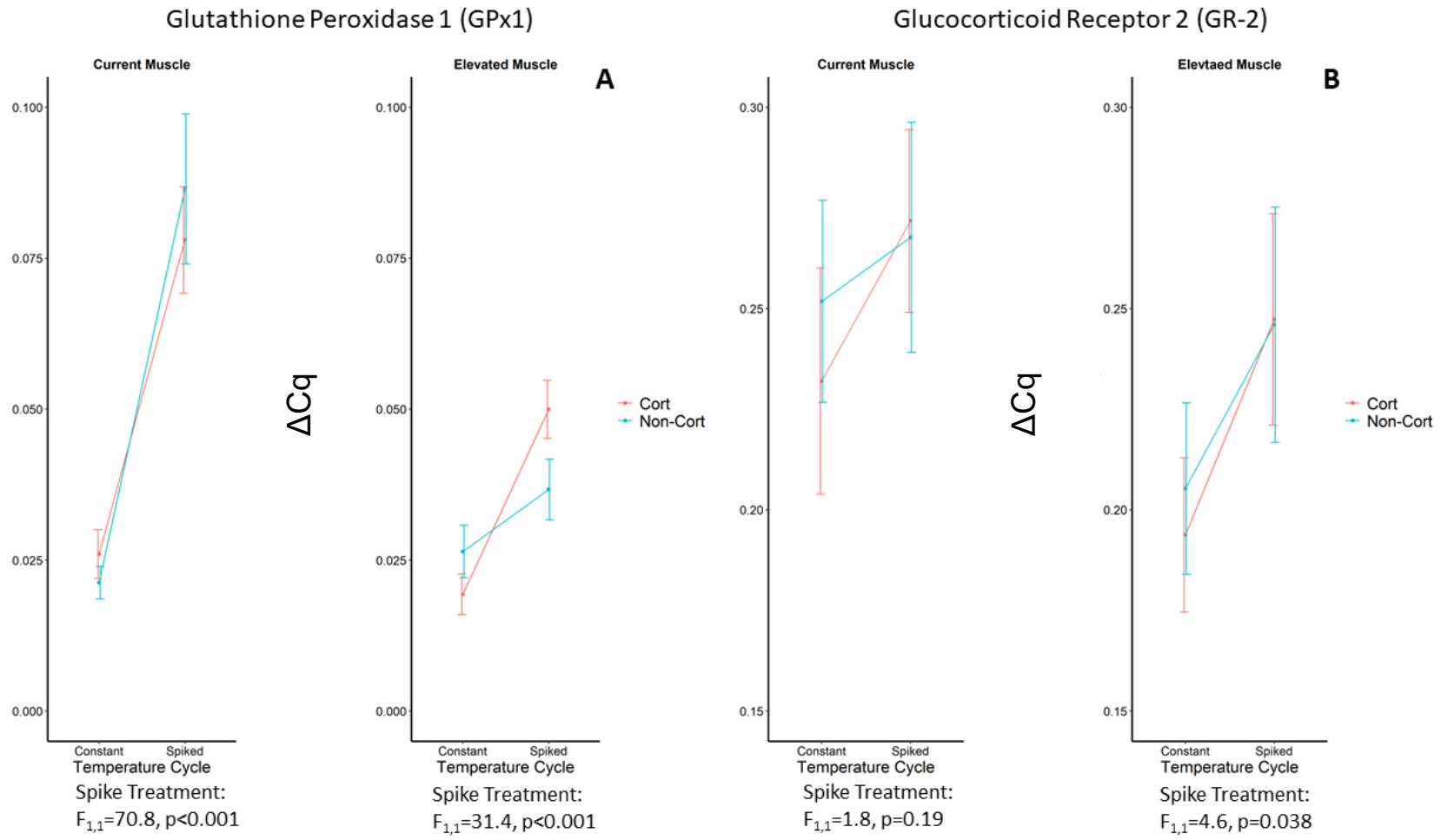
