Effects of multigenerational exposure and phenotypic variation on a freshwater fish species exposed to elevated carbon dioxide (CO_2)

By

JENNA LAUREL FLEET

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Department of Biology
Master of Bioscience, Technology, and Public Policy
The University of Winnipeg
Winnipeg, Manitoba, Canada

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DEDICATION

I dedicate my thesis to Zoey. Zoey taught me to live my life with authenticity, to love unconditionally, and the importance of living life fully, through the demonstration of her living her life this way each day.

Zoey, you're with me always.

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ABSTRACT

The amount of dissolved carbon dioxide (CO_2) and the acidity of aquatic ecosystems is increasing as atmospheric CO_2 concentrations increase due to human activities. Changes in pH and dissolved CO_2 can have considerable aversive effects on fish physiology and behaviour, which can result in negative effects on fish populations. Multigenerational studies have found that the conditions experienced by parents can have significant effects on the performance of their offspring and understanding these effects can help to predict how fish populations will cope in future conditions. Additionally, repeatable behavioural phenotypes are good predictors of trends in behaviour, can be useful predictors of other physiological and life history traits, and can be subject to selection pressures. Unfortunately, the effects of elevated CO_2 on freshwater fishes over multiple generations, and the effects of behavioural phenotypes, are poorly understood.

In my thesis, freshwater Japanese Medaka (*Oryzias latipes*) were used to investigate the influence of phenotypic variation and differences in time of exposure (generational) on biological responses to elevated CO_2. Lab-reared medaka were divided into 'responsive' and 'non-responsive' groups based on behavioural differences from the population mean during acute exposure to high CO_2 in a common shuttling and novel tank behavioural assay. Responsive and non-responsive fish in parental generation (P) were subdivided and exposed to either control (~480 ppm) or high CO_2 (~1250 ppm) conditions over a 6-week period. Following this time, eggs from this generation were collected and randomly selected into either high or control conditions, where they were hatched and reared until maturation (filial generation one (F_1), 18 weeks). Eggs from F_1 were collected and hatched and reared in the same conditions as their parents until adulthood (filial generation two (F_2), 24 weeks). Body condition (size, weight and length), behaviour (total distance moved, time spent in the outer zone of the behavioural arena, and swimming direction), reproductive (number of eggs, size of eggs, and survival to hatch)

performance, and the relative abundance of various mRNA transcripts in whole brain tissue of fish was measured across these three generations.

Behavioural phenotypes influenced reproduction for P and F_2 generation fish, and growth for F_1 and F_2 fish; suggesting that intraspecific variation in behavioural phenotypes may influence how medaka respond to elevated CO_2. However, behavioural phenotypes did not have a significant effect on mRNA abundance on genes targeted in my study. Multigenerational exposure to elevated CO_2 were shown to improve the performance of offspring in some measures and resulted in changes of mRNA abundance of several genes. Transgenerational exposure, where a parent or grandparent was exposed to elevated CO_2 but the offspring were not exposed to elevated CO_2, resulted in some deleterious effects suggesting that, generally, exposure to environmental conditions that differ from that of their parents may put fish especially at risk. In my thesis, current CO_2 exposure appeared to be the best predictor of overall condition, where fish exposed to elevated CO_2 were worse off than fish exposed to control CO_2 conditions.

The results of this research contribute to filling a current gap of knowledge in understanding how freshwater fish will respond to future conditions over an ecologically-relevant time scale. Importantly, this information will contribute to generating more informed decisions on freshwater ecosystem management and future research directions. Marine and freshwater environments offer food and water security and are of high importance to the economy and the health of our planet, making my research relevant to our broader society.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
ß-actin	Beta-actin
µatm	microatmospheres
µg	microgram
µl	microlitres
5HT	serotonin
ACTH	adrenocorticotropin
ANOVA	analysis of variance
°C	degrees Celsius
$CaCO_3$	calcium carbonate
cck	cholecystokinin
cDNA	complementary DNA
Cl^-	chloride ions
cm	centimetres
CNS	central nervous system
CO_2	carbon dioxide
CO_3^{-2}	carbonate ions
C_T	threshold quantification cycles
CRH	corticotropin releasing hormone
cyp19b	cytochrome P450 beta
DA	dopamine
DA R	Dopamine receptor
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DO	dissolved oxygen
E_2	17ß-estrogen
F_1	filial generation one
F_2	filial generation two
FSH	follicle stimulating hormone
FSHb	follicle stimulating hormone subunit b
g	grams
GABA	gamma-aminobutyric acid
$GABR_A2$	gamma-aminobutyric acid receptor subunit alpha 2
$GABR_A4$	gamma-aminobutyric acid receptor subunit alpha 4
$GABA_AR$	gamma-aminobutyric acid type A receptors
GAD1	glutamate decarboxylase 1
GAT1	GABA transporter type 1
GH	growth hormone
GlyR	glycine receptors
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptors

GREs	glucocorticoid response elements
GTHa	gonadotropin hormone alpha subunit
H^+	hydrogen ions
$H_2CO_3^-$	carbonic acid
HCO_3^-	bicarbonate ions
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HPI	hypothalamic-pituitary-interrenal
HSP	heat shock proteins
IGF	insulin-like growth factor
Insl5b	Insulin-like peptide factor 5b
IPCC	Intergovernmental Panel on Climate Change
L	litres
LH	luteinizing hormone
LHb	luteinizing hormone subunit b
m	meter
MBD2	methyl CpG binding protein 2
MC2R	melanocortin 2 receptors
mdGnRH	medaka-type gonadotropin-releasing hormone
min	minute
mg	milligrams
ml	millilitre
mm	millimetre
mM	millimolar
MR	mineralcorticotropin hormone receptor
mRNA	messanger ribonucleic acid
Na^+	sodium ions
NE	norepinephrine
NH_4^+	ammonia
NHE-1	sodium-hydrogen antiporter 1
NO_3	nitrates
NO_2	nitrites
NPY	neuropeptide Y
NTC	no-template control
OA	ocean acidification
P	parental generation
PC	principal components
PCA	principal components analysis
pCO_2	partial pressure of CO_2
PCR	polymerase chain reaction
POA	pre-optic area
POMC	proopiomelanocortin
pCO2	partial pressure of CO_2
ppm	parts per million by volume
qRT-PCR	quantitative real-time polymerase chain reaction
RBC	red blood cell
rln3b	relaxin 3b

RNA	ribonucleic acid
RPL-7	ribosomal protein L7
rxfp3-2a	relaxin Family Peptide Receptor 3 2a
S.E.	standard error
shmt	serine hydroxymethyltransferase
T	testosterone
WMO	World Meteoritical Organization

CHAPTER 1

General Background

Chapter 1: General Background

1.1 Climate Change

Where weather refers to atmospheric conditions over a short period, climate refers to the average temperature, humidity, atmospheric pressure, wind, rainfall, and other elemental and meteorological measures globally or regionally over 30 years (WMO, 2021). Climate change, therefore, refers to a significant long-term change in the weather patterns observed globally or regionally. Indicators of changing global climate include changes in global surface temperatures, ocean heat content, ocean acidification, glacier mass balance, polar sea-ice extent, sea levels, and carbon dioxide (CO_2) concentrations (WMO, 2021). These changes are all intimately interconnected to one another, and climate change in one region can significantly impact regions far distances away (Steffen et al., 2015). While there are natural causes that contribute to such changes, presently it is human activities that are the primary driver for the extreme climate changes observed on Earth (IPCC, 2014; WMO, 2021). Some of these human activities include increased greenhouse gas emissions and land-use changes.

Greenhouse gases are gas emissions from fossil fuel combustion, agricultural livestock, and industrial reactions, among others. Greenhouse gases trap heat in the atmosphere by acting like a plastic barrier in a greenhouse; allowing heat in the form of radiation energy from the sun to enter our atmosphere but reducing heat in the form of infrared radiation from escaping the atmosphere. Ultimately, this has led to global warming. Some gases considered to contribute most significantly to the greenhouse gas effect are methane, nitrous oxide, and carbon dioxide (WMO, 2021). Global mean temperatures have increased between 1.15–1.28°C since preindustrial times (WMO, 2021). This change has had drastic impacts on regional and global climate contributing to extreme weather events such as wildfires, droughts, and flooding.

The ocean acts as a heat reservoir estimating to absorb 90% of the earth's heat energy (WMO, 2021). Thus, global warming has had significant impacts on marine ecosystems, including increasing the ocean's heat content, decreasing glacier mass content, increasing sea levels, and increasing the frequency and intensity of marine heatwaves (WMO, 2021). Marine heatwaves are a short span of high aquatic temperatures that can have detrimental consequences for marine organisms. One example of a consequence of marine heat waves is coral bleaching events, which occurs when coral polyps and other organisms under heat stress evict their symbiont dinoflagellates, “zooxanthellae” (Baker et al., 2008). Corals without zooxanthellae often appear bone-white in appearance, and if heat stress conditions do not reduce in a short period, corals can starve and die (Baker et al., 2008). Without corals, the biodiversity and ecosystems they support suffer (Baker et al., 2008).

As global temperatures increase, there have also been significant effects on the cryosphere. For example, in the Arctic, winter sea ice has decreased by ~1.8 m between the years 1978–2008, with the overall volume of ice having declined by ~40% since 1991 (Vaughan et al., 2013). As global temperatures continue to increase, sea ice extent, volume, and thickness will continue to decline, with some predictions of an ice-free summer Arctic by 2040 (Vaughan et al., 2013; Polyak et al., 2010). As more ice melts, there is less white ice to reflect incoming radiation energy, which has resulted in a positive feedback cycle where more radiation is absorbed thereby increasing how quickly the Earth is warming. Reduced sea ice will cause changes in ocean circulation, climate, the frequency and severity of storms, and has the potential to alter many ecosystems and ocean productivity (Vaughan et al., 2013). Additionally, melted sea ice and glaciers are thought to be one of the major contributing factors to sea level rise and decreased salinity in some areas (WMO, 2021). The ocean's currents, temperatures, vertical cycling,

salinity, and other processes all play a crucial role in controlling climate extremes (Rahmstrof, 2002).

In addition to fossil fuel burning, land-use changes are another significant factor contributing to climate change (WMO, 2021; Paul and Rashid, 2017). Land-use change is the human transformation of a natural landscape, most often done for economic activities (Paul and Rashid, 2017). Examples of land-use change include the conversion of natural land into agricultural, urbanized, or industrial landscapes (Paul and Rashid, 2017). Land-use changes are associated with deforestation and soil degradation, and can have significant effects on ecosystems by eliminating total areas of an ecosystem and putting many others at risk (Paul and Rashid, 2017). Both climate change and land-use change have had massive impacts on biodiversity and therefore ecosystem resiliency (Rockström et al., 2009). For example, in the USA, states with high proportions of agricultural land use and destruction of open land were correlated with higher honeybee declines because of the increased difficulty for honeybees to access nutrients (Naug, 2009). Honeybees are essential for the pollination of many important plant species including those of agriculture, and their decline is a concern (Hung et al., 2018). A second example is in tropical ecosystems, where mangroves are being cleared for anthropogenic infrastructure which has left land in these areas more vulnerable to storms and has also degraded nearby reef ecosystems since mangroves serve as an important resource for neighboring reef fish reproduction and health (Mumby et al., 2004).

In summary, human activities such as burning fossil fuels and land-use changes are causing changes in the global climate. These changes increase the risk and frequency of extreme weather events such as floods, droughts, heatwaves, extreme cold, and storms. Effects of climate change are interconnected, and effects seen in regional areas on Earth can have ripple effects that cause changes in ecosystems large distances away (Steffen et al., 2015). Oceans are extremely

important for hydrologic cycling including precipitation and freshwater availability (Benedict et al., 2017). Climate changes severely impact biodiversity and ecosystems and have consequences on human health, human mobility and displacement, and water and food security (Nogués-Bravo et al., 2018; WMO, 2021).

1.2 Carbon Dioxide

Carbon is an elemental building block that exists in various forms and is cycled between the atmosphere, land, water, and living organisms (e.g., plants and animals) in a planetary 'carbon cycle'. In the atmosphere, carbon is found primarily in the form of carbon dioxide gas (CO_2). By analyzing the composition of gas trapped within ice core samples, among other sources of data, we can infer that during the past 800,000 years before the mid-1800s, atmospheric CO_2 concentrations have stayed within relatively consistent 100,000-year oscillation cycles ranging between 180 and 280 parts per million by volume (ppm) (IPCC, 2014). However, from the mid-1800s to 2007, levels of CO_2 have increased by an amount of change normally seen over 100,000 years.

The mid-1800s marks the peak of the industrial revolution, a period in human history characterized by massive increases in fossil fuel consumption. Accordingly, the primary cause of this rise of CO_2 is human usage of fossil fuels, cementing, and flaring. Secondary causes for atmospheric CO_2 are forestry and land-use changes (IPCC, 2014). This is because as plants and forests are destroyed to make products and space for humans, there is increasingly less CO_2 removed from the air by photosynthesis and the carbon cycle is disrupted (Falkowski et al., 2000). The average present-day annual concentration of CO_2 is 410.5 ppm, a concentration over 130 ppm higher than the highest concentration estimate from the past 800,000 years (WMO,

2021; McNeil and Matsumoto, 2019; IPCC, 2014). If human related CO_2 emissions continue "as is" without significant intervention and effort toward emission reduction, it is predicted that the average CO_2 concentration could reach 900 ppm or higher by the end of the century (Riahi et al., 2011; McNeil and Matsumoto, 2019). Aquatic ecosystems may be especially vulnerable to these changes as some water bodies have been known to have higher levels of dissolved CO_2 in response to high atmospheric CO_2 (e.g., Weiss et al., 2018). This is because CO_2 can dissolve in aquatic ecosystems and react with water molecules. This reaction will be explained in further detail in the following section.

1.3 CO_2 and Marine Ecosystems

The ocean is estimated to absorb between approximately 23–33% of global CO_2 emissions (WMO, 2021; Gattuso, 2015). This is because CO_2 is soluble in water and reacts with water molecules to form carbonic acid ($H_2CO_3^-$), a highly unstable state which immediately dissociates into hydrogen ions (H^+) and bicarbonate ions (HCO_3^-). Bicarbonate can then further dissociate into carbonate ions (CO_3^{-2}) and hydrogen ions ($2H^+$) ($CO_2+H_2O \rightleftharpoons H_2CO_3^- \rightleftharpoons HCO_3^-+ H^+ \rightleftharpoons CO_3^{2-} + 2H^+$; Knoche, 1980). The reaction of CO_2 dissolving into water ultimately results in a decrease of pH in the ocean, a phenomenon known as ocean acidification (OA) (Doney et al., 2009).

In response to elevated concentrations of atmospheric CO_2, ecosystems are not all affected the same. The degree to which an aquatic ecosystem can absorb atmospheric CO_2 and its sensitivity to changes in partial pressure of CO_2 (pCO_2), is variable depending on the water's temperature, salinity, dissolved inorganic carbon (sum of dissolved CO_2, carbonate ion [CO_3^{-2}], and bicarbonate ion [HCO_3^-]), and alkalinity (buffering capacity) (McNeil and Matsumoto,

2019). Some areas of the ocean that are expected to be most at risk to early exposure of hypercapnia conditions, due to their composition of these factors, include the eastern Pacific, Southern, and North Pacific oceans, in areas that tend to fall within major fishery zones that contribute largely to the 80 million tonnes of commercial fish caught and consumed for animal protein each year (McNeil and Sasse, 2016). Human exploitation is highest in ecosystems which have large biodiversity, and accordingly highly biodiverse ecosystems and their biodiversity are being disproportionately negatively affected (McIntyre et al., 2016).

Carbon dioxide and pH have major roles in many physiological processes and intracellular functions in organisms, and typically these concentrations are highly regulated in a small range for healthy organismal functioning (Gattuso, 2015). Thus, changes in these concentrations and gradients can have significant effects on organisms. Some examples include reduced calcification, growth, impaired development, and impaired reproduction (Doney et al., 2009). These changes, in part, are thought to be due to differential allocation of energy resources to acclimatize and maintain allostasis, the adaptive management of body systems in response to a stressor (Doney et al., 2009). How CO_2 specifically alters organisms, mainly fish, will be explored in Chapter 2.

1.4 Fresh Water and CO_2

Freshwater ecosystems can have especially large variability in water parameters due to strong local influences such as regional weather and substrate, runoff, and local pollution (Clair and Ehrman, 1996). Studies that address OA tend to consider ranges of CO_2 between 1000-2000 μatm, which is a normal prediction for near-future (year 2100) marine CO_2 concentration projections (Brauner et al., 2019). However, freshwater ecosystems have a large range of normal

CO_2 concentrations. For example, current temperate lakes and streams can have CO_2 concentrations as high as 4000 μatm (Brauner et al., 2019) and the current average pCO_2 concentration of freshwater lakes globally is approximately 1000 ppm and can range between 3.1-fold below to 16-fold above atmospheric pCO_2 (Cole et al., 2007; Hasler et al., 2016). Future estimates as high as 11,000 ppm by 2100 have been predicted for some freshwater ecosystems, a concentration approximately thirty-seven times higher than normal in some areas (Weiss et al., 2018). Overall, the degree of CO_2 concentration change that each freshwater ecosystem will encounter due to atmospheric CO_2 concentration elevation in the future is poorly understood (Hasler et al*.,* 2016; Clair and Ehrman, 1996). Freshwater ecosystems have been known to experience strong acidification due to acid rain and acid mine drainage, and weaker acidification resulting from elevated CO_2 is what is most expected from increases in atmospheric CO_2 (Hasler et al., 2017). Elevated atmospheric CO_2 is only one stressor of a complex interconnected suite of human caused stressors that freshwater ecosystems face (Woodward et al., 2010). Compared to marine ecosystems, freshwater ecosystems are more isolated and fragmented and thus are highly vulnerable to these stressors (Woodward et al., 2010).

Freshwater ecosystems support a disproportionally large amount of earth's biodiversity for the percentage of earth these ecosystems cover. Some studies suggest that freshwater ecosystems contain as much as one third of all vertebrate species and 6% of all biodiversity, despite only covering ~0.8% of the earth's surface (Dudgeon et al., 2006). Globally these ecosystems are experiencing major reductions in biodiversity and population sizes, with major threats including over-exploitation, water pollution, habitat degradation, flow modification, and species invasion (Dudgeon et al., 2006; Reid et al., 2019). Despite these present threats and that freshwater ecosystems are particularly vulnerable to climate change, currently, relatively little is known about how these ecosystems will respond to near-future CO_2 conditions particularly across

transgenerational timescales (Hasler et al., 2016; Brauner et al., 2019; George et al., 2019). Aquatic ecosystems offer vast biodiversity, food, and water security, and are vital components of climate and the health of our ecosystems (Dudgeon et al., 2006). Together, diversity in freshwater system responses to elevated CO_2 and high risk of human disturbances and environmental degradation (McIntyre et al., 2016) are reasons why predicting the effects of climate change and elevated CO_2 on freshwater systems is especially difficult. As some fresh-water ecosystems have already reached predicted end-of-century CO_2 concentrations, there is a sense of urgency to understand the implications of elevated CO_2 on freshwater organisms (Weiss et al., 2018).