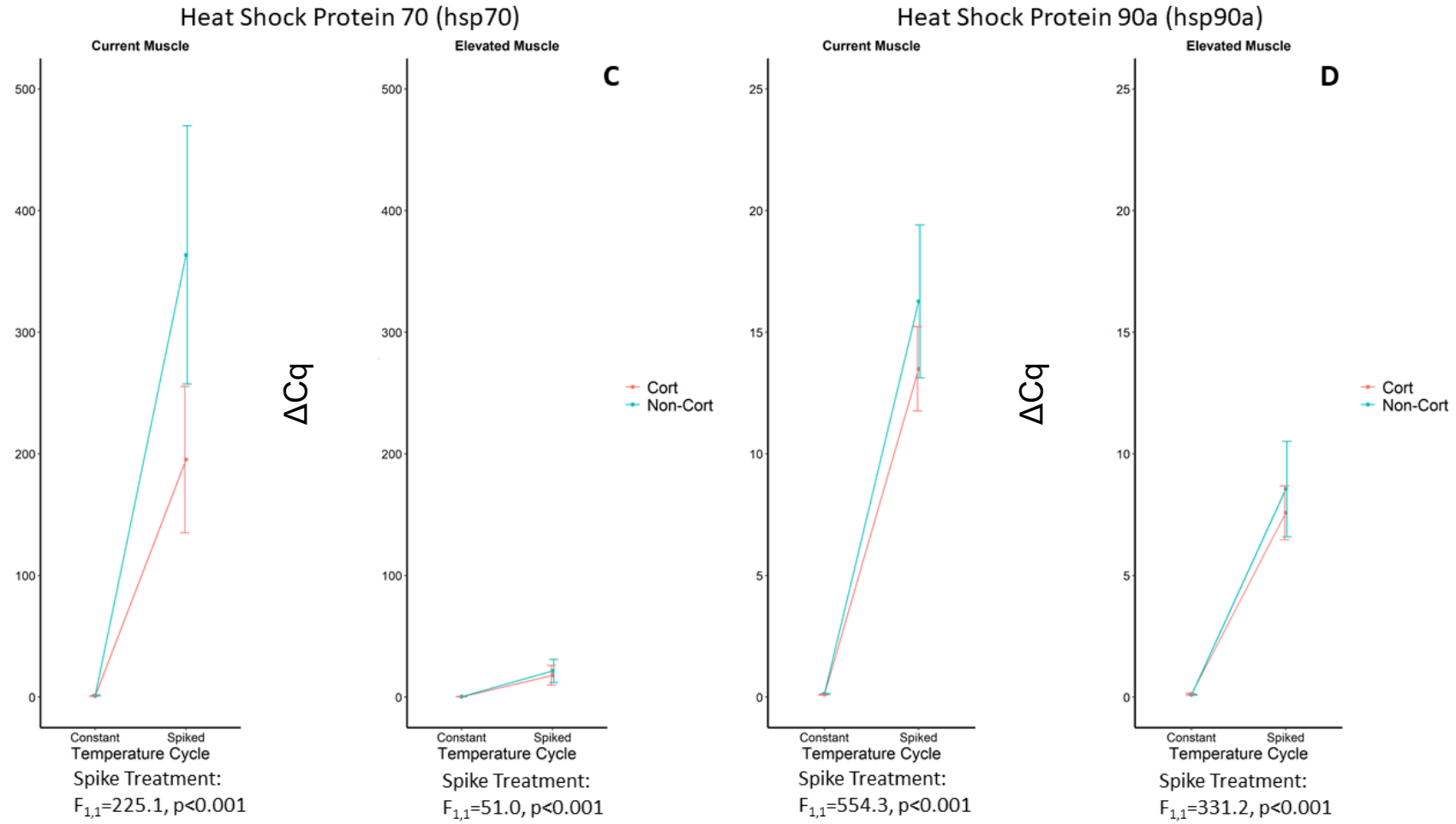


Figure 3.17 A & B – Individual gene transcriptional responses of candidate genes significantly affected by the temperature spike treatment in the immune functional group for the muscle tissue across both rearing temperatures. Both rearing temperatures NKEF were significantly affected by the spike treatment, but CAL was only significantly affected by spike treatment in the elevated reared

offspring. Lower levels of gene transcription would indicate reduced immune function in the elevated reared muscle. Y-axis is efficiency corrected ΔCq corrected with the geometric mean of both endogenous control genes.




Heat Shock Protein 70 (hsp70)
Current Muscle
Elevated Muscle
C
ΔCq
Cort
Non-Cort
Constant
Spiked
Temperature Cycle
Spike Treatment:
F1,1=225.1, p<0.001
Spike Treatment:
F1,1=51.0, p<0.001
Heat Shock Protein 90a (hsp90a)
Current Muscle
Elevated Muscle
D
ΔCq
Cort
Non-Cort
Constant
Spiked
Temperature Cycle
Spike Treatment:
F1,1=554.3, p<0.001
Spike Treatment:
F1,1=331.2, p<0.001

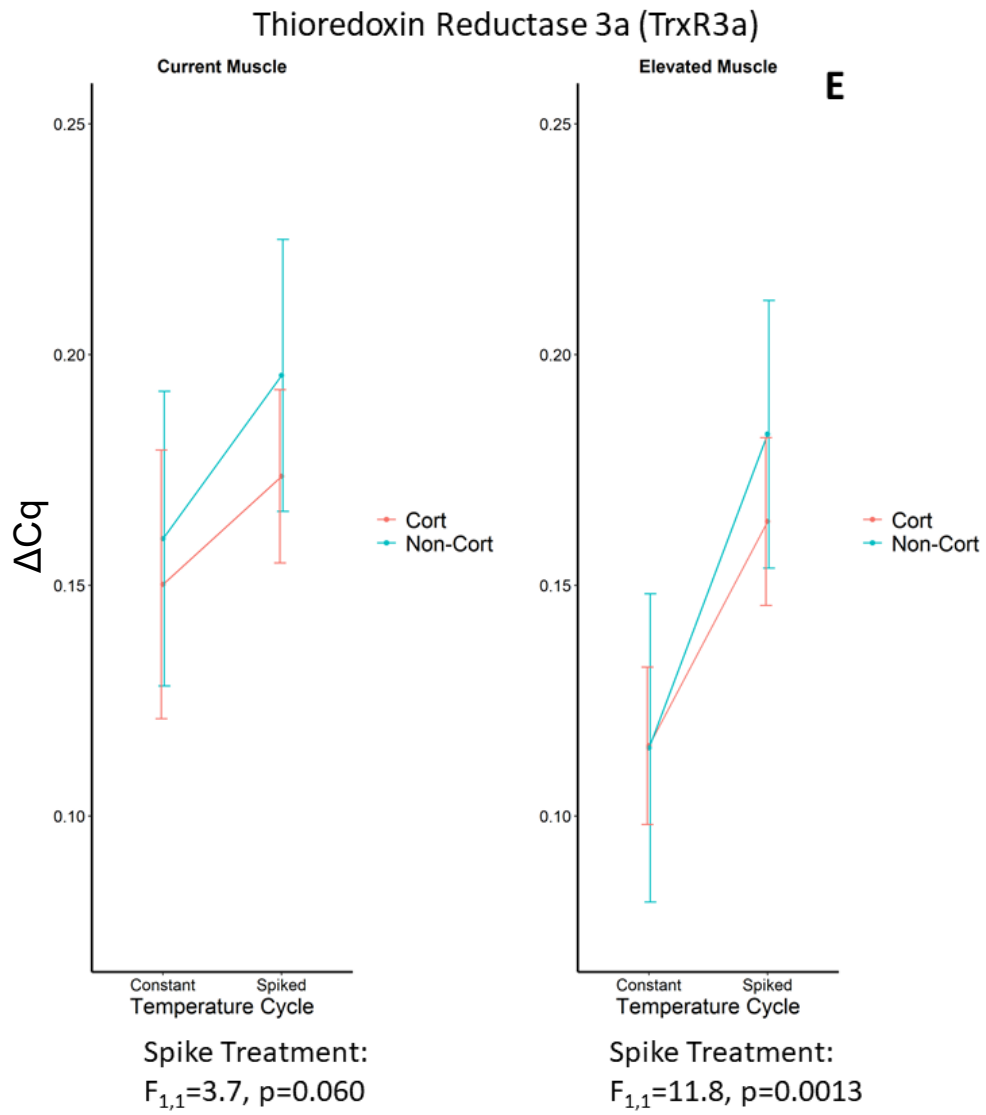


Figure 3.18 A to E – Individual gene transcriptional responses of candidate genes significantly affected by the temperature spike treatment in the stress functional group for the muscle tissue across both rearing temperatures. Overall analysis of transcriptional variation for these candidate genes indicates that the offspring reared under elevated conditions were less stressed by the temperature spike treatment than the offspring reared under current ambient conditions. Y-axis is efficiency corrected ΔCq corrected with geometric mean of both endogenous control genes.