Relatively little is known about the effects of elevated CO_2 on freshwater taxa and the majority of literature that exists best covers effects on freshwater phytoplankton and cyanobacteria (Hasler et al., 2016). Algae and phytoplankton communities are important indicators of environmental change and can influence the balance of basic nutrient availability in freshwater (Carbon, Nitrogen, and Phosphorus) and thus have influences on ecosystem and community dynamics (Hasler et al., 2016). Under elevated CO_2 conditions, several studies have observed that elevated CO_2 increases primary production (i.e., photosynthesis) (Jasson et al., 2012; Schippers et al., 2004); however, the consequence of this change is variable (Hasler et al., 2016). Little research has observed effects on freshwater macroinvertebrates and vertebrates, and research that exists finds CO_2 to affect distribution, growth, behaviour, reproduction, and survival of individuals (O'Brien and Blinn, 1999; Ou et al., 2015; Hasler et al., 2017). Communities and populations can also be affected by such changes, especially when considering differential effects in which some organisms are better adapted to changes in elevated CO_2 (Hasler et al., 2017). Because over evolutionary timescales many freshwater organisms have been exposed to extreme daily and seasonal changes in elevated CO_2 and other environmental conditions, it is possible that freshwater organisms generally have a better capacity to acclimatize to such changes relative to

marine organisms (Hasler et al., 2016). My thesis will explore how a freshwater fish responds to elevated CO_2 (1000–2000 μatm), with particular attention towards within and across generation variation.

CHAPTER 2

Introduction, Research Gaps, Objectives, and Hypotheses

Chapter 2: Introduction, Research Gaps, Objectives, and Hypotheses

2.1 Elevated Carbon Dioxide, Fish Physiology, and Stress

Freshwater fishes are known to be sensitive to elevated CO_2 and studies have found dose dependent effects on reproduction, physiology, and behaviour (Hasler et al., 2016; Ou et al., 2016). For example, Davidson (1933) observed over 5,000 pink salmon, bullhead catfish and trout suffocate and die over 30 mins because of a brief period of elevated CO_2 (5.6 pH). Another study found avoidance behaviour to elevated CO_2 (Kates et al. 2012) even after individuals were acclimated to elevated CO_2 conditions (Dennis et al., 2016). Gills have a large surface area, small diffusion distance, and counter-current blood flow, which intimately links them with changes in their environment (Willmer et al*.,* 2005). As aquatic pCO_2 increases, the ability of CO_2 to diffuse from the fish's gills into the surrounding environment is decreased due to the reduced concentration gradient that normally drives CO_2 from high to low concentrations (Fick's Law). Resultingly, pCO_2 will increase in a fish's body as it increases in the environment, which can be detrimental to a fish.

Changes in CO_2 and hydrogen ions (H^+) are directly linked to changes in pH (Henderson-Hasselback equation: $pH = pK' + \log([HCO_3^-]/\alpha CO_2 \bullet pCO_2)$). pH is a logarithmic measure of the concentration of H^+ ions. The higher the H^+ ions, the higher the acidity, and the lower the pH value ($pH = -\log [H^+]$). As such, as extracellular pCO_2 and H^+ concentrations increase, pH is decreased (acidified). Changes in pH can have significant effects on protein folding and normal cellular functions, and thus maintaining stable H^+ and pH levels is essential for life (Tresguerres et al., 2019). For example, a fish's primary oxygen carrying pigment is hemoglobin. As pH decreases (acidifies) or pCO_2 increases, hemoglobin's oxygen binding affinity is reduced (Bohr effect). Under normal conditions, this is adaptive as it aids in the unloading of oxygen in tissues,

especially during high metabolic activity (Willmer et al., 2005). However, under elevated environmental pCO_2 conditions this means that fish have a reduced ability to bind oxygen at their gills and will begin experiencing hypoxia (inadequate oxygen supply) (Willmer et al., 2005). It is therefore extremely important for fish to maintain a stable pH.

To maintain a pH supportive of their biological functions, the primary way fish compensate for systemic acidosis is to increase extracellular bicarbonate (HCO_3^-) concentrations and reduce H^+ ion concentration (Heuer and Grossell, 2014). This is accomplished both by the pK of this buffer system which under physiological pH favors HCO_3^- production when CO_2 is added to a body system, and by ionocyte cells, which are gill cells specialized for ion transportation. Ionocytes excrete H^+ in exchange for sodium ions (Na^+), and chloride ions (Cl^-) in exchange for bicarbonate (HCO_3^-) (Tseng et al. 2013). Additionally, absorption of HCO_3^- may be increased in the intestine and kidneys (Brauner et al., 2019). The higher the CO_2 concentration a fish experiences, the more HCO_3^- is required to buffer the CO_2 and maintain a stable pH (Brauner et al., 2019). Fish have an apparent physiological threshold for the maximum HCO_3^- they can achieve, which is determined by the point at which it is not possible to reduce extracellular anions (i.e., Cl^-) any further (Brauner et al., 2019). Under OA CO_2 concentrations (1000-2000 μatm), it is expected that the concentration of HCO_3^- required to buffer this acidity is within physiological limits for most fish (Brauner et al., 2019). However, the degree to which an increase in atmospheric CO_2 impacts an aquatic ecosystem is heavily influenced by other factors of that ecosystem including temperature, salinity, dissolved inorganic carbon and alkalinity (Section 1.3). Additionally, fish can have long term adaption and acclimation strategies that also influence how a species or an individual will respond to changing CO_2 conditions. Some of these strategies are: the increased production of carbonic anhydrase at the gills (an enzyme that catalyzes the conversion of CO_2 to HCO_3^- or the reverse), decreased concentration of organic phosphate

concentrations in red blood cells (improves H^+ buffering by reducing oxygen-hemoglobin binding affinity) and increasing red blood cell count (Power, 1980). Some acclimatization strategies may also be heritable through transgenerational plasticity, making some individuals respond differentially to a stressor based on multigenerational exposure (Munday et al., 2014). Therefore fish can have different sensitivities to elevated CO_2, in which some species are found to be highly affected at OA CO_2 concentrations, while others have no apparent response (Heuer and Grosell, 2014). Because fish and aquatic ecosystems can have significant difference in response to increases of CO_2 and have large variability in their "normal" CO_2 exposure conditions, for the purpose of this introduction, "elevated CO_2" refers generally to CO_2 concentrations that are higher than the normal CO_2 concentrations experienced by the organism of a study. For most prior studies, elevated CO_2 conditions are above 2000 ppm and there is generally less research on OA concentrations of CO_2 (~1000-2000ppm).

Elevated CO_2 is considered a stressor for fish (Bernier and Randall, 1998; Tucker et al., 2019). Studies have found high elevated CO_2 exposure (~48,000 ppm) causes elevated circulating cortisol, an indicator of stress (Bernier and Randall, 1998). Through multiple stressor effects, CO_2 exposure can also cause exasperated negative effects when a fish is exposed to other stressors such as elevated temperatures (Bernier and Randall, 1998). Stress is defined as the physiological response to aversive or stressful stimuli. The physiological responses to stress have been categorized into three phases (Barton, 2002; Norris and Carr, 2013). The first phase includes the release of stress hormones: corticosteroids and catecholamines. The second phase of physiological response is responses that occur because of elevated corticosteroids and catecholamines. Some examples of secondary responses are metabolic changes such as an increase of blood glucose and lactate, heat shock or stress proteins (HSPs), changes in osmoregulatory ion concentration (chloride, sodium, water balance), hematological factors, and

immune function (e.g., antibody or lysozyme production) (Barton, 2002). Finally, tertiary responses refer to whole-organism effects of stress such as changes to growth, condition, resistance to disease, behaviour, and reproduction.

Elaborating on the primary stress response phase: when a fish is exposed to a stressor catecholamines are released rapidly, followed by a slightly delayed response from the hypothalamic—pituitary—interrenal (HPI) axis that stimulates the release of corticosteroids (Barton, 2002). Specifically, sympathetic innervations of the central nervous system (CNS) stimulate chromaffin tissue (adrenal medulla homologue) that is located mainly in the anterior region of the kidneys, which induces the release of catecholamines (Barton, 2002; Norris and Carr, 2013). The primary catecholamines released during stress in teleost fishes are epinephrine and norepinephrine. In the body, epinephrine causes immediate "fight-flight" responses. For example, epinephrine regulates cardiovascular and respiratory functions to maintain adequate oxygen supply to the body during stress (Reid et al., 1998). Catecholamines also drive the breakdown of energy stores (glycogen) into a form of energy that cells can more readily use (glucose), thereby allowing the body to meet increased energy demands that occur during a stressor (Reid et al., 1998).

The HPI axis is activated when stress simulates hypothalamic neurons in the preoptic area of the brain to release corticotropin-releasing hormone (CRH). CRH in turn stimulates corticotropic cells in the anterior pituitary to secrete adrenocorticotropin (ACTH) which is cleaved from proopiomelanocortin (POMC) protein (Alsop and Vijayan, 2009). ACTH circulates in the blood and acts on interrenal cells (adrenal cortex homologue) *via* melanocortin 2 receptors (MC2R) to stimulate the production and release of corticosteroids into circulation (Alsop and Vijayan, 2009). Cortisol, a corticosteroid, can transverse cell membranes and bind to intracellular glucocorticoid receptors (GR) in cells throughout the body. GRs are nuclear hormone receptors

that regulate gene expression by binding to intracellular glucocorticoid response elements (GREs) that are transcriptional regulator sites in genes relevant to glucose metabolism, ion regulation, immune function, and behaviour (Alsop and Vijayan, 2009). Mineralcorticoid receptor (MR) in teleost fishes have also been implemented as contributing to the stress axis (Faught and Vijayan, 2018). In zebrafish (*Danio rerio*) MR were involved in stress-related behaviours and stress regulation (Faught and Vijayan, 2018).

The acute stress response is adaptive and highly conserved across species (Barton, 2002). Stress allows an animal to maintain allostasis, which is the process where the appropriate level of adrenal responses (i.e., homeostasis) is determined given a specific time or situation to maintain adaptable body regulation (Romero et al, 2009). This accounts for the normal daily and seasonal fluctuations seen in circulating cortisol and blood glucose (Tucker et al., 2019). Additionally, the magnitude of a stress response is variable and dependent on the type of stress, the magnitude of stressor, how long a stressor is present for, and what coping strategies are available to the organism (Barton, 2002; Tucker et al., 2019). While acute stress is adaptive, chronic and prolonged stress conditions are thought to be detrimental, particularly when considering tertiary stress responses.

When stress is chronic, the negative feedback mechanisms that typically inhibit a stress response after the stressor has passed become less effective, and glucocorticoid receptor resistance can occur (Mariotti, 2015). This can lead to a maintenance of a chronic elevated circulating cortisol. Chronically elevated stress hormones can have a number of negative of effects on the brain and body. Some of the effects include dendritic atrophy and reduced spine density in the prefrontal cortex and limbic system, reduced reproductive success, reduced growth, reduced immune responses, reduced cognition and memory, and elevated inflammation, to name a few (Yaribeygi et al., 2017; Mariotti, 2015). Because cortisol acts through changes in gene

expression, these effects can have cascading effects on future generations (Nestler, 2016). For example, several studies on mice have found that early life stressors can impact many future generations and the expression of genes in their HPA axis, even when the organism does not encounter the stressor itself (Nestler, 2016). These transgenerational effects are mediated by epigenetic means.

In response to elevated CO_2 exposure, fish have been observed to increase hyperventilation, have a reduced blood oxygen concentration, and have acid-base physiological disturbances (Bernier and Randall, 1998). Additionally, when used for euthanasia and anesthetic practices, elevated CO_2 exposure has been observed to cause prolonged struggling and stress in fishes and has a higher non-recovery rate relative to other anesthetic methods (Gräns et al., 2015). Generally, research observing effects of elevated CO_2 on stress measures have found that fish exposed to acute elevated CO_2 have significant elevated circulating cortisol concentration, while chronic CO_2 exposure responses tend to be more varied based on species, life stage of exposure, and concentration of CO_2 exposure (Tucker et al., 2019; Petochi et al., 2011). For example, in one study, cortisol significantly differed in high CO_2 groups (18,000–50,000 ppm) versus control CO_2 fish during acute exposures but was not significantly different over a chronic 45-day period in European sea bass (*Dicentrachus labrax*) despite hypoxemia and nephrocalcinosis present in the fish exposed to chronic elevated CO_2 conditions (Petochi et al., 2011). Overall, elevated CO_2 conditions can cause significant activation of the HPI axis in fish, and acclimation strategies, which allow a fish to adjust to changes in the environment, appear to be more successful for some fishes over others, varying on species, time of exposure, length of exposure, concentration, among others (Bernier and Randall, 1998; Tucker et al., 2019; Petochi et al., 2011).

2.2 Carbon Dioxide effects on Fish Growth, Feeding, and Condition

Environmental factors that have been found to significantly affect growth include temperature, dissolved oxygen, salinity, day length, and CO_2 (Moyle and Cech, 2004). In response to elevated CO_2, uncompensated acidosis in the body is thought to lead to metabolic depression, which can result in reduced size and growth rate (Tseng et al., 2013). However, research examining effects of elevated CO_2 on fish growth, mortality and development is highly variable amongst life states, species, length, and the concentration of CO_2, among others. Where growth of some fish species are significantly affected by elevated CO_2 conditions, other fishes are highly resistant to growth effects even in extremely elevated CO_2 conditions (50,000 μatm; Heuer and Grosell, 2014). Likely some variability is due to nutrient availability to compensate for effects and energy constraints (Heuer and Grosell, 2014). Larvae are thought to be most susceptible to metabolic depression, while adults have a better ability to acclimatize to the elevated CO_2 conditions (Tseng et al., 2013). In a study on transgenerational effects of elevated CO_2, parental CO_2 exposure reduced or reversed deleterious effects on length and weight which was observed in offspring from control parents that were raised in OA CO_2 conditions (1,000 μatm) (Miller et al., 2012; Heuer and Grosell, 2014). Developmental delays have also been observed in some species including Japanese ricefish (*Oryzias latipes*) at 1,200 μatm, although this delay occurred only during development and was not observed to impact final length measures (Heuer and Grosell, 2014; Tseng et al., 2013). In their study, metabolic compensation was found to have occurred, as measured by an upregulation of genes associated with the glycolysis pathway, Krebs cycle, and electron transport chain in fishes exposed to elevated CO_2 (Tseng et al., 2013). Several studies have found that experimental condition impacts on metabolism can be buffered by a fish's ability to compensate its metabolic load increase by increasing their food intake (Miller et al., 2012). Additionally, when a stressor occurs on a

chronic timeline, anatomical and physiological changes can occur to maximize nutrient extraction, including shortened evacuation time. (Moyle and Cech, 2004).

Nutrient uptake and growth are affected by a wide variety of environmental, nutritional, central and peripheral factors (Canosa et al., 2007; Rønnestad et al., 2017). In the pituitary of the brain, nutrient uptake and growth are influenced by a variety of hormones including growth hormone and steroid hormones (eg. sex hormones, stress hormones) (Moyle and Cech, 2004). For example, norepinephrine (NE), serotonin (5HT), glutamate, and GABA (gamma-aminobutyric acid) all inhibit secretion of growth hormone (GH) in teleost (Canosa et al., 2007) while dopamine (DA) and GnRH are thought to stimulate GH secretion (Peng and Peter, 1997). Growth hormone (GH) and insulin like growth factor (IGF) are two of the major hormones directly responsible for growth and development in fishes (Wilkinson et al., 2006). Typically, an increased circulation of these hormones positively correlates with an increase of body size and growth (Wilkinson et al., 2006). GH and IGF have also been found to play a role in acid base regulation, lipid metabolism, behaviour, reproduction, and immune function (Wilkinson et al., 2006; Reinecke et al., 2005; Canosa et al., 2007).

Growth is also related to feeding, since a large amount of energy is required to facilitate growth (Peng and Peter, 1997). Food energy ingested can be used for metabolism (i.e., digestion, body maintenance, movement), growth, or excreted (feces, ammonia) (Moyle and Cech, 2004). Feeding behaviours in fishes is regulated by a variety of central or peripheral factors that can have appetite-stimulating (orexigenic) or appetite-inhibiting (anorexigenic) effects (Volkoff et al., 2009). Neuropeptide Y (NPY) is an example of a CNS appetite-simulating peptide and has been found to have a dose dependent increase on food intake and weight gain in several species of both freshwater and saltwater fish (Volkoff et al., 2009; Narnaware et al., 2000; Rønnestad et al., 2017). Neuropeptide Y is also linked to simulating release of GH, thereby promoting growth

(Peng and Peter, 1997; Narnaware et al., 2000). Cholecystokinin (CCK), is an appetite-inhibiting peptide that has also been found in the brain and is partially responsible for simulation of fat and protein digestion in fish (Peng and Peter, 1997; Hoskins and Volkoff, 2012). Other factors included in feeding behaviours include relaxin-like peptides. Relaxin-like peptides are a family of peptides that are implicated in reproduction, neuroendocrine regulation and growth (Kusakabe et al., 2014; Good et al., 2012). Relaxin 3b (rln3b) is one member of this family which has also been implicated in osmoregulatory actions including vasopressin release, the stress response, and has been found in the brain of teleost fishes (Good et al., 2013; Kusakabe et al., 2014). Insulin-like peptide 5b (insl5b) is another example of a member of this family and is implicated in glucose metabolism (Kusakabe et al., 2014).

In response to elevated CO_2, fishes of all life stages, especially those in development, have been found to have a reduced survival, growth rate, and overall body condition (Baumann et al., 2012; Alves et al., 2020; Miller et al., 2012; Jutfelt et al., 2013). Although the exact mechanism for why growth and survival are affected by elevated CO_2 is currently not known, an increased energy expenditure required to acclimatize to elevated CO_2 conditions is one hypothesis for why such changes in growth and condition have been observed (Baumann et al., 2012; Alves et al., 2020; Sundin et al., 2019). Growth, as measured by changes in length and or weight, is an indicator of individual and population health and are important measures to study (Moyle and Cech, 2004).

2.3 Carbon Dioxide effects on Fish Behaviour

Rising CO_2 and its effects on fish behaviour is a current topic of study due to climate change (Clements and Hunt, 2015). Fish behavioural responses to elevated CO_2 are variable and include changes in sensory systems (olfactory, auditory, vision), behavioural lateralization,

learning, and activity. For example, some studies have observed impaired anti-predator response learning (Chivers et al., 2014), reduced retinal reaction speed to visual stimuli (Chung et al., 2014), and reduced response to alarm cues (Tix et al., 2017). In one study, two-spotted goby larvae (*Gobiusculus favescens*) demonstrated a stronger phototactic response when brooded in elevated CO_2 (1400 μatm) relative to those brood in control CO_2 (Forsgren et al., 2013). A proposed physiological mechanism for behavioural changes observed in fish, and a topic of significant debate, is the reversal of ionic influx through the gamma-aminobutyric acid type A receptors, one of the major inhibitory neurotransmitter receptors in the brain ($GABA_AR$) (Nilsson et al., 2012; Hamilton et al., 2014; Lai et al., 2015).

GABA receptors are a ligand-gated/ionotropic receptor which consists of four hydrophobic transmembrane domains (TM1-4) which make up a pentameric (5 subunit ring) structure forming the channel pore with a large extracellular N-terminus for binding GABA (Chuang and Reddy, 2018). GABA receptors are primarily permeable to chloride ions (Cl^-) and to bicarbonate ions (HCO_3^-) to a lesser extent. Under normal conditions, GABA receptors are inhibitory and the Cl^- concentration is higher outside of nerve cells. When a GABA receptor channel is opened, there is an inward flow of Cl^- into the nerve cell, hyperpolarizing it (inhibitory effect). As previously described, one pH buffering strategy under hypercapnic conditions is to increase HCO_3^- concentrations by upregulating ion transporters that exchange HCO_3^- for Cl^- (section 2.1). This can lead to intracellular Cl^- being of lower concentration outside of the cell in comparison to the inside of the cell which results in an outflow, or polarizing, effect on the cell when GABA receptor channels are open. Studies have measured extracellular and intracellular ion concentration changes in blood in response to elevated CO_2 conditions (1,900 μatm) are consistent with this hypothesis (Heuer et al., 2016; Regan et al., 2016). Nilsson et al. (2012) and others have further tested the reversal of $GABA_AR$ from inhibitory to excitatory potential in the

brain by treating fish with gabazine (antagonist) after they were exposed to elevated CO_2 and found a reversal of elevated CO_2-related behavioural impairments after gabazine treatment. Notably, other studies found that high CO_2-mediated behavioural impairments were not reversed after exposure to gabazine suggesting multiple mechanisms for these behavioural changes (Hamilton et al., 2014), possibly related to glycine receptors (GlyR) (Squire et al. 2008, Tresguerres and Hamilton, 2017).

$GABA_AR$ and GlyRs are closely related as both are major inhibitory receptors in vertebrate central nervous systems, both have similar structures, and both are primarily permeable to Cl^- and to a lesser extent HCO_3^- (Squire et al. 2008, Tresguerres and Hamilton, 2017). Thus, it is possible that like $GABA_AR$, normally inhibitory GlyR also becomes excitatory under high CO_2 conditions. This may explain a mechanism of effect for some behavioural effects mediated by high environmental CO_2, especially in studies in which gabazine did not completely reverse behavioural impairments (Tresguerres and Hamilton, 2017; Heuer et al., 2019). Glycine has been found to contribute to the control of motor and reflex synchronization and generation, and processing of sensory stimuli, further supporting the hypothesis for mode of behavioural change (Low et al., 2018). Thus, measuring both $GABA_AR$ and GlyR relevant genes in the brain is important in understanding how inhibitory receptors in the brain are affected by elevated CO_2 and behaviour in response to elevated CO_2 (Tresguerres and Hamilton, 2017).

Genes expressed in the brain which are relevant to $GABA_AR$ and GlyR include GABA transporter type 1 (GAT1), Glutamate decarboxylase 1 (GAD1), gamma-aminobutyric acid receptor subunit alpha 4 and 2 (GABRA4, GABRA2), and serine hydroxymethyltransferase 2 (shmt) (see figure 2.1). GAT1 is a GABA transporter that removes GABA from the synaptic cleft and is hypothesized to be downregulated in elevated CO_2 conditions which would increase the availability for GABA to bind to the postsynaptic cleft (Schunter et al., 2018). GAD1 is the gene

responsible for the synthesis of GABA and is expected to be upregulated in fish exposed to elevated CO_2 which would reflect an overall increase in GABA production (Shunter et al., 2018). GABRA4 and GABRA2 are GABA receptors, both of which are anticipated to be upregulated during exposure to elevated CO_2 (Shunter et al., 2018). Finally, serine hydroxymethyltransferase (shmt), the gene for an enzyme that converts serine into glycine in the brain is also expected to increase in fish under elevated CO_2 conditions (Schunter et al., 2018).

The effects of elevated CO_2 on behaviour are debated, as other studies have been unable to replicate similar significant results or do not find meaningful differences in behaviour (e.g., Clark et al., 2020). When taken together, there appears to be a consensus of there being large variance on behaviour change depending on the species, the timing of exposure, length of exposure, and CO_2 concentration during exposure (Heuer et al., 2019). Additionally, there is need for more consistent and transparent research in behavioural sciences, beyond just studies that seek to address the effects of rising CO_2 (Clark et al., 2020).

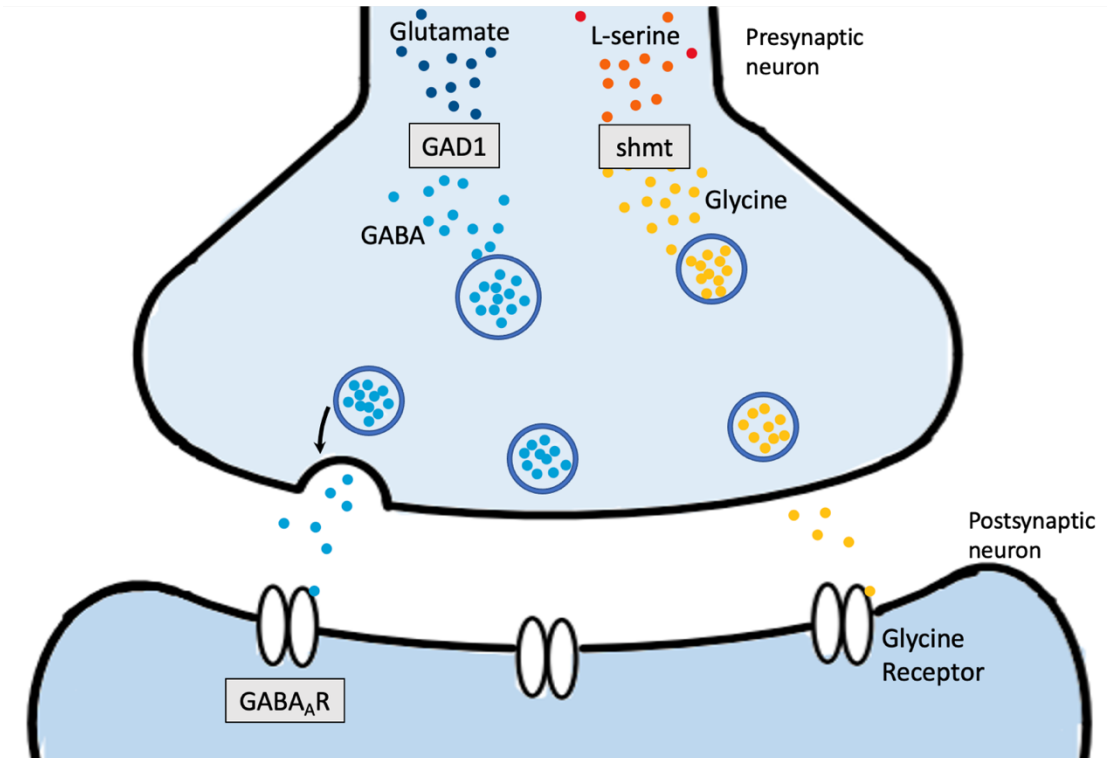


Figure 2.1 Visualization of a synaptic cleft for gamma-aminobutyric acid (GABA) and glycine related pathways, which are neurotransmitters in the brain. Glutamate decarboxylase 1 (GAD1) is an enzyme that catalyzes the synthesis of GABA from glutamate. $GABA_AR$ are GABA type A receptors which GABA binds to on the post synaptic cleft. Finally, serine hydroxymethyltransferase (shmt) is an enzyme that converts serine into glycine in the brain. In this study the relative abundance of mRNA for GAD1, shmt, and $GABA_AR$ (marked with grey boxes) were measured.

2.4 Carbon Dioxide effects on Fish Reproduction

In vertebrates, there are three major structures involved in the endocrine regulation of reproductive functions: the hypothalamus, the adenohypophysis of the pituitary, and the gonads (Norris and Carr, 2013). Together, these structures compose the hypothalamic-pituitary-gonadal (HPG) axis. In teleost fish, environmental stimulants such as pheromones, visual cues, and circadian cycles, can stimulate hypophysiotropic neurons in the pre-optic area (POA) to generate gonadotrophic releasing hormone (GnRH) (Wootton and Smith, 2015). Once stimulated by GnRH, gonadotroph cells in the pituitary secrete follicle stimulating hormone (FSH), and luteinizing hormone (LH) (Wootton and Smith, 2015). These hormones stimulate gametogenesis and steroidogenesis in gonadal tissues (Wootton and Smith, 2015).

Hormone availability and number of receptors available for binding, are general regulatory factors that will result in either stimulatory or inhibitory effects on the HPG axis (Wootton and Smith, 2015, Norris and Carr, 2013). These factors can be influenced by several means including neurotransmitter gamma-aminobutyric acid (GABA), norepinephrine (NE), endogenous opioid peptides, and testosterone (T) (Wootton and Smith, 2015; Norris and Carr, 2013). GABA acts by directly stimulating production of GnRH and indirectly promotes its production by inhibiting dopamine (DA), which has an inhibitory function on GnRH production in the hypothalamus and gonadotropin hormone cells in the pituitary (Figure 2.2). GnRH production is also induced by normal levels of estrogen (i.e., some forms of estradiol) which are produced by the conversion of testosterone by aromatase in the brain, cytochrome P450 19b (cyp19b) (Norris and Carr, 2013).

At the level of the pituitary, GnRH binding is the main stimulatory factor on gonadotrophic production (Wootton and Smith, 2015; Norris and Carr, 2013). Moreover, GnRH pulsatile rhythm, which is mainly influenced by calcium cycling and autocrine feedback of

GnRH neurons, will determine whether LH or FSH release is favored (Norris and Carr, 2013). Production of LH and FSH is inhibited mainly by gonadotrophic inhibiting hormone (Norris and Carr, 2013). Steroidogenesis in the gonads will be impacted by the availability of enzymes and proteins required for each step in the steroidogenic pathway (Wootton and Smith, 2015; Norris and Carr, 2013).

In medaka, there are three forms of GnRH: gnrh1 (medaka-type GnRH/mdGnRH), gnrh2 and gnrh3. mdGnRH is the form thought to be directly responsible for HPG axis functioning, while gnrh2 and gnrh3 are thought to have indirect effects to reproduction such as through reproductive behaviour (Kinoshita et al., 2009). FSH and LH are heterodimer glycoproteins consisting of an alpha (GTHa) and a beta subunit (FSHb or LHb) (Zhang et al., 2008). In female medaka, it is thought FSH stimulates thecal cells in the ovaries to produce testosterone and granulosa cells to convert the testosterone to 17ß-estrogen (E_2) under the catalyst aromatase. Aromatase in the gonads is produced via the gene cytochrome P450 alpha, while in the brain aromatase is transcribed by cyp19b (Kinoshita et al., 2009; Kuhl et al., 2005). In the liver E_2 is converted to vitellogenin which is a precursor for egg yolk protein and stimulates the synthesis of choriogenin the egg envelope precursor protein (Kinoshita et al., 2009). In fish, LH typically stimulates ovulation and maturation of oocyte and later stages of spermatogenesis.

Currently, little literature observes how OA and elevated CO_2 will affect fish reproduction (Heuer and Grosell, 2014). Research that exists on this topic holds variable results. For example, one study found an increase of reproductive output and an increase weight of juveniles in adult anemonefish (Miller et al., 2013) while in other studies, elevated CO_2 has been linked with reduced reproductive success and effort in fishes (Miller et al., 2012). In one study on intergenerational effects of CO_2 acidification on a guppy species (*Poecilia reticulata*), two of six pregnant females exposed to CO_2 aborted dead underdeveloped or morphologically compromised

fry, while no such observations were found under control pH (George et al., 2019). While many studies focus on maternal investments, other studies have found evidence for paternal effects such as reduced sperm motility (Ingermann et al., 2002). Additionally, early life stages in fish are suggested to be particularly vulnerable to elevated CO_2 conditions (Brauner et al., 2019). For example, marine medaka (*Oryzias melastigma*) exposed to elevated CO_2 during early life stages had abnormal development and increased mortality (Lee et al., 2018). Because reproductive responses of fish can be used to assess the potential for long-term impacts on populations, these studies are indicative of CO_2 having potential long-term population effects (Gormley and Teather, 2003). These studies are also relevant to aquaculture and global fisheries, where elevated CO_2 conditions as high as 10,000–24,000 ppm have been observed in some intensive aquaculture settings (Brauner et al., 2019).

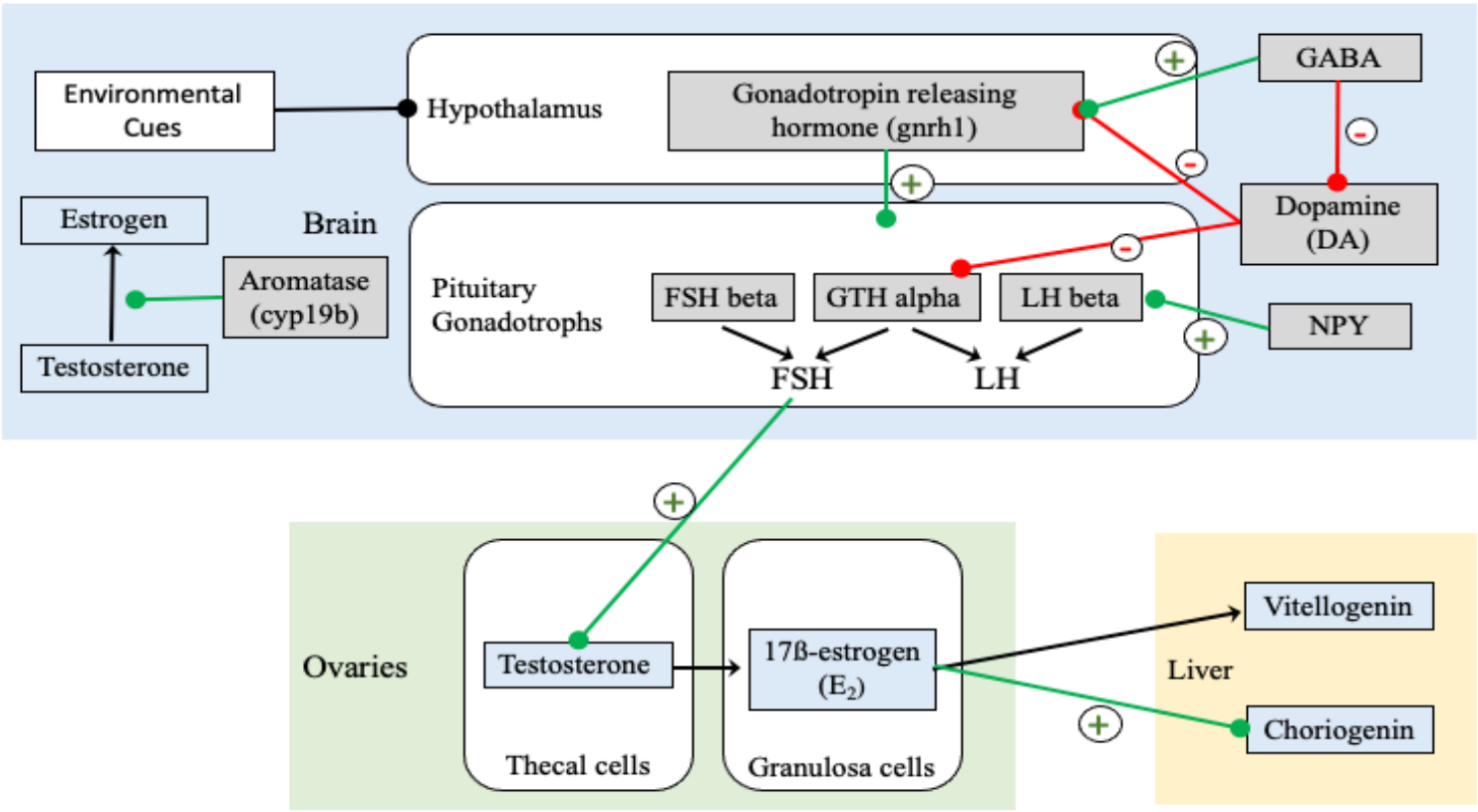


Figure 2.2. Hypothalamic-Pituitary-Gonadal Axis (HPG Axis) schematic in a female medaka fish highlighting, in grey boxes, genes of which expression was measured in the current study in context with the function these genes play in the reproductive axis. Round end arrows represent stimulation of a tissue where green represents excitation and red represents inhibition. Black pointed arrows represent conversion or reactions. Abbreviations are as follows: neuropeptide Y (NPY), follicle stimulating hormone beta subunit (FSHb), glycoprotein hormones alpha chain (GTHa), lutenizing hormone beta subunit (LHb), gamma aminobutyric acid (GABA).

2.5 Research Gap 1: Transgenerational and Multigenerational Studies

At present, most research measuring the effects of elevated CO_2 on aquatic organisms is conducted over short-term elevated CO_2 exposures ranging from hours to weeks (Schunter et al. 2018). Short term exposures cannot accurately predict an animal's response to elevated CO_2 because they do not account for long-term acclimation especially over multiple generations as is expected in ecologically-relevant changes to elevated CO_2 (Schunter et al., 2018). This lack of research has left a major gap in literature on the effects of elevated CO_2 on individuals, populations, and communities over a timescale that is gradual and long term as is expected with climate change (Doney et al., 2009). Multigenerational studies are also important because they can capture the effects of epigenetic inheritance. Considering epigenetics is important because they link life experiences and environment with changes in physiology and overall gene expression (Bollati and Baccarelli, 2010).