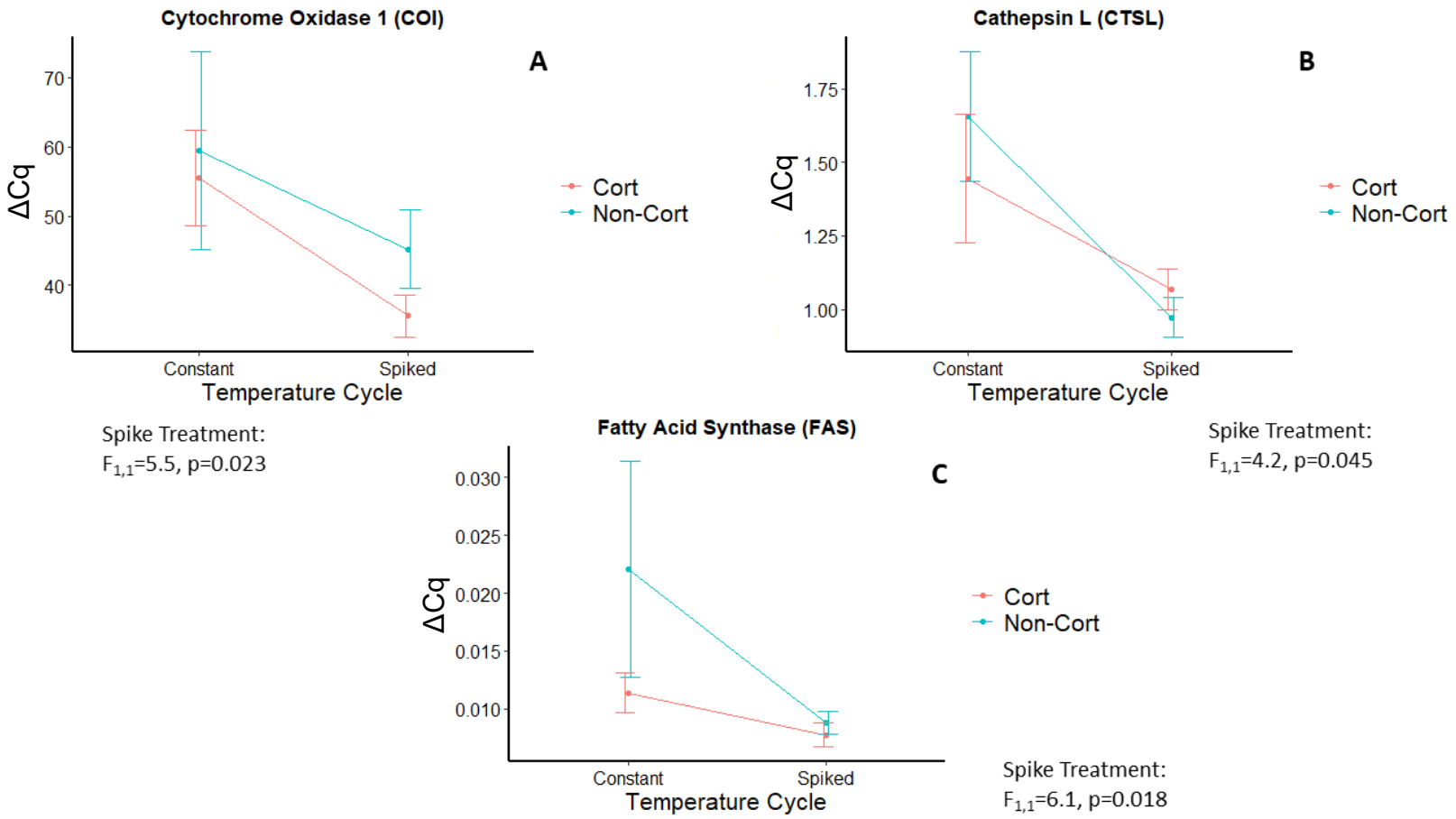


Figure 3.19 A to C – Individual gene transcriptional responses of candidate genes significantly affected by the temperature spike treatment in the metabolism functional group for the muscle tissue for offspring reared under current ambient rearing temperatures. Offspring reared under elevated rearing conditions did not exhibit significantly different functional response for metabolic candidate genes, possibly indicating greater thermal tolerance in the elevated group to continue similar metabolic activity as benign conditions. Y-axis is efficiency corrected ΔCq corrected with geometric mean of both endogenous control genes.

Chapter 4 – GENERAL DISCUSSION

Summation of Thesis Results

My graduate research found no capacity for prenatal cortisol to modulate gene transcriptional phenotypes for developing Chinook salmon offspring under current and future predicted thermal water conditions as well as in response to multiday, acute thermal fluctuations that approach the maximum limits of the species thermal range later in life. In **Chapter 2**, we explored the effects of prenatal cortisol and rearing temperature as environmental stressors to evaluate the individual capacities for both to alter gene transcription across multiple developmental stages (i.e., eyed-egg, alevin, and fry). Our approach used an applied setup to quantify variation in gene transcription under the evolutionary framework of the Environmental-Matching Hypothesis (Love et al., 2013) to evaluate to what degree prenatal cortisol or chronic thermal stress altered developmental plasticity. Results of the analysis indicated that prenatal cortisol had no significant ability to modify gene transcriptional profiles at any developmental stage, across either thermal rearing conditions for key biological functions (i.e., growth, metabolism, immune function, and stress response). However, differences in thermal rearing conditions did alter gene transcription profiles significantly for the developmental stages eyed-egg and alevin. Specifically, the metabolic functional gene group for both stages and the stress functional gene group for the eyed-eggs were significantly affected by the elevated rearing conditions. No individual candidate genes were significantly altered by the chronically elevated thermal rearing conditions which would indicate that the thermal stressor altered overall metabolic and stress response without resulting in an extreme response of a singular gene. We suggest that these significant effects of rearing

temperature on the earliest developmental stages negative effects associated with heat stress due to higher mortality rates for eyed-eggs under elevated rearing conditions. Additionally, prenatal cortisol did not rescue offspring fitness (i.e., survival from fertilization to fry) when experiencing predicted thermal environments (i.e. higher rearing temperatures) which would give more credibility that prenatal cortisol is not an effective modifier of developmental plasticity to cope with a thermal stressor. Likewise, in **Chapter 3** prenatal cortisol also had no capacity to alter gene transcriptional profiles at the complete gene set or functional gene group levels the biological functions examined in the Chapter 2 (i.e., growth, metabolism, immune function, and stress response) at a later life history stage (i.e., parr) to an acute thermal stressor of +9°C over a nine hour period for three consecutive days. Again, this would indicate that the prenatal cortisol administered to the offspring immediately post-fertilization is not an effective signal to modulate transcriptional responses that persist into later life history stages to alter transcriptional plasticity. Differences in gene transcription across rearing temperatures in response to an acute thermal stressor indicated that being reared under elevated thermal conditions had some capacity to modify transcriptional responses. Furthermore, rearing temperatures results in significantly different transcriptional response intensities, across both individual candidate genes and candidate gene relationships (see Chapter 3 Results for which genes differed across rearing temperatures), suggesting that rearing conditions may play a key role in what degree offspring respond plastically at later life history stages in response to acute thermal stress.

What could this mean for hatcheries and fisheries?

Prenatal cortisol has been previously shown to alter developmental phenotypes of Pacific salmonid offspring (e.g., reduced size at key life history stages (Sopinka et al., 2017), behavioural changes (Sopinka et al., 2015), physiological functions (Li & Leatherland, 2012; McNeil et al., 2016; Sloman, 2010)), but often does not result in reduced mortality under chronic mild thermal stress conditions (Eriksen et al., 2006; Eriksen et al., 2007; Warriner et al., 2020). Our results would strengthen these previous findings in that prenatal cortisol did not rescue offspring survival when experiencing predicted chronic thermal stress rearing conditions in this experiment as well. Therefore, prenatal stress's capacity to alter developmental phenotypes, in relation to thermal stress, does not appear to be adaptive under the context of predicted future thermal environmental conditions of +3°C. Without further analysis indicating the opposite, prenatal cortisol would not be an advised mechanism in preparing future offspring for environmental conditions they may experience based on the current capacity for the populations ability to respond.

Chronically elevated rearing temperatures elicited possible adaptive and maladaptive phenotypic responses in juvenile salmon. During early development, the differences in rearing conditions did alter gene transcription profiles significantly, but the biological functions affected (metabolism and stress response) compounded with the increased mortality under elevated thermal conditions would indicate that they did not rescue the offspring from the negative effects of the elevated thermal conditions. However, the offspring that survived the early developmental stages had altered transcriptional responses to the acute thermal stressor later in life, some of which could indicate greater thermal tolerance for the offspring reared under the elevated thermal

conditions (see Chapter 3 for specific examples). Therefore, fish offspring reared under chronically elevated thermal conditions may actually be better prepared for more severe thermal stressors later in life, an effect reported previously (Comte & Olden, 2017; Corey et al., 2017). Further fitness analysis would need to be completed to identify if these variations are associated with an adaptive response under acute thermal environmental conditions. If there is some adaptive capacity by preparing current populations for future acute thermal environments through manipulation of elevated thermal rearing environments, hatcheries may consider implementing a similar strategy to increase stocking success as we progress towards more deteriorating thermal environments for Pacific salmonids.

What can this tell us about future Pacific salmon population health?

Gene transcription is now becoming an important tool for biologist to examine population health due to its ability to provide cellular details of variation across key biological systems when exposed to various and varying intensities of environmental stressors (Balenger et al., 2015; Lewis et al., 2010; Maes et al., 2013; Peretz et al., 2007). Monitoring norms of reaction to environmental change, both benign and stressful, in natural and lab settings can provide important baseline metrics that could indicate what kind of stressors, and to what intensity, populations are experiencing at different timepoints in their natural environments (Aubin-Horth & Renn, 2009; Cossins, 2006). Therefore, the results from this experiment are important for evaluating current capacities of Chinook salmon to modulate their molecular processes under current and future thermal conditions. However, future populations might have different responses to these future stressors, compared to what we observed, due to evolutionary processes selecting

for traits that might enhance individual's adaptive plastic capacity to respond to the future environmental conditions (Scheiner, 1993; Via, 1993). While survival appears to decrease at higher temperatures during early development, those individuals that do survive through the chronic stress may have greater plasticity to respond and therefore future generations might share this enhanced ability to cope more rapidly with stress. In order to evaluate if this enhanced generational plasticity exists and if so, to what extent the plastic responses affect fitness, more transgenerational transcriptional analysis will need to be completed.

What are the next steps for evaluating pre- and post-natal stress?