Where genetic inheritance refers to the transmission of DNA sequences from parents to offspring, epigenetic or "non-genetic inheritance" refers to any heritable effect on an offspring's phenotype that is due to factors other than DNA sequences (Bhandari, 2016). Non-genetic inheritance illustrates how an offspring's fitness can be influenced by the environmental conditions experienced by their parents (Munday et al., 2014). Mechanisms of non-genetic inheritance include selective DNA methylation, histone modification, and small RNAs (Schunter et al., 2018; Bhandari, 2016). DNA methylation is the addition of a methyl group (CH_3) to a DNA molecule (Brander et al., 2017). Methyl-CpG binding domain protein 2 (Mbd2) is one example of a methylation factor, which tends to bind preferentially to promotor regions of genes (Fan and Hutnick, 2005) which makes it more difficult for transcription factors to bind to this region, thereby reducing the expression of said gene (Brander et al., 2017). Mbd2 has been found to significantly influence multigenerational effects of stress and is implicated in persisting

through the germ-line (Crudo et al., 2012). Non-genetic inheritance also includes the transmission of cytoplasmic or somatic factors such as hormones, proteins, lipids, and RNA which can be passed to offspring in several ways including in gamete membrane or cytoplasm, or by transfer of parental hormones, waste products, parasites, or symbionts (Bonduriansky and Day, 2009). One example is nutrient provisioning, which refers to both the quantity and quality of metabolic resources provided to an offspring from its parent(s). In fish, this can include the investment in egg yolk nutrients that can be measured by egg weight and or size (Vijendravarma et al., 2010).

Generally, non-genetic inheritance can influence an organism's resilience or susceptibility when it is exposed to the same or different stressor as its relative (Nestler, 2016). Transgenerational effects are those of which differences are observed in offspring who were not directly exposed to a stressor, but who were instead affected via epigenetic tags through the germline (Brander et al., 2017). Whereas multigenerational effects are those when the offspring is exposed to the same conditions as their relatives. When considering fish exposed to elevated CO_2, it may be possible that fish with parents or grandparents exposed to chronic elevated CO_2 conditions will contain epigenetic characteristics that allow them to be better acclimated to the same high CO_2 conditions. This acclimation could have significant implications for fish population fitness (Munday et al., 2014). Several studies have found evidence for non-genetic inheritance in fishes (Salinas et al., 2013; Miller et al., 2012). For example, in some studies, maternal environments had significant impacts on the effects of acidification on fish growth (George et al., 2019). In a study on a marine fish, parental exposure to elevated CO_2 mediated metabolic and growth impacts from elevated CO_2, as offspring of fish exposed to elevated CO_2 did not demonstrate higher metabolic rates, lower growth rates, or lower survival, which were observed when only juveniles (and not their parents) were exposed to elevated CO_2 (Miller et al.,

2012). Overall, studying trans- and multi- generational effects can help more accurately predict how fish will cope with future conditions.

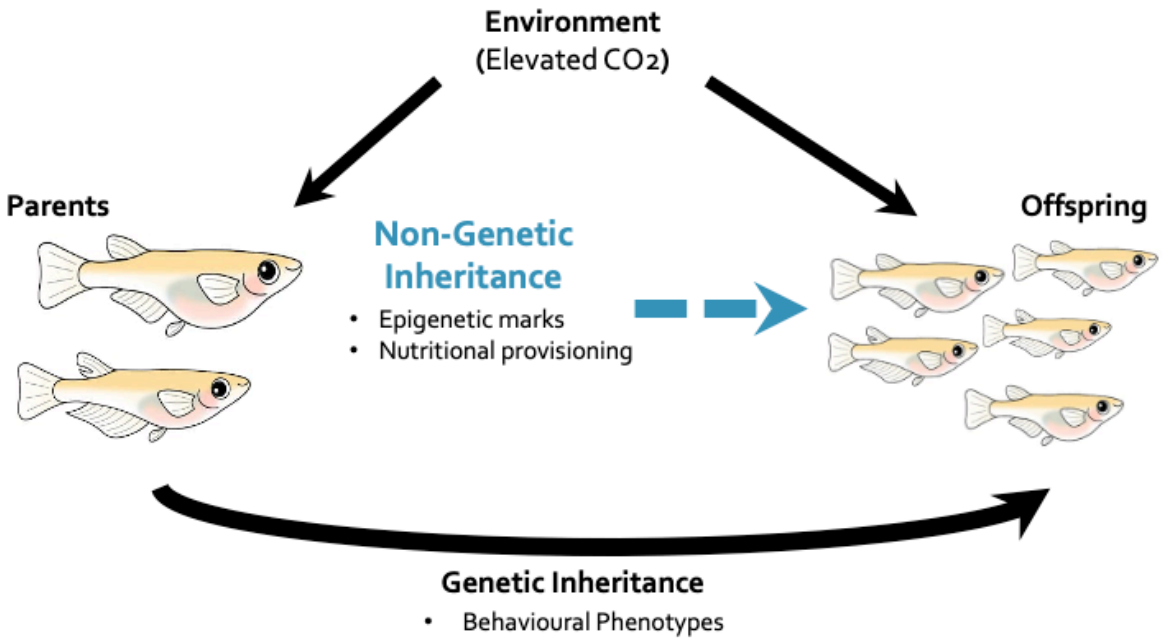


Figure 2.2 Illustration of two modes of inheritance from parents. Genetic inheritance refers to the transmission of DNA sequences from parents to offspring, and epigenetic or "non-genetic inheritance" refers to any heritable effect on an offspring's phenotype that is due to factors other than DNA sequences (Bhandari, 2016). Illustration adapted from Munday, P. L. 2014. Transgenerational acclimation of fishes to climate change and ocean acidification. *F1000 Prime Rep.* **6**, 99–106.

2.6 Research Gap 2: Behavioural Phenotyping

The second gap in literature is repeatable behavioural phenotypes or "animal personality". Animal personality is a population metric that refers to repeatable within-individual behavioural tendencies, while behavioural flexibility refers to the degree of within-individual variation exhibited (Dingemanse and Wolf, 2010; Stamps and Groothuis, 2017; Bell et al., 2009). Behavioural phenotypes have been identified across many taxa, and some common assays for determination of a phenotype is responsiveness towards novel objects, novel environments, or predator responses (Brown et al., 2011). When considering animal personality from an evolutionary perspective, repeatable behaviours that persist over time must have a heritable component (Dingemanse, et al., 2002).

Behaviours are observable actions of an animal, which in fish are typically measured to quantify feeding, mating, parental care, predator avoidance, dispersal, habitat-use, or social behaviours (Brown et al., 2011). Behaviour results from a cumulative interaction between environmental, physiological, developmental, molecular, and evolutionary processes, and behaviour is acted on by natural selection (Willmer et al., 2005; Brown et al., 2011; see figure 2.3). Behaviour is also plastic and can allow an organism to respond to environmental stressors without the need for significant molecular or physiological acclimatization (Willmer et al., 2005; Brown et al., 2011). For example, when fish are exposed to temperature stress, they move to deeper, cooler, water, or a different geographical location, if it permits (Willmer et al., 2005). Other environmental variables can also affect behaviour (e.g., Heuer and Grosell, 2014).

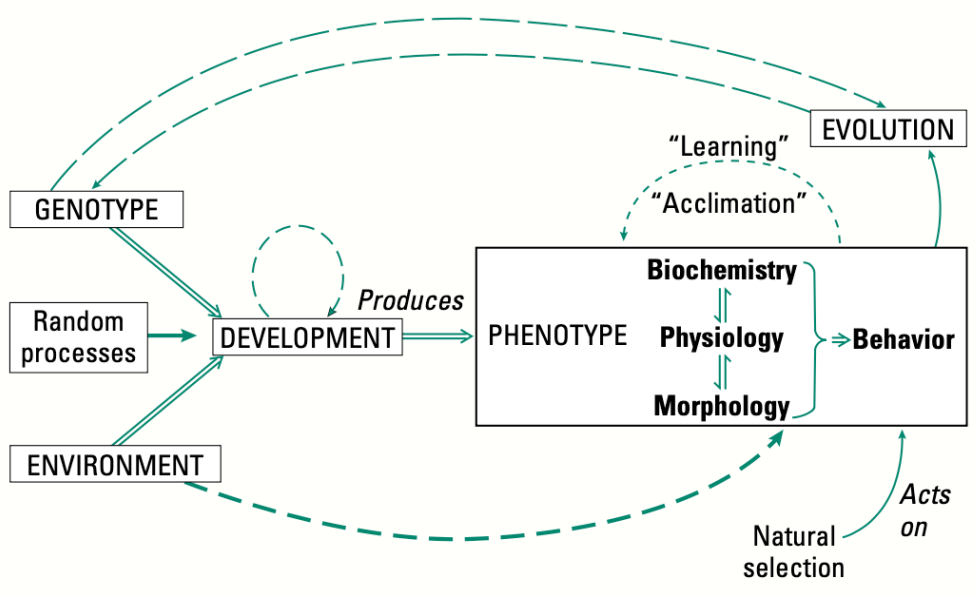


Figure 2.3. Schematic diagram of the interaction of behaviour with environment, development, physiology, biochemistry, and morphology of an organism (Willmer et al., 2005).

Reactive versus proactive coping style preference is one personality dichotomy which refers to repeatable within individual behavioural difference in response to a stressor (Baker et al., 2018). These coping styles have been explored across a broad range of taxa in literature and have been corresponded to a suite of differences between animals (Coppens et al., 2010; Baker et al., 2018). Animals with a reactive coping style tend to react more substantially to a novel stressor, have higher glucocorticoid responses to stress, but show higher behavioural plasticity (Archard et al., 2012; Baker et al., 2018). Whereas proactive animals tend to be bolder, more aggressive, have low glucocorticoid responses to stress, tend to be more reproductively successful, but favor patterns and routine, and are less behaviorally flexible (Coppens et al., 2010; Archard et al., 2012; Baker et al., 2018). In prior literature, studies that selectively bred individuals that showed different behaviour in response to stress result in offspring that react similarly to the same stimuli, suggesting heritability (Archard et al., 2012; Baker et al., 2018).

Stress responses can be context specific and can depend on other physiological states of an organism (Ricklefs and Wikelski, 2002). In a study measuring zebrafish coping styles, time spent in the center of a tank, time spent stationary, and swimming speed were all repeatable and reliable behaviours in an open field test and these behaviours were consistent with differences in gene expression in the brain (Wong et al., 2015). Variation and diversity in a population are evolutionarily beneficial as it can allow a population to be more adaptive to a variety of stressors (Wolf et al., 2008). Understanding phenotypic diversity is also important for understanding population dynamics. For example, dispersion of an invasive mosquitofish (*Gambusia affinis*) was significantly different depending on the fish's behavioural phenotype, which can have significant implication for invasive species management (Cote et al., 2010).

In their study, Schunter et al. (2018) found that the offspring of parents behaviorally more 'tolerant' or 'sensitive' to CO_2 had significantly different variations in their transcriptomes (messenger ribonucleic acid, mRNA) and proteins. Thus, repeatable individual behavioural variation in response to elevated CO_2 exposure may have a molecular or physiological basis that affects the individual's ability to acclimatize to the elevated CO_2 conditions (Schunter et al., 2018). Historically, hesitancy around naming "personality" traits in animals has existed especially regarding fishes and other non-primate animals, and thus personality in organisms such as fishes has been understudied (Brown et al., 2011). Repeatable behavioural phenotypes are good predictors of trends in behaviour, can be useful predictors of other physiological and life history traits, and can be subject to selection pressures, thus studying effects of behavioural phenotypes in response to environmental change is important (Fleeson, 2014; Coppens et al., 2010; Schunter et al., 2018). Repetitive behavioural variation 'personality' in response to CO_2 therefore may be a predictor of underlying physiological differences in acid-base regulation adaptions (Schunter et al., 2018).

2.7 Objectives and Hypotheses

In my study, I aimed to understand the influence of phenotypic variation and transgenerational acclimation on the biological responses of medaka exposed to elevated CO_2. To do this, I completed both within and across generation comparisons between fish exposed to control CO_2 conditions and fish exposed to either acute or chronic elevated CO_2 conditions. Briefly, the biological responses quantified were: growth and development (i.e., length, weight, size, sex ratio), behaviour (total distance moved, swimming direction, time spent in outer versus inner zone of arena), reproductive outputs (egg size, number of eggs per female per day, hatch success), and the relative abundance of various mRNA transcripts in whole brain tissue.

My first objective was to examine phenotypic variation in fish (i.e., high behavioural responder vs low behavioural responder) exposed to acute elevated CO_2, and to quantify the effects a 6-week elevated CO_2 exposure had on growth, behaviour, and reproduction relative to their determined phenotype. I hypothesized that behavioural phenotypes would influence how a fish responds to elevated CO_2 because repeatable behavioural variation has previously been linked to differences in an animal's physiology and life history traits which can impact how an organism responds to environmental stressors. Therefore, I predicted fish that do not change their behaviour significantly when exposed to elevated acute CO_2 (non-responsive fish) would demonstrate tendencies consistent with low plasticity in responses to elevated CO_2 conditions as demonstrated by reduced behavioural differences, reduced reproductive measures, reduced growth measures, and changes in relative mRNA abundance consistent with such deleterious effects (e.g., downregulation of HPG axis relevant genes). I also predict that responsive fish would be more plastic in their response after a 6-week exposure to elevated CO_2 as demonstrated by no measured differences in behaviour, reproduction, or growth relative to control CO_2 exposed

fish, and difference in relative gene expression consistent with acclimatization to elevated CO_2 conditions (e.g., higher relative abundance of mRNA for appetite simulating genes).

The second objective of my thesis was to investigate acclimation by medaka to elevated CO_2 conditions across multiple generations. I hypothesized that elevated CO_2 exposure would affect a fish's growth, reproduction, and behaviour due to differential energy allocation strategies which would prioritize acid-base acclimatization efforts. Therefore, I predicted that fish in elevated CO_2 conditions would have a decline in weight/body condition, reduced reproductive outputs, and would display differences in behaviour consistent with increased anxiety behaviour, reduced cognition, and reduced overall health of a fish. Finally, I predicted that the relative mRNA abundance for whole brain tissue genes of interest would reflect changes consistent with the previous listed predictions.

Chapter 3: Methodology

3.1 Study Animal, Husbandry, and Holding Conditions

Japanese medaka (*Oryzias latipes*) is a teleost fish native to eastern Asia (Figure 3.1) where it typically lives in rice paddies, ponds, and slow-moving freshwater (Sasado et al., 2010). Medaka belong to the order Beloniformes and the family Adrianichthyidae, which contains four genera, one of those being *Oryzias* (Figure 3.2; Kinoshita et al., 2009). For my thesis I used the Hd-rR strain of medaka, which is an inbred strain originating from a Southern Japanese population and a stock (d-rR) from Nagoya University (Sasado et al., 2010; Kinoshita et al., 2009; see Figure 3.1).

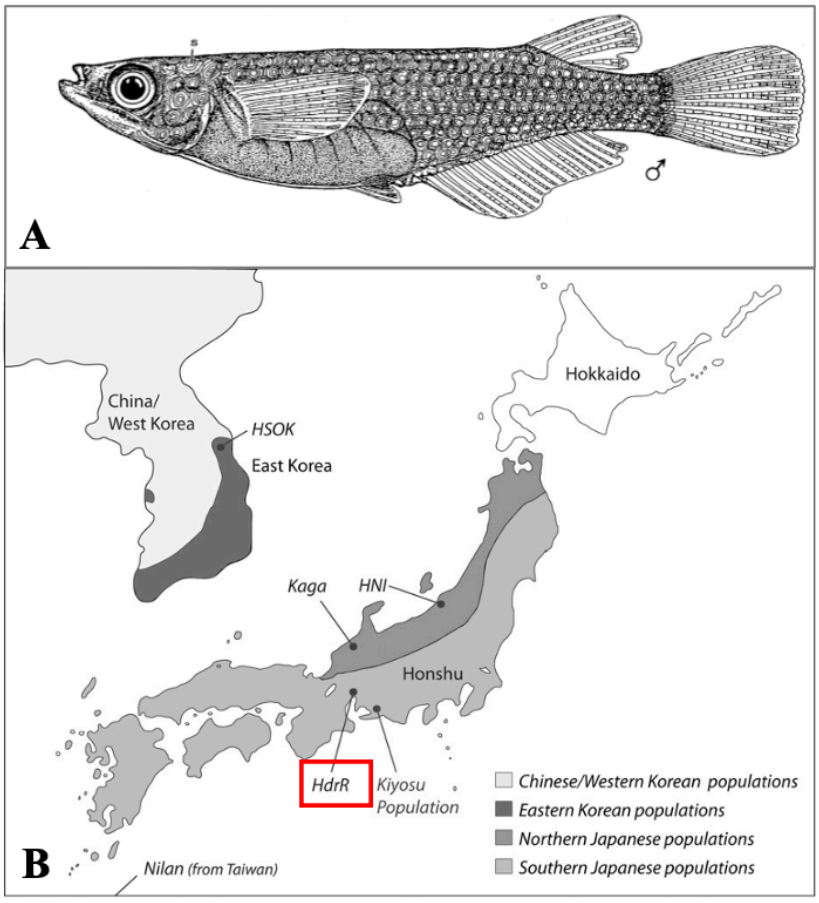


Figure 3.1 A. Illustration of adult male medaka fish (Iwamatsu, 2004). B. Geographic distribution of the native populations of common laboratory medaka *(Oryzias latipes*) strains. Image modified from that published by Kirchmaier et al. (2015).

Populations of medaka (Southern, Northern, Eastern, Chinese/Western Korean) are genetically distinct, and southern populations have 2n, 48 chromosomes (Kinoshita et al., 2009). The Hd-rR strain is fast-growing and reaches adulthood in one month when held at 27°C in a 14 hour-light:10-hour dark cycle (Kinoshita et al., 2009). Medaka are oviparous and reach sexual maturation at the approximate age of 2–3 months. Healthy reproductive females on average

spawn approximately 20–40 eggs per day (Kinoshita et al., 2019). Medaka are a common model organism in biological research, are easily maintained and bred in controlled laboratory conditions, and have been shown to respond to elevated CO_2 (Sasado et al., 2010; Tseng et al., 2013; Wang et al., 2017; Lee et al., 2018; Lin et al., 2016). A whole-genome sequence for the Hd-rR strain is publicly available for medaka and a reliable phylogeny has been constructed (Kinoshita et al., 2009; Narus et al., 2000; Figure 3.2).