While we did not identify any instances of prenatal cortisol modifying gene transcriptional profiles across our candidate genes, analysis could still be done with different dosages of cortisol (i.e., dose-response analysis) to identify if there is a concentration that does alter gene transcription of important biological functions. Previous work by Capelle et al. (2016) identified differing effects of varying cortisol concentration dosages that actually enhanced survival of offspring at the lowest concentrations – which were identical to the concentrations we used - but they used only benign environments. Therefore, there may be a concentration that is an optimal signal for future chronic and acute thermal conditions that we currently have not identified. Unfortunately, even if we do identify a cortisol dosage that is affective at producing adaptive responses to future thermal conditions that enhance offspring fitness, egg concentrations we observed are consistent with naturally occurring signals offspring currently experience during periods of extreme stress on females migrating to spawn, in a semelparous species. Positive results at higher concentrations, if any are present, would

indicate that any manipulation that is adaptive under chronic and acute thermal stressors associated with prenatal cortisol are possibly not attainable naturally and would need to be administered under controlled settings like hatcheries or labs. Also, while we did not identify any significant maternal effects on gene transcription throughout development in this thesis research, previous literature has identified significant alterations to offspring phenotypes and plasticity of early developmental stages that can be attributed to parental inheritance (Nettle & Bateson, 2015; Wadgymar et al., 2018; Warriner et al., 2020). Those observations may be a result of varying quality of parental genetic material that alter how offspring respond to environmental stressors adding complexity in identifying what dosage of cortisol is necessary to elicit the desired phenotype to increase offspring fitness. Do offspring from lower quality parents require more/less/equal concentrations of egg cortisol to elicit adaptive plasticity? Does a maternal stress signal offer more benefits to offspring from higher quality parents because of better genetic material to work with or is it a signal honed for lower quality offspring to perform better under worse circumstances? How much cortisol is deposited in the egg prior to the cortisol solution being administered to make sure that the correct dosed is administered to give the desired egg cortisol concentration? Again, these are all questions that would easier to evaluate under controlled settings like hatcheries or labs if a correct cortisol concentration could be identified that enhances offspring survival under chronically and acutely elevated thermal environments.

Implementing adaptive management plans for species at risk can be enhanced by understanding how organisms are responding to stressors at the cellular level (Connon et al., 2018; Williams et al., 2015), gene transcriptional analysis gives only part of the

picture for how organisms are responding to environmental stressors. Using gene transcription in conjunction with physiological or behavioural phenotype alteration would give a more holistic evaluation of how species are reacting to environmental conditions. Additionally, while we did not identify significant modulation to gene transcriptional profiles by prenatal cortisol, downstream processes like peptide production may be significantly altered to produce the phenotypes often associated with prenatal stress signals identified in previous literature (Liu et al., 2016). Therefore, proteomic evaluation is the logical next step of evaluating if any minute changes in gene transcription of our candidate genes, that may not be significantly significant but may be biologically relevant, are amplified at future cellular processes.

Finally, while we did not identify any effects of prenatal cortisol to modulate gene transcriptional profiles within a single generation of offspring reared under these conditions, continued alterations to environmentally-induced phenotypes associated with these two stressors may have a greater potential to drive evolutionary adaptations than mutations (Moczeck et al., 2011; West-Eberhard, 2005). Therefore, analysis of continued transgenerational effects of cortisol or rearing conditions in response to future predicted deteriorating thermal conditions might indicate some adaptive capacity for future generations whose previous generations endured continued environmental stress.

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APPENDICES

Appendix A1 - Candidate Gene List for Chapter 2

Gene	Forward Primer	Reverse Primer	Probe	Amplicon Length	Accession Number
Endogenous Control Genes					
Acidic Ribosomal Phosphoprotein (ARP)	aggaccacgtggaagtccaa	cgccgacaatgaaacatttg	tatgaaaatcatccaattgc	108 bp	AY685220.1
Elongation Factor-1a (EF-1α)	aataccctcctcttggtcgtttc	cttgtcgacggccttgatg	tgcgtgacatgaggc	80 bp	AF498320.1
Growth Genes					
C-FOS	ccgcaacaggaggaaggaa	ctccagctcgtcggtttca	acgagctggaggacg	58 bp	AB11053.1
Early Growth Response Protein 1 (EGR-1)	cgaacatctgaatggagatacattacc	caggctccagggtgaacct	ctatcggctgtgacaagt	128 bp	NM_001141824.1
Growth Hormone Receptor (GH-R)	ccccactaaagagtcccgatt	ctaaacccaaggcagcaaaga	ccagttactgtcctgctt	62 bp	NM_001124731.1
Insulin-like Growth Factor 1 (IGF-1)	atttcagtaaaccaacgggctatg	cgtccacaataccacggttatg	ccagttcacgacggtc	66 bp	U14536.1
Insulin-like Growth Factor Binding Protein (IGFBP2b)	caactgtcccgaggaacctaag	ctccagctcctgtgcacaag	cccagcagcccatga	64 bp	HM358881.1
Myostatin-1A (MYO1a)	gggaaatgatctggccgtta	tctgaaatcgtcacctccatga	agaaggactgcaaccc	75 bp	EU009952.1
Tumor Suppressor p53 (p53)	cagtccagcacagccaagtc	cgccaactggcagaacaact	acttgcacatactcgc	72bp	AF071574.1
Metabolic Genes					
Carnitine Palmytol Transferase 1 (CPT1)	gaagggcctgatcaaaaagtgt	tccccttgtccctgaagtga	cttcatccagatcgc	86 bp	AJ620357.1
Cytochrome Oxidase 1 (COI)	ggcagcaggcattactatgttactc	gcctgccgggtcaaaga	cggaccgaaatcta	67 bp	KP720599.1
Cytochrome p450 Family 1A (CYP1a)	tcttccttcctgccgttcac	gaagtagccattgagggatgtgt	ccacactgcacgatc	66 bp	M21310.1
Elongation of Very Long Chain Fatty Acids Protein 7 (ELOVL7)	ttatgccatgcgtatgacagttagt	cggaggcgaaaaccaaaat	tgctttctcactgggtgtg	69 bp	XM_024418089.1
Fatty Acid Synthase (FAS)	ccaggtctgtacggtcttcca	cgaaccggctgatgtcctt	agaggaacggcaagct	58 bp	XM_014179800.1
Hexokinase 1 (HK1)	gggtctggacagcacatgtg	ggacacggtgctgcatacct	cgacagtatcatcgtcaag	61 bp	AY864082.1
Insulin Induced Gene 1 (INSIG1)	ctgctgttgttggcctgcta	ttgtgtggctctccgagatg	acccctgcatcgacag	58 bp	XM_024393097.1
Immune Genes					
Calmodulin (CAL)	cagacagcgaggaggagatca	taaccgttcccatccttgtca	agaagcgttccgtgtct	61 bp	BT074280.1
Chemokine 1 (CK-1)	tcctggctgctctgttctctct	acagcagtccgctgattgtg	ctcatcatcaccctcatt	68 bp	AF093810.1
Interleukin 1B (IL1B)	atgggaaccgagttcaagga	tccacagcactctccagcaa	aaggacctgctcaactt	59 bp	NM_001124347.2
Interleukin 8 (IL-8)	agacggagagcagacgtatcg	cgagctgggagggaacatc	taggctcattaagaaggtg	62 bp	DQ778949.1
Natural Killer Enhancing Factor (NKEF)	tgaggtcattggtgcctctgt	gaggtgtgttggtccaagca	attcccacttctgccatc	92 bp	AF250193.1
Tumor Necrosis Factor-α (TNF-α)	cccaccatacattgaagcagatt	ggattgtattcaccctctaaatgga	ccggcaatgcaaaa	70 bp	DQ778945.1

Stress Genes					
Glucocorticoid Receptor 2 (GR-2)	agcaccgtgccaaaagatg	gccttccccaactccttga	ctcatcaaacactgcctg	83 bp	AY495372.1
Glutathione Peroxidase 1 (GPX1)	acccagatgaacgagctccat	ggcacccccagaatcactag	cggtacgccgacaag	62 bp	NM_001124525.1
Heat Shock Protein 70 (HSP70)	tcaacgatcaggtcgtgcaa	cgtcgctgaccaccttgaa	ccgacatgaagcactg	60 bp	U35064.1
Heat Shock Protein 90a (HSP90a)	agatcttccttagggagctcatctc	tgtcaagctctcgtatctgatcttg	aactcttcagatgctttgg	71 bp	U89945.1
Metallothionein A (metA)	gctccaaactggatcttgcaa	tggtgcatgcgcagttg	tgcggtggatcctg	62 bp	DQ139342.1
Thioredoxin Reductase 3a (TrxR3a)	caggacgcccgcaagtt	tcatcgtctcccagttgtgttt	tgggaggtgcctgagg	67 bp	HF969246.1