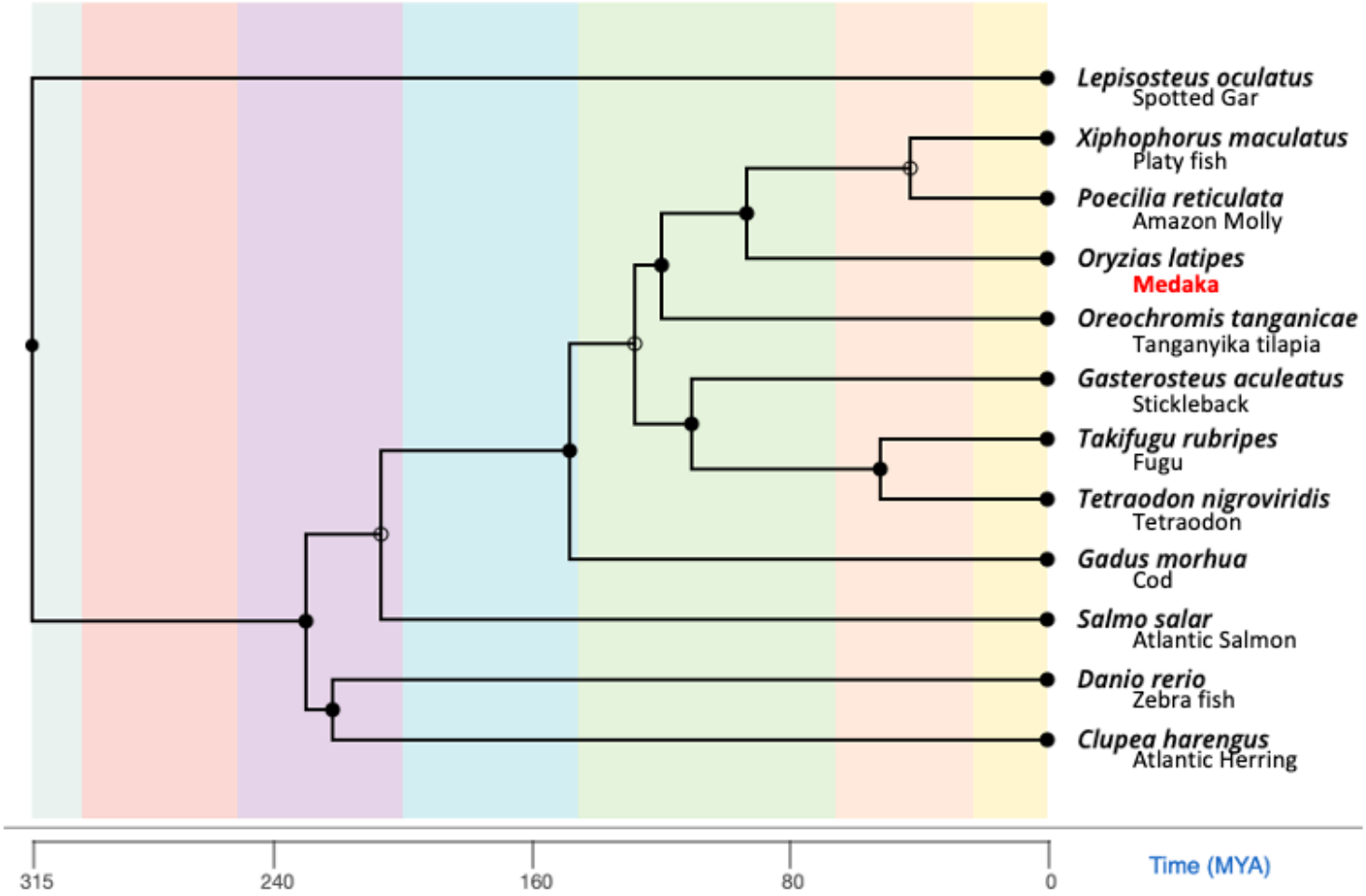


Figure 3.2 Evolutionary relationship between Medaka (*Oryzias latipes*) and other teleost fishes, using spotted gar as an outgroup which did not undergo a whole-genome duplication (3R) as did teleost fishes (c.f. TimeTree.org).

Medaka in my study were housed at the fish facility in the animal complex at The University of Winnipeg in two stand-alone aquarium systems (Aquaneering, Inc., San Diego CA, USA). Fish experienced a photoperiod of 14 h of light, and 10 h of dark, and the temperature of the aquarium systems was maintained between 26–28°C. This temperature and light/dark cycle are consistent with the environmental conditions a native medaka would experience during June and July when spawning occurs, thus medaka in this study experienced constant environmental conditions supportive for reproduction (Kinoshita et al., 2009). Adult medaka (90+ days of age) were fed to satiety twice daily (10:00 and 15:00) with flakes (O.S.I. Spirulina Flake Food, Golden Pearls, and Zeigler Adult Zebrafish Diet). Embryos were generated by natural cross-fertilization and collected from tanks using a plastic dropper. Embryos were then counted in a petri dish and transferred to a larval tube on one of the two aquarium systems (treatment-dependent). Juvenile and larvae fish were fed four times daily. Juveniles (21–90 days of age) were fed Zeigler Larval Diet AP 100 (150–450 micron) and Golden Pearls. Larvae (fish up to 21 days of age) were fed Zeigler Larval Diet AP 100 (<100–150 micron) and live paramecium. After reaching a size of approximately 1 cm in length, larvae were transferred out of the larval tube and into a standard tank. All animal care and experimental protocols were reviewed and approved by The University of Winnipeg Animal Care Committee.

Various water parameters were monitored throughout my study. Alkalinity, ammonia (NH_4^+), hardness (calcium carbonate ($CaCO_3$)), nitrates (NO_3), nitrites (NO_2), and pH in the aquarium systems were measured daily using 5-way test strips (API ®, MARS). Conductivity and dissolved oxygen (DO) were measured with a handheld Hach portable multi-parameter meter (HQ40d, London ON, Canada). Water parameter ranges for the three generations were as follows: conductivity, 300–450 µS/cm; ammonia, < 0.5 mg NH_4^+ /L; nitrite, < 0.1 mg NO^-_2 /L; nitrate, < 0.1 mg NO^-_3 /L; total hardness, 0–40 mg $CaCO_3$/L. These parameters are in an

approximate appropriate range for this species, with exception of ammonia levels in which suggested levels would be below 0.2 mg NH^{+}_{4} /L (Kinoshita et al., 2009). To measure experimental variables pH and CO_2 in this study (process described below in Section 3.3), pH was measured using a pH probe (American Marine Inc., Ridgefield, USA), and a modified infrared handheld CO_2 probe was used to measure the partial pressure of CO_2 (GM70, Vaisala, Helsinki, Finland) (Johnson et al., 2010). A range of 6.5–7.2 pH is suitable for medaka, with an ideal pH of 6.8 (Kinoshita et al., 2009).

3.2 Determination of parental phenotypes

3.2.1 Elastomer Tagging

To behaviourally phenotype parental fish based on their response to elevated CO_2, 80 medaka from a single generation had a visible implant elastomer tag manually injected into the dorsal musculature using an insulin syringe (Northwest Marine Technology, Inc., Shaw Island, WA, USA). The tag was inserted ~ 2 mm into the musculature, anterior to the dorsal fin and posterior to the spinal cord. Prior to the insertion, the fish were anesthetized in 50 mg/L of buffered tricaine methanesulfonate (MS222) before tagging. To reduce chances of post-procedure tag loss, during needle retraction the elastomer was not expressed so that ~ 1 mm of the injection site was free of elastomer tag (Hohn and Petrie-Hanson, 2013). The injection site was then wiped with a dampened sterile gauze pad and tagged fish were moved to an aerated recovery tank held within a portable animal intensive care unit, which was maintain at ~ 27°C (ThermoCare, Paso Robels, CA, USA). Following recovery from anesthesia, fish were returned to the stand-alone holding systems and monitored for 48 h. No experiments were conducted for 14-d post-procedure to allow for optimal recovery. My process has been shown to permit the identification of

individual medaka for over 8 weeks (Hechter and Hasler, 2019). Following tagging recovery, fish were divided into 'high-response or 'low-response'. The division was determined by each fish's behavioural variation from the population mean after acute exposure to high CO_2 in common shuttling (Kates et al., 2012) and novel tank diving (Egan et al., 2019) assays.

3.2.2 Shuttling Assay

The shuttling assay, which measured preference to control or elevated CO_2 by allowing a fish to choose between swimming arenas with each CO_2 condition, was conducted using a shuttle box arena (Loligo Inc., Hobro, Denmark) consisting of two circular, identically sized tanks (10.6 radius x 4 height cm) connected through a gated narrow central channel (~ 3.3 width x 5.5 length x 4.0 height cm) (see Figure 3.3). Each tank had a connected external buffer chamber where the water was treated with either air or CO_2. To treat the chambers, water was circulated between each tank and its associated chamber by a multi-channel peristaltic pump (BT100-1L, 4 channels, Longer Precision Pump Co., Ltd, Hebie, China). Before each trial 2L of water from the aquarium holding system was added to the shuttle box arena and to each of the buffer chambers, for a total of 6L of water. Once the water had leveled out between the two circular tanks, the gate between the tank channels was closed to minimize the mixing of water between the tanks. The water for each buffer chamber was then randomly selected and treated for either control or elevated CO_2 conditions. The elevated CO_2 condition chamber and its corresponding shuttle box tank was

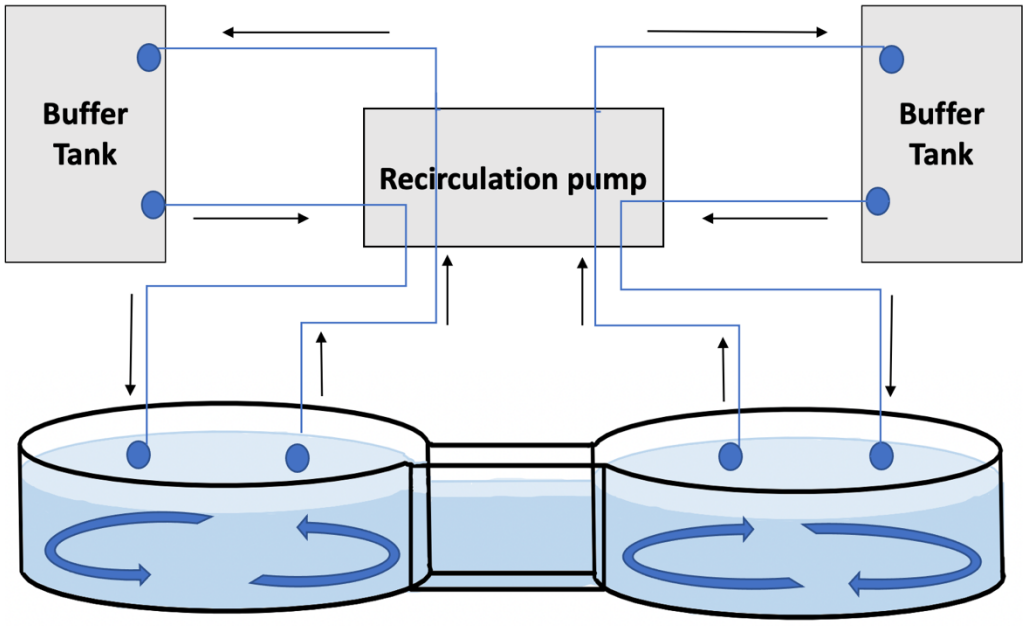


Figure 3.3 Diagram illustrating shuttle box arena and overviewing the cyclical flow of water from each side of the shuttle box arena into its respective treated buffer tank, and back into its respective arena.

treated and maintained at 6.1 pH (±0.1 pH) using a pH pinpoint controller that regulates CO_2 by using a solenoid valve attached to a compressed CO_2 gas tank (American Marine Inc., Ridgefield, USA). The CO_2 concentration maintained by this pH averaged at 3,460 ppm (S.E. ± 215.2). Air was bubbled into the control condition tank buffer chamber as a control. Individual fish were placed into the elevated-CO_2 treated tank in the shuttle box and left to habituate for 3 min. The gate separating the shuttle box tanks was then removed and fish behaviour was recorded for 10 minutes using a web camera (Logitech, Newark CA, USA). To reduce experimenter influence on behaviour, the experimenter remained out of the assay room for the duration of the test. A camera (Logitech BRIO-4K Ultra HD Webcam, Lausanne, Switzerland) was connected to a computer in

a separate room *via* a portal hole so that videos could be recorded and monitored remotely (see Figure 3.4). Additionally, the table holding arenas was encircled, including the top, by a white curtain to reduce other visual environmental stimuli.

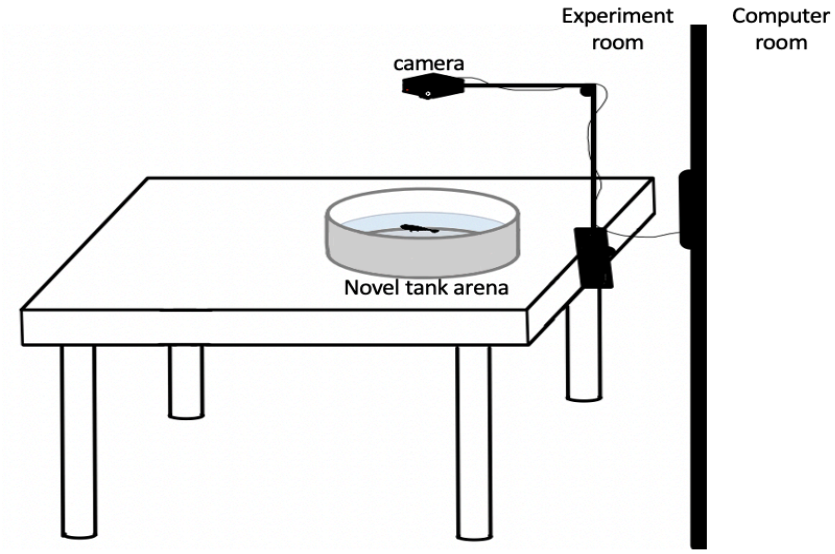


Figure 3.4 Visualization of behaviour recording room and novel tank arena recording set up. To reduce experimenter influence on behaviour, the experimenter remained out of the assay room for the duration of the test. A camera was connected to a computer in a separate room *via* a portal hole so that videos could be recorded and monitored remotely. The table holding arenas was encircled, including the top, by a white curtain to reduce other visual environmental stimuli.

3.2.3 Novel Diving Tank Assay

Approximately one week after the shuttling assay, each fish underwent a novel tank diving assay adapted from toxicological studies (e.g., Egan et al. 2019; Figure 3.5). This novel tank diving assay was used to measure exploration behaviour, which is a measure that has previously been used to quantify responses to stress and anxiety and determine repeatable behavioural phenotypes

(Egan et al., 2019). First, a separate large basin containing water from the experimental fish's housing aquarium system was treated with compressed CO_2 and maintained at 6.1 pH (± 1 pH) using the controller described above (mean 6,102 ppm [± 84]). I targeted a 6,000 ppm CO_2 to ensure the elevated CO_2 was high enough to elicit a behavioural response on the acute time scale of this behavioural assay, and so that the behaviour after exposure could be measured. A circulation pump (969 L/h) circulated the basin's water to maintain a consistent CO_2 concentration throughout (Aquatop., Brea CA, USA). To be treated with CO_2, each fish was netted and transferred to a 600 mL beaker containing 400 mL of treated basin water, and held in this beaker for 3 min. Immediately following treatment, fish were placed in a 1.5 L trapezoidal tank (15 height × 26 top × 23.5 bottom × 7.5 width cm; Aquaneering, Inc., San Diego CA, USA) filled 2.5 cm below the inner edge of the tank with water from experimental fish's housing aquarium system. The activity of the fish was recorded for 15 min. To reduce stress influencing behaviour, fish were identified by its unique elastomer tag after the assay was complete.

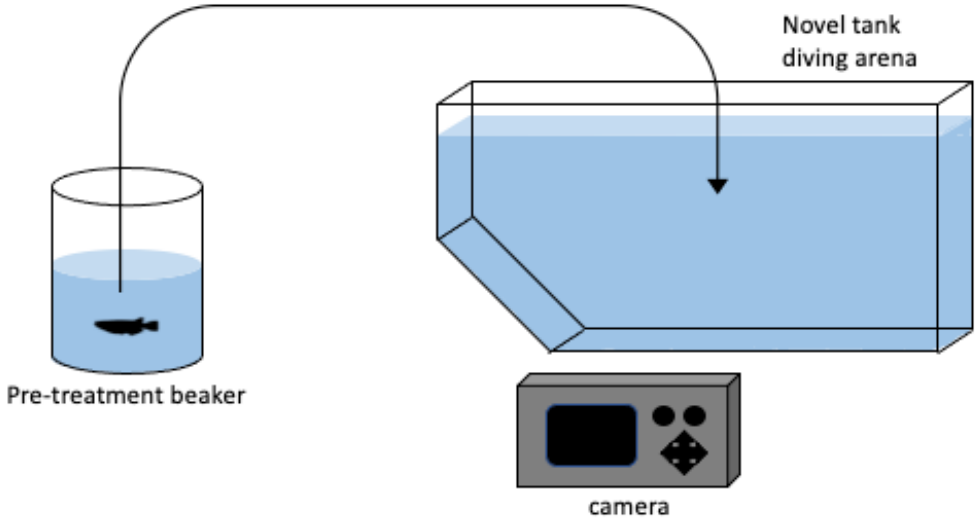


Figure 3.5 Novel tank diving arena illustration and recording approximation set up. To be treated with CO_2, each fish was placed in 400 mL of elevated CO_2 treated water in a 600 mL beaker for 3 min. Immediately following treatment, fish were be placed in a 1.5 L trapezoidal tank (15 height × 26 top × 23.5 bottom × 7.5 width cm; Aquaneering, Inc., San Diego CA, USA) filled 2.5 cm below the inner edge of the tank with water from experimental fish's housing aquarium system.

3.2.4 Phenotyping

Shuttle box and novel tank assay videos were manually tracked using metrics least likely to be subject to observer bias (EthoVision® XT software, Noldus, Wageningen, Netherlands). Fish behaviour in the shuttling assay was measured by calculating the amount of time (duration [s]) the entire fish spent in the high-CO_2 shuttle tank and the number of movements between tanks the entire fish made (frequency). To measure fish behaviour in the novel tank diving assay behaviour, the tank area was divided into six subsections using the tracking software (EthoVision ®XT, Noldus, Wageningen, Netherlands). This included a horizontal division that divided the

upper and lower portions of the tank, and two vertical divisions which divided the tank into left, middle, and right portions. For each subsection, the number of times a fish entered each section (frequency) and the amount of time the anterior end of the fish spent in each section (duration) during the test were measured. For each test, a mean Pearson's Chi-square statistic was calculated and summed for each fish. Fish were then rank-ordered and fish with the lowest chi-square statistics displayed behaviour on average that varied the least from the population mean, while fish with the highest chi-square statistics displayed behaviour that on average varied the most from the population mean. Note, this method means that consistent freezing and darting behaviours in a fish to CO_2 were captured as variance from the population mean. This is important because animals may display both freezing and darting behaviour in response to a stressor, with both indicating physiological stress responses (Archard et al., 2012). Therefore, using consistent behavioural differences from the population average as a parameter rather than behavioural extremes, allowed us to capture more accurately 'non-responder' and 'responder' behavioural responses to CO_2.