Appendix A2 - Candidate Gene List for Chapter 3

Gene	Forward Primer	Reverse Primer	Probe	Amplicon Length	Accession Number
Endogenous Control Genes					
Acidic Ribosomal Phosphoprotein (ARP)	aggaccacgtggaagtccaa	cgccgacaatgaaacatttg	tatgaaaatcatccaattgc	108 bp	AY685220.1
Elongation Factor-1a (EF-1α)	aataccctcctcttggtcgtttc	cttgtcgacggccttgatg	tgcgtgacatgaggc	80 bp	AF498320.1
Growth Genes					
C-FOS	ccgcaacaggaggaaggaa	ctccagctcgtcggtttca	acgagctggaggacg	58 bp	AB11053.1
Early Growth Response Protein 1 (EGR-1)	cgaacatctgaatggagatacattacc	caggctccagggtgaacct	ctatcggctgtgacaagt	128 bp	NM_001141824.1
Growth Hormone Receptor (GH-R)	ccccactaaagagtcccgatt	ctaaacccaaggcagcaaaga	ccagttactgtcctgctt	62 bp	NM_001124731.1
Insulin-like Growth Factor 1 (IGF-1)	atttcagtaaaccaacgggctatg	cgtccacaataccacggttatg	ccagttcacgacggtc	66 bp	U14536.1
Insulin-like Growth Factor Binding Protein (IGFBP2b)	caactgtcccgaggaacctaag	ctccagctcctgtgcacaag	cccagcagcccatga	64 bp	HM358881.1
Myostatin-1A (MYO1a)	gggaaatgatctggccgtta	tctgaaatcgtcacctccatga	agaaggactgcaaccc	75 bp	EU009952.1
Tumor Suppressor p53 (p53)	cagtccagcacagccaagtc	cgccaactggcagaacaact	acttgcacatactcgc	72bp	AF071574.1
Metabolic Genes					
Carnitine Palmytol Transferase 1 (CPT1)	gaagggcctgatcaaaaagtgt	tccccttgtccctgaagtga	cttcatccagatcgc	86 bp	AJ620357.1
Cathepsin L (CTSL)	cgagactggctttgtggacat	gccacagccttcatcatagca	ccagtggcaaggag	60 bp	AF358668.1
Cytochrome p450 Family 1A (CYP1a)	tcttccttcctgccgttcac	gaagtagccattgagggatgtgt	ccacactgcacgatc	66 bp	M21310.1
Cytochrome Oxidase 1 (COI)	ggcagcaggcattactatgttactc	gcctgccgggtcaaaga	cggaccgaaatcta	67 bp	KP720599.1
Fatty Acid Synthase (FAS)	ccaggtctgtacggtcttcca	cgaaccggctgatgtcctt	agaggaacggcaagct	58 bp	XM_014179800.1
Elongation of Very Long Chain Fatty Acids Protein 7 (ELOVL7)	ttatgccatgcgtatgacagttagt	cggaggcgaaaaccaaaat	tgctttctcactgggtgtg	69 bp	XM_024418089.1
Insulin Induced Gene 1 (INSIG1)	ctgctgttgttggcctgcta	ttgtgtggctctccgagatg	acccctgcatcgacag	58 bp	XM_024393097.1
Immune Genes					
Calmodulin (CAL)	cagacagcgaggaggagatca	taaccgttcccatccttgtca	agaagcgttccgtgtct	61 bp	BT074280.1
Chemokine 1 (CK-1)	tcctggctgctctgttctctct	acagcagtccgctgattgtg	ctcatcatcaccctcatt	68 bp	AF093810.1
Complementary Component 3 (C3)	attggcctgtccaaaacaca	agcttcagatcaaggaagaagttc	tggaatctgtgtgtctgaacccc	85 bp	AF271080.1
Complementary Component 5 (C5)	cgattactccggccttcaag	gctgcgagcatggaagtagat	caacatgtcagcattc	64 bp	AF349001.1
Natural Killer Enhancing Factor (NKEF)	tgaggtcattggtgcctctgt	gaggtgtgttggtccaagca	attcccacttctgccatc	92 bp	AF250193.1
Tumor Necrosis Factor-α (TNF-α)	cccaccatacattgaagcagatt	ggattgtattcaccctctaaatgga	ccggcaatgcaaaa	70 bp	DQ778945.1
Stress Genes					
Glucocorticoid Receptor 2 (GR-2)	agcaccgtgccaaaagatg	gccttccccaactccttga	ctcatcaaacactgcctg	83 bp	AY495372.1
Heat Shock Protein 70 (HSP70)	tcaacgatcaggtcgtgcaa	cgtcgctgaccaccttgaa	ccgacatgaagcactg	60 bp	U35064.1

Heat Shock Protein 90a (HSP90a)	agatcttccttagggagctcatctc	tgtcaagctctcgtatctgatcttg	aactcttcagatgctttgg	71 bp	U89945.1
Metallothionein A (metA)	gctccaaactggatcttgcaa	tggtgcatgcgcagttg	tgcggtggatcctg	62 bp	DQ139342.1
Glutathione Peroxidase 1 (GPX1)	acccagatgaacgagctccat	ggcacccccagaatcactag	cggtacgccgacaag	62 bp	NM_001124525.1
Thioredoxin Reductase 3a (TrxR3a)	caggacgcccgcaagtt	tcatcgtctcccagttgtgttt	tgggaggtgcctgagg	67 bp	HF969246.1

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