3.3 Parental and Transgenerational CO_2 Treatments

After phenotyping each fish, the 20 fish with the lowest and highest-ranking values within the first and fourth quartile of chi-square statistics were sexed, and male and female groups were selected into either control or high CO_2 conditions (described below; Table 3.1). The outcome of this selection led to four groups of ten fish (5 males, 5 females) in either low response + high CO_2, low response + control CO_2, high response + high CO_2, or high response + control CO_2 conditions. A 1500 ppm CO_2 concentration elevated from an aquatic ecosystem at ambient CO_2 (atmospheric pressure, ~450 ppm) is considered a reasonable projection for near-future CO_2

elevation in freshwater ecosystems and was the target average CO_2 concentration for this study (Weiss et al. 2018). The control CO_2 target was a ppm less than 600 (~ 400 ppm) which is a CO_2 concentration consistent with ambient CO_2 (atmospheric), which was the condition the fish had been exposed to since the removal of their medaka strain of origin (d-rR) from the wild in 1952 (Sasado et al., 2010), and is the level typical for larger inland lakes and reservoirs (Cole et al., 2007). A stand-alone aquarium system was treated for either elevated-CO_2 or control conditions. The CO_2 concentrations in the elevated-CO_2 condition were held within the target range using a pH controller and probe which regulated the amount of CO_2 bubbled into the aquarium system basin by controlling a solenoid valve on a compressed CO_2 gas tank (American Marine Inc., Ridgefield, USA; Dennis et al., 2016). These pH and ppm were recorded daily. Additionally, pH probes were regularly re-calibrated and dissolved CO_2 concentration was confirmed using a titration (digital titrator CO_2 test kit, Hach Company, Loveland, Colorado).

Table 3.1 Carbon dioxide (CO_2) concentrations by parts per million (ppm) and acidity (pH) of control and elevated CO_2 condition aquarium system basins by the generational start of exposure to tissue dissection. The interquartile range (IQR) is a measure of the difference between the 75th and 25th percentile of the pH or ppm measure distribution.

Generation	**Treatment**	**Mean pH (± S.E.)**	**pH IQR**	**Mean CO_2 (± S.E.) (ppm)**	**CO_2 IQR (ppm)**
Parental	Control	7.15 (±0.03)	0.14	424.1 (±30.7)	107.8
	High	6.94 (±0.01)	0.14	1016 (±114.9)	1016
F_1	Control	6.86 (±0.02)	0.32	510.6 (±5.2)	80
	High	6.46 (±0.03)	0.46	1261 (±42.1)	636.75
F_2	Control	6.72 (±0.02)	0.34	516.6 (±4.1)	80.5
	High	6.0 (±0.03)	0.47	1598.3 (±42.14)	774

Parental fish were exposed to their treatment conditions for 6 weeks ($n_{control/responder}$ = 10, $n_{control/non\text{-}responder}$ = 10, $n_{high/responder}$ = 10, $n_{high/non\text{-}responder}$ = 9). In previous studies, 6–8 weeks has been demonstrated to be a sufficient time in CO_2 conditions to show physiological changes including ion transportation mechanisms in gills (Dennis et al., 2016; Deigweiher et al. 2008). Fish were acclimatized to high CO_2 conditions by increasing CO_2 in small increments over one week. Following the six-week treatment period, parental generation (P) eggs were collected, measured ($n_{control/responder}$ = 245, $n_{control/non\text{-}responder}$ = 291, $n_{high/responder}$ = 157, $n_{high/non\text{-}responder}$ = 63), and assigned to filial generation one (F_1) control CO_2 or high CO_2 conditions for rearing and maturation (approximately 18 weeks post-hatch) before reproductive, molecular, and behavioural measures were taken for this generation ($n_{control/responder\rightarrow control}$ = 60, $n_{control/responder\rightarrow high}$ = 30,

$n_{control/non\text{-}responder \rightarrow control} = 62$, $n_{control/non\text{-}responder \rightarrow high} = 30$, $n_{high/responder \rightarrow control} = 31$, $n_{high/responder \rightarrow high} = 53$, $n_{high/non\text{-}responder \rightarrow control} = 17$, $n_{high/non\text{-}responder \rightarrow high} = 25$). Following the 18 weeks, F_1 generation medaka eggs were collected and measured ($n_{control/responder \rightarrow control} = 98$, $n_{control/responder \rightarrow high} = 97$, $n_{control/non\text{-}responder \rightarrow control} = 100$, $n_{control/non\text{-}responder \rightarrow high} = 128$, $n_{high/responder \rightarrow control} = 144$, $n_{high/responder \rightarrow high} = 120$, $n_{high/non\text{-}responder \rightarrow control} = 164$, $n_{high/non\text{-}responder \rightarrow high} = 160$), and filial generation two (F_2) larvae were reared to maturity in their respective parental condition for 24 weeks before reproductive, molecular, and behavioural measures were taken for this generation ($n_{control/responder \rightarrow control \rightarrow control} = 79$, $n_{control/responder \rightarrow high \rightarrow high} = 71$, $n_{control/non\text{-}responder \rightarrow control \rightarrow control} = 67$, $n_{control/non\text{-}responder \rightarrow high \rightarrow high} = 53$, $n_{high/responder \rightarrow control \rightarrow control} = 75$, $n_{high/responder \rightarrow high \rightarrow high} = 28$, $n_{high/non\text{-}responder \rightarrow control \rightarrow control} = 73$, $n_{high/non\text{-}responder \rightarrow high \rightarrow high} = 57$) including egg size ($n_{control/responder \rightarrow control \rightarrow control} = 327$, $n_{control/responder \rightarrow high \rightarrow high} = 188$, $n_{control/non\text{-}responder \rightarrow control \rightarrow control} = 254$, $n_{control/non\text{-}responder \rightarrow high \rightarrow high} = 320$, $n_{high/responder \rightarrow control \rightarrow control} = 270$, $n_{high/responder \rightarrow high \rightarrow high} = 62$, $n_{high/non\text{-}responder \rightarrow control \rightarrow control} = 122$, $n_{high/non\text{-}responder \rightarrow high \rightarrow high} = 244$). A visual representation of respective generations and assignments to elevated CO_2 or control CO_2 conditions can be seen in Figure 3.6.

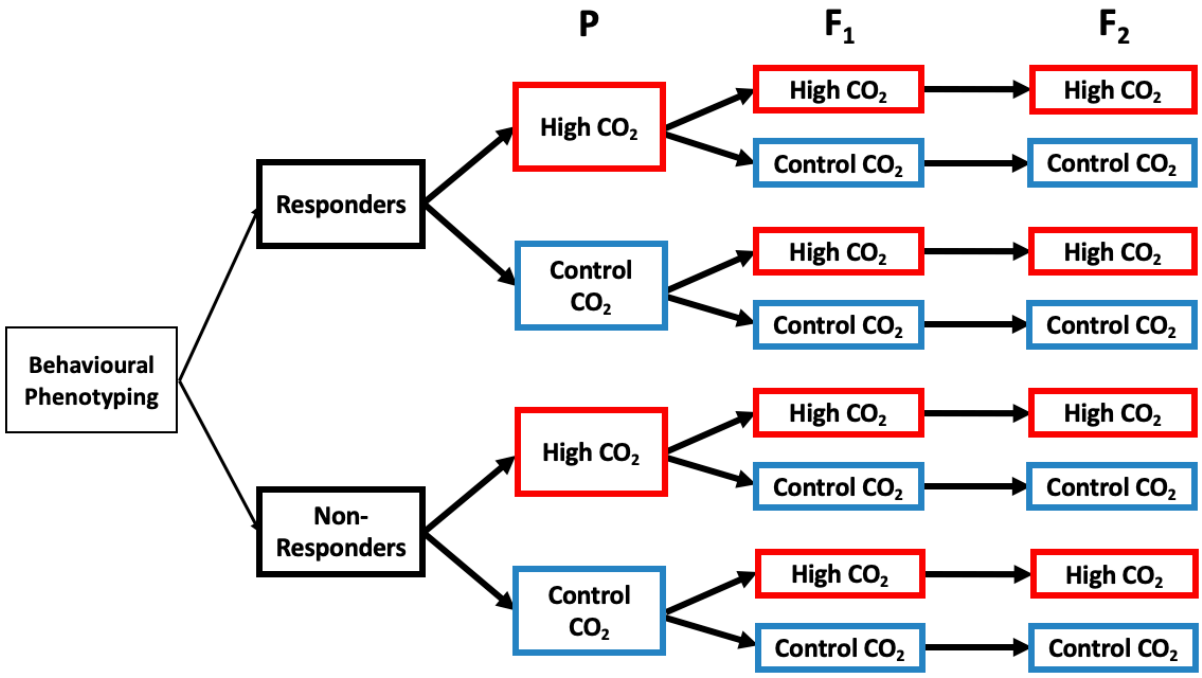


Figure 3.6 Generational overview of experimental design. First and four quartiles of a group of fish were selected based on responses to elevated CO_2 where non-response fish were those that responded on average most closely to the population mean and responders responded most differentially from the population mean. Parental generation (P) fish were then sexed and randomly selected into elevated CO_2 or control CO_2 conditions ($n_{control/responder} = 10$, $n_{control/non-responder} = 10$, $n_{CO2/responder} = 10$, $n_{CO2/non-responder} = 9$). P generation eggs were collected and randomly selected into either high or control CO_2 conditions, where these eggs were hatched and raise until adulthood and made filial generation one (F_1) ($n_{control/responder→control} = 60$, $n_{control/responder→high} = 30$, $n_{control/non-responder→control} = 62$, $n_{control/non-responder→high} = 30$, $n_{high/responder→control} = 31$, $n_{high/responder→high} = 53$, $n_{high/non-responder→control} = 17$, $n_{high/non-responder→high} = 25$). F_1 eggs (filial generation two fish) were hatched and reared until adulthood in the same elevated CO_2 conditions ($n_{control/responder→control→control} = 79$, $n_{control/responder→high→high} = 71$, $n_{control/non-responder→control→control} = 67$,

$n_{control/non\text{-}responder \rightarrow high \rightarrow high} = 53$, $n_{high/responder \rightarrow control \rightarrow control} = 75$, $n_{high/responder \rightarrow high \rightarrow high} = 28$, $n_{high/non\text{-}responder \rightarrow control \rightarrow control} = 73$, $n_{high/non\text{-}responder \rightarrow high \rightarrow high} = 57$).

3.4 Reproductive and developmental outcomes

Medaka eggs from P, F_1, and F_2 generations were collected, and number, size, and survival to hatch rate were assessed to evaluate maternal investment and egg viability. Due to limitations resulting from COVID-19 restrictions, F_2 generation was the endpoint for the study, and a third generation was not reared. As a result, eggs from generation F_2 were not incubated, and thus survival to hatch rate was not collected for this generation. The number of eggs was determined by counting the eggs produced in each tank within a 72-hour or 48-hour period and averaging the eggs per female per day. This collection was repeated three times for each generation for at least two fish tanks. Egg size was measured using a light microscope and its digital ruler to determine diameter in millimetres. To measure survival to hatch for P and F_1 generations, a known number of eggs from each treatment group was added to a larval tube held within the egg generation's prospective CO_2 exposure, and the number of hatched larvae was counted fourteen days following incubation. Survival to hatch success was then calculated by dividing the number of hatched larvae from the known number of eggs that were incubated.

3.5 Novel Tank Behavioural Assays

Following the 6-week exposure for P, the 18-week rearing for adult F_1 generation and the 24-week rearing for F_2, adult fish underwent a novel-tank behavioural assay while in their treatment CO_2 concentration water (P: $n_{control/responder} = 10$, $n_{control/non\text{-}responder} = 10$, $n_{high/responder} = 10$, $n_{high/non\text{-}responder} = 9$, F_1: $n_{control/responder \rightarrow control} = 20$, $n_{control/responder \rightarrow high} = 19$, $n_{control/non\text{-}responder \rightarrow control} =$

20, $n_{control/non\text{-}responder \rightarrow high}$ = 17, $n_{high/responder \rightarrow control}$ = 20, $n_{high/responder \rightarrow high}$ = 20, $n_{high/non\text{-}responder \rightarrow control}$ = 20, $n_{high/non\text{-}responder \rightarrow high}$ = 20; F_2: 10 fish for each group. Additionally, in groups where there were enough fish available, filial generation one and two fish also underwent a novel tank behavioural assay under acute elevated CO_2 conditions (F_1: $n_{control/responder \rightarrow control}$ = 10, $n_{control/responder \rightarrow high}$ = 10, $n_{control/non\text{-}responder \rightarrow control}$ = 11, $n_{control/non\text{-}responder \rightarrow high}$ = 10, $n_{high/responder \rightarrow control}$ = 11, $n_{high/responder \rightarrow high}$ = 10, $n_{high/non\text{-}responder \rightarrow high}$ = 5; F_2: $n_{control/responder \rightarrow control \rightarrow control}$ = 6, $n_{control/responder \rightarrow high \rightarrow high}$ = 6, $n_{control/non\text{-}responder \rightarrow control \rightarrow control}$ = 5, $n_{control/non\text{-}responder \rightarrow high \rightarrow high}$ = 6, $n_{high/responder \rightarrow control \rightarrow control}$ = 6, $n_{high/responder \rightarrow high \rightarrow high}$ = 6, $n_{high/non\text{-}responder \rightarrow control \rightarrow control}$ = 7, $n_{high/non\text{-}responder \rightarrow high \rightarrow high}$ = 6).

Behavioural measures in a novel tank exploratory assay were used to quantify behavioural differences in locomotion (total distance moved), time spent in the peripheral area of the arena (thigmotaxis; Schnörr et al., 2012), and swimming direction. Behavioural measures have previously been found to have significant effects in fish after exposure to high CO_2 and can be important predictors of ecological and evolutionary outcomes mechanisms (Schneider et al., 2018; Tresguerres and Hamilton, 2017). Locomotion is a measure that has been used in many environmental pollutant studies and is a good predictor of toxic effect and the health status and survival of fish (Chiffre et al., 2014). Time spent in the periphery of an arena is a measure of anxiety behaviour and has been measured using similar behavioural arenas (Hamilton et al., 2014; Chiffre et al., 2014; Schnörr et al., 2012). Lateralization was also measured based on swimming direction preference. Behavioural lateralization is the preference to either the right or left directionality and it has been assumed to be related to hemispheric asymmetry in the brain (Lai et al., 2015). Lateralization is thought to allow a fish to focus on two tasks more effectively leading to improved schooling performance, orientation and cognition, and escape behaviour (Lai

et al., 2015; Brown, 2011). In prior research, Chiffre et al., (2014) used pharmaceuticals to demonstrate how biphasic behavioural responses in medaka larvae can be measured using a circular novel tank arena, and a similar arena has also been used to measure thigmotaxis behaviour (Schnörr et al., 2012) and thus a similar arena style was chosen for our behaviour assays.

In the acclimatized CO_2 novel tank behaviour assays, fish were placed in a circular white plastic arena with a diameter of 34 cm and walls ~ 16 cm in height holding 2 L of water from the experimental fish's holding aquarium system (Hamilton et al., 2017). Each fish's behaviour was recorded for 5 min and videos were later tracked using automated tracking software (EthoVision® XT, Noldus, Wageningen, Netherlands) to measure total distance moved, swimming directionality, and time spent in outer zone (periphery) of the behaviour arena.

In the acute CO_2 novel tank assays a separate subset of fish were used. The assay was completed in a similar manner, but the water added to the behavioural arena was treated with CO_2. Specifically, a basin of water from the respective fish's housing aquarium system was treated with compressed CO_2 and maintained at 6.1 pH (± 0.2 pH) using the pH controlling methods described above. The pCO_2 in the basin averaged 9,780 (S.E. = 179.3). A circulation pump (256 gallons/hour) circulated the basin's water to maintain a consistent CO_2 concentration throughout (Aquatop., Brea CA, USA). Water from this CO_2-treated basin was used in the behavioural arena. The pH and CO_2 concentration (ppm) were recorded before the water was added to the experimental arena to ensure consistency across assays.

3.6 Measuring mRNA Abundances

Adult P, F_1, and F_2 fish were euthanized in MS-222 (500 mL aquarium water, 0.5 g sodium bicarbonate ($NaHCO_3$), and 1.0 g tricaine methane sulfonate). Spinal cords were then severed as a secondary euthanasia method. Fish total length and weight were measured, and sex was determined based on gonad observation during each dissection. The gill, liver, and brain tissues were collected from each fish. Gill was collected so that ion transporters responsible for acid-base regulation (i.e., transporters excreting H^+ in exchange for sodium ions (Na^+), and chloride ions (Cl^-) in exchange for bicarbonate (HCO_3^-)) along with those that regulate the chemoelectrical gradient of a cell could be measured to determine what acid-base strategies may be occurring. Liver tissue was collected because the liver is the site for many metabolic functions, such as sugar and fat storage, and is the site of vitellogenin, a precursor protein of egg yolk in medaka (Kinoshita et al., 2009). Brains were collected because the brain, especially the pituitary, is the primary site of regulation of hormones that influence growth, behaviour, reproduction, and stress (Kinoshita et al., 2009). Finally, gallbladders were also dissected for F_1 and F_2 fish because visual difference in the size and coloration of gallbladders were observed during dissection, and gallbladders have been associated with social stress (Earley et al., 2003). Tissues were placed into a 2 mL microcentrifuge tube with 100 µl of RNAlater for RNA preservation, left at 4°C overnight to saturate tissue with RNAlater, and then stored at -80°C. Note, due to the COVID pandemic restrictions, only brain tissues were used for measuring mRNA abundances for this thesis.

Molecular assays in this study were designed and optimized in Dr. Sara Good's laboratory (University of Winnipeg) using the publicly available sequence information in the NCBI (National Center for Biotechnology Information) repository. To obtain 2 µg of RNA, 3 whole-brain samples were pooled to generate pooled "biological" replicates, and 3 biological replicates

were obtained for each CO_2 exposure and phenotype combination for parental generation, for each parental exposure, current exposure, and each sex (male and female) combination for F_1, and for each grandparent CO_2 exposure and phenotype and current CO_2 exposure for F_2 (see appendix Table A.1).

Total RNA was extracted from adult brain tissues using the RNeasy Plus Universal Mini Kit (QIAgen, Toronto, ON, CA) containing QIAzol®, according to the manufacturer's protocol. Briefly, brains were placed in a 2mL round bottom tube containing 900 µl of QIAzol Lysis Reagent and homogenized using a handheld 150 homogenizer (Fisher Scientific) for 40 s at medium speed. To maximize RNA yields, QIAshredders® (QIAgen) was then used following homogenization of the tissues with a handheld homogenizer. Homogenate was left at room temperature (~ 23°C) for 5 minutes before 100 µl gDNA Eliminator Solution and 180 µl chloroform was added to the tube and shaken vigorously for 15 s following the addition of each. Homogenate mixture was left at room temperature for 3 min before being centrifuged at 12,000x g for 15 min at 4°C which separated the mixture into three phases: an upper colorless aqueous phase containing RNA, a white interphase containing DNA, and a red aqueous organic phase, containing proteins. The upper phase (approximately 400 µl) containing RNA was transferred to a new 1.5 mL microcentrifuge tube to which an equal volume of 70% ethanol was then added and mixed by pipetting up and down. Half of the sample was then transferred to a RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 15 s at 12,000x g at room temperature, and the flow-through was discarded. This step was repeated once more after adding the remaining half of the sample. Next 700 µl Buffer, then 500 µl of Buffer RPE, and finally a second 500 µl of RPE was added to the column separately and centrifuged for 2 min at 12,000x g between each edition. RNA was eluted into a new 1.5 mL collection tube twice each time with 30

µl of RNase-free water and centrifuge for 1 min at 12,000x g to create a total RNA collection of 60 µl.

The RNA concentration and purity of each sample were measured using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, see appendix table A.2) and samples were stored at -80°C until use. To ensure high-purity RNA, the absorbance ratio (A260/A280) had to be above at least 1.8 or higher to be used. A260/230 ratios from the samples ranged between 0.777 and 2.29 and with impurities were found to be contaminated with less than 5.5 mM of guanidine impurity. Previous literature has found that concentrations of guanidine, a salt present at high concentration in the extraction process, below 100 mM do not compromise downstream applications (Ahlfen and Schlumpberger, 2010). The extracted RNA samples were converted to complementary DNA (cDNA) using iScript™ cDNA Synthesis Kits (Bio-Rad®). Each reaction had a total starting quantity of 2 µg and the final volume for each reaction was 40 µl. Samples were stored at -20°C and diluted 5-fold before qPCR.

Primers were designed using Primer 3 software (www.primer 3.ut.ee). The parameters for primer design were set so that the product size was between 100–120 base pairs, and the primers spanned introns (exon-exon) to control for genomic DNA contamination. Primers were successfully designed for *cytochrome P450 19b (cyp19b), follicle stimulating hormone beta subunit (fshb), glycoprotein hormones alpha chain (gtha), dopamine receptor (dar), serine hydroxymethlytransferase (shmt2), glucocorticoid receptor alpha (gra), lutenizing hormone beta subunit (lhb), medaka type gnrh (mdgnrh), glutamate decarboxylase 1 (gad1), methyl CpG binding protein 2 (mbd2), gamma-aminobutyic acid receptor subunit alpha-2 (gabra2), mineralcorticotropin hormone receptor (mr), and sodium-hydrogen antiporter 1 (nhe-1)* (see Table 3.2). Primer efficiencies were tested for each primer by performing qPCR on a 5-point 10-fold serial dilution of template cDNA in duplicate. Efficiencies were calculated using the slope of

a standard curve from a plot of the log of dilution factor (x) against the average number of cycles required to pass the threshold concentration (i.e., the number of quantification cycles (C_T). Primers whose efficiencies fell between the acceptable range of 90-110% were included in the study, while those outside of the range were excluded or redesigned (Taylor et al., 2010). Primers *neuropeptide y (npy), cholecystokinin (ccka), insulin-like peptide 5b (insl5b), relaxin 3b (rln3b), relaxin family peptide receptor 3-2a (rxfp-2a),* and *growth hormone (gh)* were designed by Kellie Zelmer and two of these primers (*ccka* and *rln3b*) fell outside the 90–110 % efficiency range (Zelmer, 2015).

The relative abundance of mRNA of selected genes in the brains of adult fish from parental generation and F_1 and F_2 were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). Medaka genes were measured for mRNA expression using qRT-PCR in duplicate for each sample. Using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), each reaction had a total volume of 15µl composed of 0.5µl cDNA, 0.5 µl 10 µM forward primer, 0.5 µl 10µM reverse primer, 1.5 µl water, and 7.5 µl SYBR. Reactions were prepared in Bio-Rad Hard-Shell® 96-Well thin wall white PCR Plates and sealed with Bio-Rad Microseal® 'B' Adhesive Optical Seals. The sealed plates were then run on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The thermal cycling conditions were determined according to previously used standard qRT-PCR protocols (see Table 3.3).

Table 3.2 Gene abbreviation, designed primer sequence, accession number and measured efficiency for genes measured.

Gene Name	Gene	Primer Sequence 5'-3'	Accession number	Efficiency
Cytochrome P450 19B	CYP19B	F: TCAGAGCGATTGTGGTGGAC R: TGCTTGCCAGGTCTCAAAGT	ENSORLT00000006986.2	96%
Follicle Stimulating Hormone beta subunit	FSHb	F: TGCGTCCACACCACCATATG R: CAGTCCCCACTGCAGATCTT	ENSORLT00000044905.1	95%
Glycoprotein hormones alpha chain	GTHa	F: CTTCTTTGCACAGCCGACAC R: GGTAGACCGGTTTACCTTCCC	ENSORLT00000031582.1	94%
Dopamine D2 type receptor	DA R	F: TCAGGCTGTGCACCATGATT R: GCTCCAGATCTTCCCCATCG	ENSORLT00000009424.2	94%
serine hydroxymethyltransferase 2	shmt2	F: CACAAGTCCCTCAGAGGAGC R: CGGAGAAGTTGACCCTGTCC	ENSORLT00000006971.2	103%
glucocorticoid receptor alpha	GRa	F: GGGCTGGAGGTCTTACCAAC R: TTGCTCAAACTGGTCAGCCA	ENSORLT00000039061.1	102%
luteinizing hormone beta subunit	LHb	F: CCCGGGTTAGCAGAGTGATG R: TTGACTGGCTGGCAGTAAGG	ENSORLT00000004425.2	104%
medaka type gonadotropin-releasing hormone	mdGnRH	F: CTGGAGGGAAGCGAGAACTG R: CTCAAGTCACTGCAGGGTGT	ENSORLT00000017870.2	97%
glutamate decarboxylase 1	GAD1	F: CAGATCCTGGTGGACTGCAG R: CTCCTGCCAACCCTACGATG	ENSORLT00000021606.2	103%
methyl CpG binding protein 2	MBD2	F: GCTGGAAAGAGCGACGTCTA R: GCGGAAATCGAAACATGCCA	ENSORLT00000040966.1	94%
gamma-aminobutyric acid receptor subunit alpha-2	GABRA2	F: ACCCTGTTCACACGCATCTT R: GGTCCAAAGCTGGTGACGTA	ENSORLT00000011902.2	97%
mineralcorticotropin hormone receptor	MR	F: AACAACACCATGCCCACATC R: ATGCCGAGTGAATGACATCG	ENSORLT00000042724.1	95%

sodium-hydrogen antiporter 1	NHE-1	F: GAGCTGCTGCATATCCTGGT R: ACTGTTCCTTCGTGGGCAAA	ENSORLT00000035013.1	104%
Neuropeptide Y	NPY	F:GCCTTGGAGCCTTAACAGAGG R: TCTCAGGACTGGACCTCTTC		99%
Cholecystokinin	CCKa	F:GAATCAGCCCCAGAACAGCCT R: AGGATCTGGTCTGGAGGGAT	ENSORLG00000005949	117%
Insulin-like 5b	insl5b	F: TTCTGAGGGCGTTGGTGT R: GGTGCCGCTGTTCTCTTCT	No ID	109%
Relaxin 3b	rln3b	F: GGAGGTTCAAGATGGAAACG R: ACGCTCCACCACAAAGTTCT	ENSORLG00000011777	113%
Relaxin Family Peptide Receptor 3 2a	rxfp3-2a	F: TTCCCCCTGACTGTGTGTCT R: CCCGGCTCTGTACRCTCTTC	ENSORLG00000014985	99%
Growth Hormone	GH	F: GGACCTACGAACTGCTAGCT R: TAGCCACAGTCAGGTAGGTC	ENSORLG00000019556	98%
Beta-actin	ß-actin	F: TCCACCTTCCAGCAGATGTG R: AGCATTTGCGGTGGACGAT	DQ118296	101%
Ribosomal Protein L7	RPL-7	F:CGCCAGATCTTCAACGGTGTA T R:AGGCTCAGCAATCCTCAGCAT	S74868	105%

Table 3.3 RT-PCR thermal cycling conditions.

Phases	Temperature (°C)	Time
1. Initial Denaturation	95	2 min
2. Denaturation	95	5 sec
3. Annealing and Extension	60	30 sec
4. Plate Read		
5. Repeat steps 2-5, 39 times		

A no-template control (NTC), containing no cDNA, was used with each gene to control for contamination and primer dimers. QRT-PCR was also used to quantify levels of mRNA of the two reference genes, RPL-7 and ß-actin, which were identified by Zhang and Hu (2007). The relative fold difference in mRNA abundance of a gene was measured using an adapted $2^{-\Delta}$CT method (Livak and Schmittgen, 2001) where the expression of a gene of interest was quantified relative to the reference genes. Specifically, the number of cycles required to pass the threshold concentration (C_T) of RPL-7 and and ß-actin was averaged for each group of fish, and this number was subtracted from the C_T values of each sample for a gene of interest. A calibrator was not used as a second relative comparison so that directionality of differential expression relative to the reference genes could be more accurately considered.

3.7 Data analysis

For gene expression, broad patterns of differential transcript expression between groups were analyzed using principal components analysis (PCA) performed using GraphPad Prism version 9.1.1 for Macintosh (San Diego, California USA). Principal components (PCs) that

contributed to 85% of total explained variance of relative gene abundance was used in further analysis. Principal components, reproductive responses, body condition measures, and behavioural responses were all analyzed using multiple linear regression models using GraphPad Prism version 9.1.1 for Macintosh (San Diego, California USA). Multiple linear regression models were conducted for current CO_2 exposure condition of fish, the parent or grandparent CO_2 exposure condition, interaction effect of current CO_2 exposure and parent or grandparent CO_2 exposure conditions, and finally current, parent or grandparent behaviour phenotype. Sex effects were measured for PCs for generation one, and current CO_2 exposure and a behavioural phenotype interaction was considered for parental generation fish. Assumptions for model normalities and variance was tested for using Anderson-Darling, D'Agostino-Pearson omnibus, Shapiro-Wilk, and Kolmogorov-Smirnov tests. If a data set did not meet normalities, the data was log or square root (sqrt) transformed. If the data still did not meet normalities, a non-parametric rank transformation was used. The level of significance (α) was 0.05. Data figures for significant models were all created using RStudio Team (2018).

When a model was found to be significant, TukeyHSD tests were used to compare groups of interest (Bates, 2010; RStudioTeam, 2018). Comparisons of interest in parental generation was current CO_2 exposure and behavioural phenotype interaction effects. For F_1 generation, developmental effects were measured by comparing P control CO_2 → F_1 control CO_2 fish with P control CO_2 → F_1 high CO_2 to measure effects of exposure to high CO_2 during rearing in the absence of parental exposure. Parental only exposure effects were measured by comparison P control CO_2 → F_1 control CO_2 fish with fish whose parents were exposed to elevated CO_2, but who were not exposed to elevated CO_2 themselves (P high CO_2 → F_1 control CO_2). Two generation additive effects were measured for parental plus developmental exposure effects by comparing F_1 control-control fish with fish who had themselves as well as their parents had both

been exposed to elevated CO_2 conditions (P high CO_2 → F_1 high CO_2). In filial generation two (F_2) multiple generation exposure to high CO_2 was explored by comparing fish exposed to chronic high environmental CO_2 over three generations (P high CO_2 → F_1 high CO_2 → F_2 high CO_2) with F_2 control fish (P control CO_2 → F_1 control CO_2 → F_2 control CO_2). Transgenerational effects of exposure to high CO_2 was also measured by comparing fish whose grandparents had been exposed to elevated CO_2, but whose parents were not exposed to elevated CO_2 and who had not been exposed to CO_2 exposure themselves (P high CO_2 → F_1 control CO_2 → F_2 control CO_2) with F_2 control fish (P control CO_2 → F_1 control CO_2 → F_2 control CO_2). Each experimental comparison will also compare the CO_2 responder behavioural phenotype lineage to the CO_2 non-responder lineage to understand the importance of parental phenotype in offspring responses to elevated CO_2.

Chapter 4

Results

Chapter 4: Results

4.1 Parental Generation Phenotyping

Behavioural phenotyping was completed for 80 fish. The twenty fish with the lowest average chi-square values (non-responsive) for behavioural assay variables and the twenty fish with the highest average chi-square values (responsive) were selected as parental generation (P). An ANOVA test comparing the difference in chi-square values found significant difference between assigned responsive and non-responsive behavioural phenotype fish ($F_{1,37} = 48.68$; $p < 0.0001$; Figure 4.1). An example of a behaviour captured for the shuttle-box assay was that fish with responsive phenotypes spent either the most or the least amount of time in the elevated CO_2 exposure tank, while non-responsive fish spent average time in the elevated CO_2 tank (see Appendix, Figure A.1). A novel tank behaviour was that fish of responsive behavioural phenotype spent either the most or the least amount of time at the bottom of the tank, while non-responder fish spent an average amount of time at the bottom of the tank (see Appendix Figure A.2).

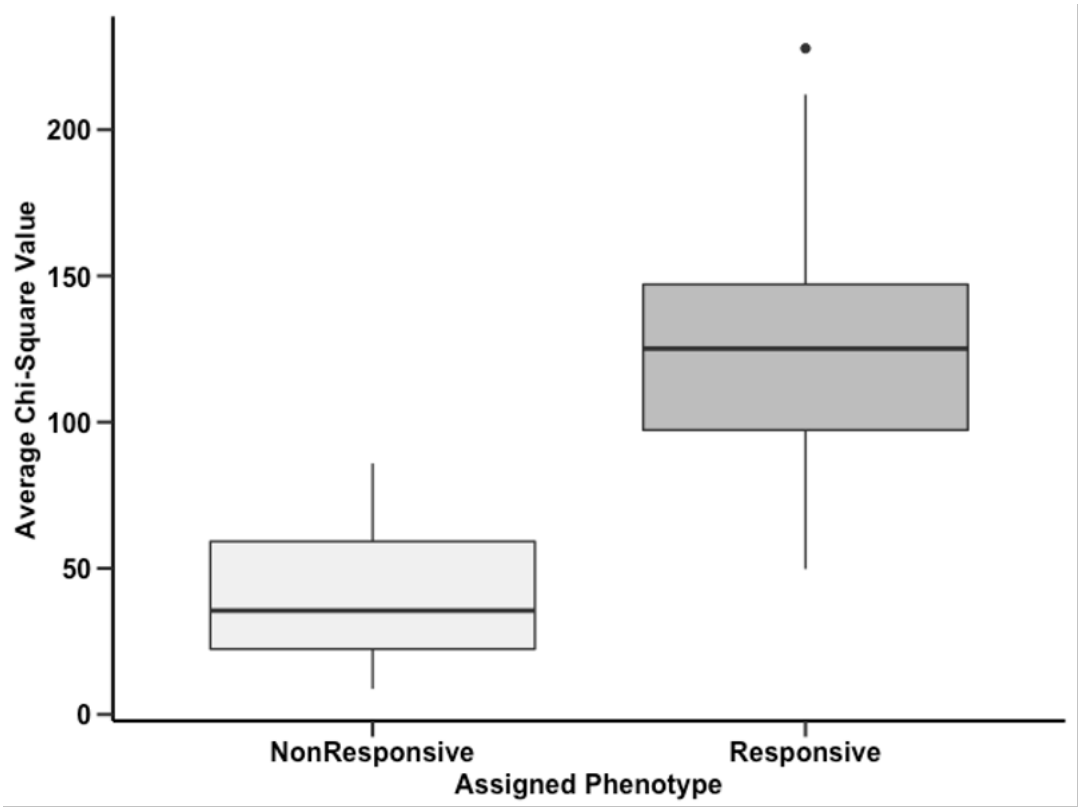


Figure 4.1 Parental generation (P) average chi-square value by assigned behavioural phenotype, an ANOVA calculated for these values for responsive and non-responsive found significant differences between these groups($F_{1,37} = 48.68$; $P < 0.0001$).

4.2 Parental Generation and 6-week CO_2 Exposure

Following a 6-week CO_2 exposure, growth and development measures (length, weight, size (weight/length)), behaviour (total distance moved, swimming direction, time spent in outer versus inner zone of arena), reproductive outputs (egg size, number of eggs per female per day, hatch success), and the relative abundance of various mRNA transcripts in whole brain tissue were measured in fish from the P generation. To assess possible effects, statistical models compared current CO_2 exposure, behavioural phenotype, and a current CO_2 and behavioural phenotype interaction term.

Growth and developmental measures did not significantly differ for CO_2 exposure or behavioural phenotype in the P generation (Table 4.1). The average weight of all P generation fish was 0.35 g (S.E. ± 0.12). The average length for parental fish was 3.09 cm (S.E. ± 0.31). The average size for parental fish was 0.026 g/cm (S.E. ± 0.026).

Behavioural measures, total distance moved and time spent in the outer zone, did not significantly differ by CO_2 exposure or behavioural phenotype (Table 4.2). There was a significant interaction effect for directionality; however, the full model was not significant ($F_{3,35}$ = 1.887; P = 0.1497; Figure 4.2) and no trend was discernable between any groups (Tukey HSD; $P < 0.05$). The average total distance moved for fish in parental generation was 926.3 cm (S.E. ± 340.1). The average percentage of time spend in the outer zone of the arena was 89.7 % (S.E. ± 12.7). The average direction parental generation fish swam 73.3° (S.E. ± 13.1).

Reproductive measures, egg size and average eggs per female, had significant variance in the P generation (Table 4.3). Parental generation egg size significantly differed by current CO_2 exposure but was dependent on behavioural phenotype ($F_{3,752}$ = 18.22; P = < 0.0001; Table 4.3; Figure 4.3). For fish in control CO_2 conditions, fish with a responder phenotype had eggs that were on average 3 % larger in diameter than the non-responder phenotype, while non-responder

fish had 2 % smaller in diameter eggs when in control conditions (Table 4.3; Figure 4.3). The interaction between current CO_2 and behavioural phenotype was significant for number of eggs per female ($F_{3,4} = 10.82$; $P = 0.0217$; Figure 4.4). Non-responder fish held in elevated CO_2 conditions had 72 % less eggs per female per day on average relative to fish held in control CO_2 conditions (TukeyHSD; $P = 0.0191$). No significant effects were found post-hatch survival in the P generation. The average hatch success rate for the parental generation was 65.4 % (S.E. ± 4.5) and the average eggs per female per day for parental generation fish was 5.1 (S.E. ± 2.3).

Four principal components were found to explain 85 % proportion of variance in the P generation's relative mRNA abundances (Table 4.4). Specifically, PC1 explained 46.71 % of variance in the dataset, PC2 explained 11.38 %, PC3 explained 5.79 %, and PC4 explained 5.45 %. Current CO_2 exposure, behavioural phenotype, and the interaction term had no significant effect on PC1, PC3, or PC4. PC2 significantly differed by current CO_2 exposure ($F_{3,8} = 1.959$, $P = 0.1988$; Table 4.5). Genes with loading factors $\geq \pm 0.5$ that were considered to have significant contribution to PC2 variance included genes associated with the reproductive axis and growth and feeding related genes (Table 4.4). Specifically, fish in elevated CO_2 had reduced relative abundances of mRNA for *medaka type gonadotropin-releasing hormone* (*mdgnrh*) and *relaxin 3b* (*rln3b*), and increased relative abundances of mRNA for *follicle stimulating hormone beta subunit* (*fshb*), *lutenizing hormone beta subunit* (*lhb*), *glycoprotein hormones alpha chain* (*gtha*), *dopamine receptor* (*dar*), and *growth hormone* (*gh*) (Table 4.5); however, no statistical differences were found for these genes and current CO_2 exposure when they were independently analyzed (Figure 4.5).

Table 4.1 Parental (P) generation multiple linear regression model statistical outputs for length, weight, and size by current CO_2 exposure level, behavioural phenotype, and an interaction term. Weight ($F_{3,35} = 0.2747$; $P = 0.8433$), length ($F_{3,35} = 0.8189$; $P = 0.4922$), and size ($F_{3,35} = 0.2822$; $P = 0.8379$) of P generation fish measured.

Variable/Term	Estimate	Standard error	|t|	*P* value
Total Length (cm)				
Intercept	**3.030**	**0.09917**	**30.55**	**<0.0001**
Current CO_2 exposure	0.1700	0.1441	1.180	0.2460
Phenotype	0.1000	0.1402	0.7130	0.4805
Current CO_2 : Phenotype	-0.3000	0.2011	1.492	0.1447
Weight (g)				
Intercept	**-0.4803**	**0.04374**	**10.98**	**<0.0001**
Current CO_2 exposure	0.03336	0.06356	0.5248	0.6030
Phenotype	-0.005059	0.06186	0.08178	0.9353
Current CO_2 : Phenotype	-0.05175	0.08869	0.5835	0.5633
Size (g/cm)				
Intercept	**0.1113**	**0.008462**	**13.15**	**<0.0001**
Current CO_2 exposure	0.005901	0.01230	0.4799	0.6343
Phenotype	-0.003520	0.01197	0.2942	0.7704
Current CO_2 : Phenotype	-0.006683	0.01716	0.3895	0.6993

Table 4.2 Parental generation (P) multiple linear regression model statistical outputs for total distance moved, percent time spent in outer zone of the novel tank, and average directionality by current CO_2, behavioural phenotype and an interaction term.

Variable/Term	Estimate	Standard error	$\|t\|$	*P* value
Total Distance Moved (cm)				
Intercept	**1014**	**108.9**	**9.311**	**<0.0001**
Current CO_2	-207.6	154.0	1.348	0.1863
Phenotype	-99.33	154.0	0.6449	0.5232
Current CO_2 : Phenotype	267.9	220.8	1.213	0.2331
Time Outer Zone (%)				
Intercept	**75.03**	**4.081**	**18.38**	**<0.0001**
Current CO_2	-7.820	5.772	1.355	0.1842
Phenotype	-2.415	5.772	0.4184	0.6782
Current CO_2 : Phenotype	14.26	8.275	1.723	0.0938
Directionality (°)				
Intercept	33.06	34.99	0.9449	0.3512
Current CO_2	-71.84	49.48	1.452	0.1554
Phenotype	-48.11	49.48	0.9724	0.3375
Current CO_2 : Phenotype	**158.3**	**70.94**	**2.232**	**0.0321**

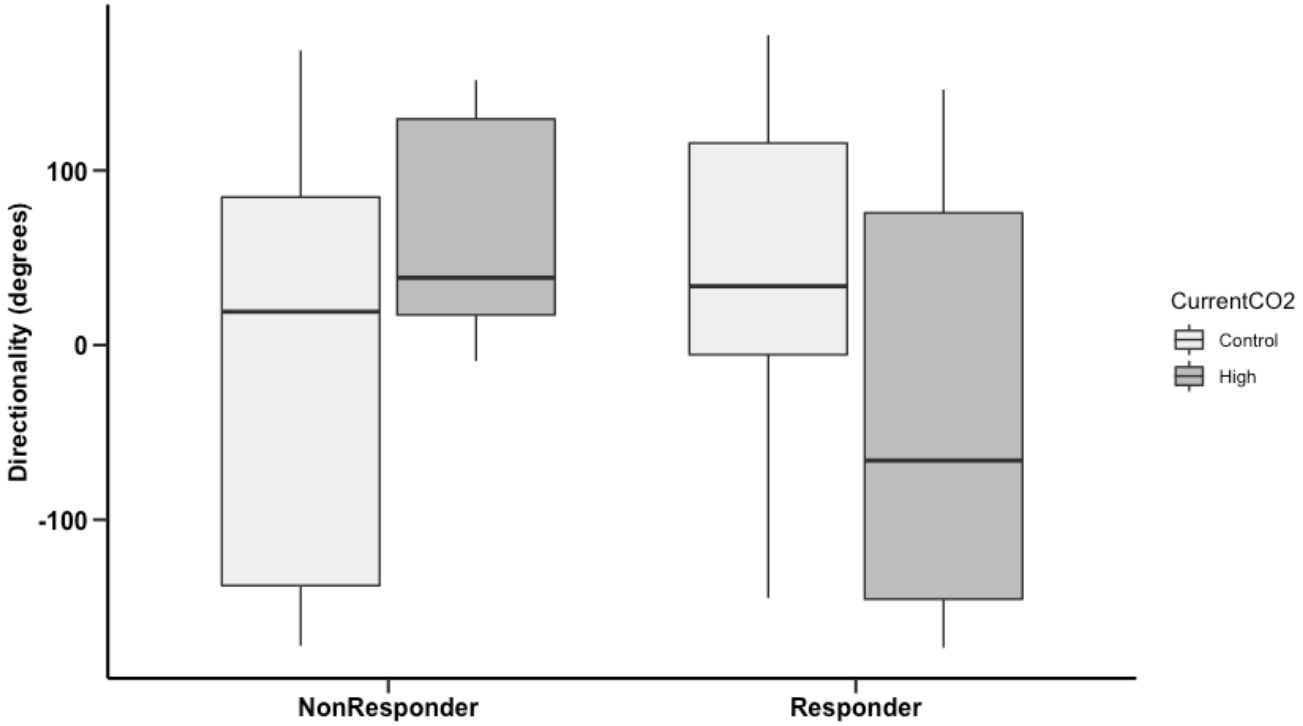


Figure 4.2 Parental (P) generational box plot for directionality by the interaction of behavioural phenotype and current CO_2 exposure ($F_{3,35} = 1.887$; $P = 0.1497$). No discernable trend was found (Tukey HSD; $P = 0.05$).

Table 4.3 Parental (P) generation multiple linear regression model statistical outputs for egg size, average eggs per female, and hatch success by current CO_2 exposure, behavioural phenotype, and an interaction term.

Variable/Term	Estimate	Standard error	$\|t\|$	*P* value
Egg Size (rank) Variables				
Intercept	**409.5**	**26.59**	**15.40**	**<0.0001**
Current CO_2 Exposure	**-82.06**	**29.33**	**2.798**	**0.0053**
Phenotype	**-67.18**	**31.48**	**2.134**	**0.0331**
Current CO_2 : Phenotype	**194.1**	**36.41**	**5.331**	**<0.0001**
Average Eggs Per Female Variables				
Intercept	**2.038**	**0.6842**	**2.978**	**0.0408**
Current CO_2 Exposure	**2.869**	**0.9677**	**2.965**	**0.0414**
Phenotype	**5.238**	**0.9677**	**5.413**	**0.0056**
Current CO_2 : Phenotype	**-4.019**	**1.368**	**2.937**	**0.0425**
Hatch Success Variables				
Intercept	**61.50**	**15.25**	**4.034**	**0.0157**
Current CO_2 Exposure	8.500	21.56	0.3942	0.7135
Phenotype	7.000	21.56	0.3247	0.7617
Current CO_2 : Phenotype	-15.50	30.49	0.5083	0.6380

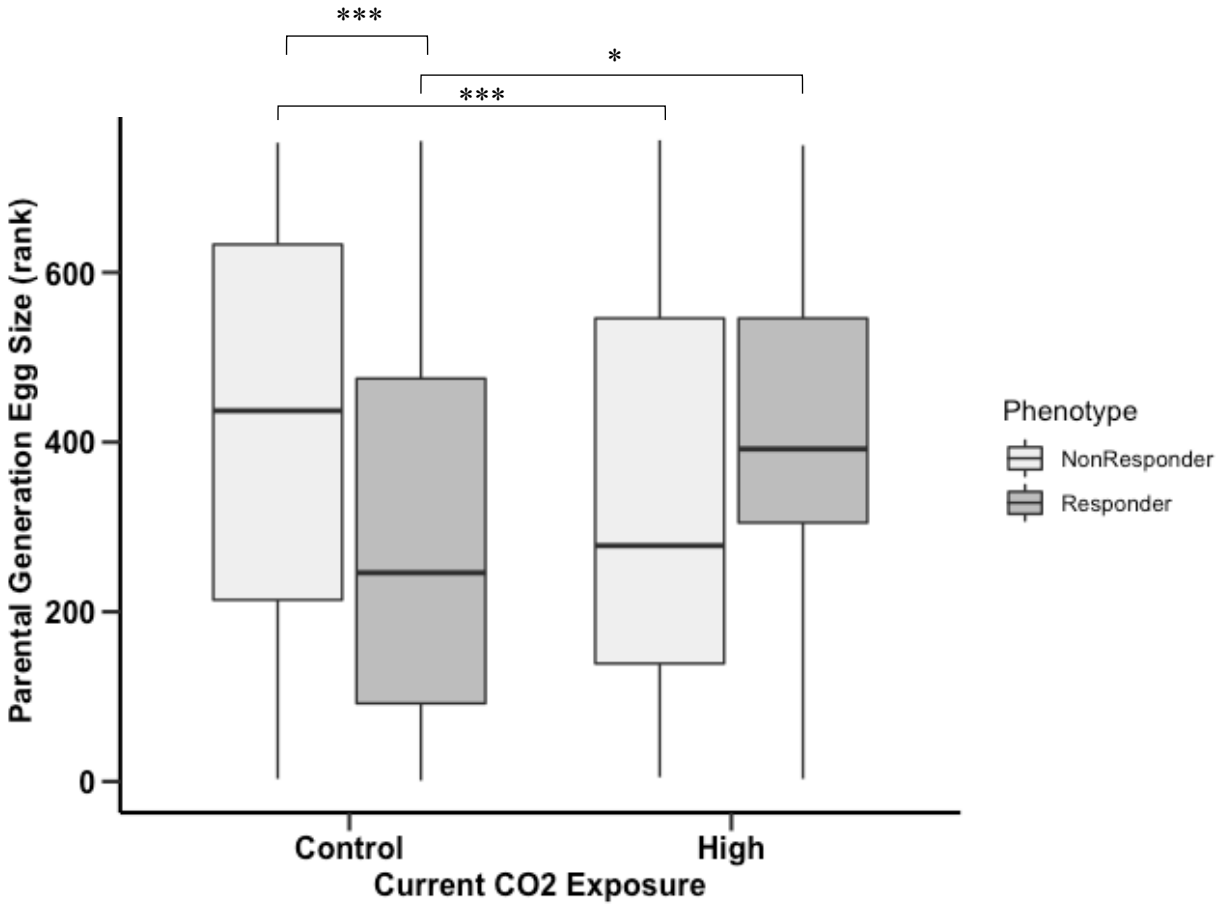


Figure 4.3 Parental (P) generation boxplot for egg size (diameter) by current CO_2 exposure and phenotype interaction term ($F_{3,752} = 18.22$; $P = < 0.0001$). Control CO_2 exposure fish significantly differed by responder or non-responder behavioural phenotype (TukeyHSD; $P < 0.0001$). Responder behavioural phenotype fish differed when exposed to elevated CO_2 or control CO_2 exposure condition (TukeyHSD; $P < 0.0001$). Non-responder behavioural phenotype fish also had significantly different egg diameter when exposed to elevated CO_2 exposure conditions, versus control CO_2 conditions (TukeyHSD; $P = 0.0292$). Asterisks and connecting bars indicate statistical differences between the boxplots.

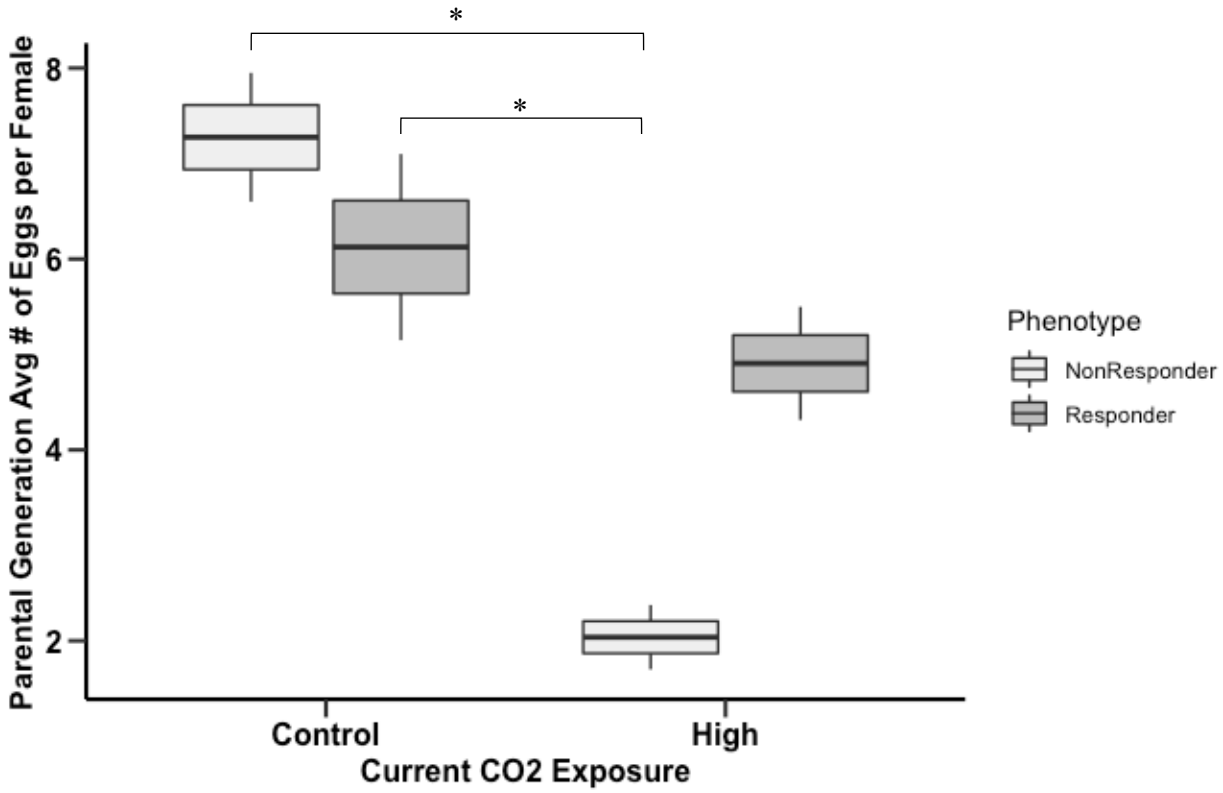


Figure 4.4 Parental (P) generation boxplot for number of eggs per female by current CO_2 exposure and phenotype interaction term ($F_{3,4} = 10.82$; $P = 0.0217$). Non-responder behavioural phenotype fish number of eggs per female per day differed significantly when exposed to elevated CO_2 conditions versus control CO_2 conditions (TukeyHSD; $P = 0.0191$). Fish with responder behavioural phenotypes held in control CO_2 conditions significantly differed from fish with non-responder behavioural phenotypes held in elevated CO_2 conditions (TukeyHSD; $P = 0.0444$). Asterisks and bars indicate statistical differences between the boxplots.

Table 4.4 Parental (P) generation loading factors for relative abundance of genes in whole brain tissue of adult medaka fish selected based on 85% percent of total explained variance ($F_{3,8}$ = 1.959, P = 0.1988). Variables with factor loadings ≥ ±0.5 are shown in bold.

Gene	PC1	PC2	PC3	PC4
cyp19b	-0.4692	0.211	**-0.801**	0.069
npy	-0.482	-0.395	-0.084	**-0.716**
fshb	-0.258	**0.692**	**0.652**	0.014
gtha	-0.268	**0.775**	**0.552**	-0.035
dar	**-0.601**	**0.607**	-0.238	-0.170
shmt2	**-0.528**	-0.257	0.328	0.191
gra	**-0.965**	-0.129	0.091	-0.003
ccka	**-0.639**	0.018	0.379	0.042
insl5b	**-0.662**	0.140	-0.289	0.133
rln3b	**-0.535**	**-0.639**	0.316	0.213
rxfp3-2a	**-0.955**	0.055	0.008	0.061
gh	-0.059	**0.730**	-0.411	0.038
lhb	-0.207	**0.854**	0.177	-0.305
mdgnrh	**-0.552**	**-0.611**	0.155	-0.474
gad1	**-0.937**	0.143	-0.181	-0.070
mbd2	**-0.940**	0.091	-0.088	0.267
gabra2	**-0.951**	-0.200	-0.125	-0.006
mr	**-0.948**	0.196	-0.005	-0.020
nhe1	**-0.868**	-0.360	0.0384	0.227

Table 4.5 Parental (P) generation multiple linear regression outputs for principal components explaining 85% of variance, for current CO_2, phenotype, and a current CO_2 and phenotype interaction term.

Variable/Term	Estimate	Standard error	$\|t\|$	*p* value
PC1				
Intercept	1.153	1.813	0.6363	0.5424
Current CO2	-1.002	2.563	0.3907	0.7062
Phenotype	-3.230	2.563	1.260	0.2432
Current CO2 : Phenotype	3.850	3.625	1.062	0.3193
PC2				
Intercept	-1.349	1.032	1.307	0.2276
Current CO2	**3.380**	**1.460**	**2.315**	**0.0493**
Phenotype	0.7995	1.460	0.5475	0.5989
currentCO2 : Phenotype	-2.963	2.065	1.435	0.1892
PC3				
Intercept	0.2962	0.9479	0.3124	0.7627
Current CO2	0.2470	1.341	0.1843	0.8584
Phenotype	-0.7520	1.341	0.5609	0.5902
Current CO2 : Phenotype	-0.1748	1.896	0.09220	0.9288
PC4				
Intercept	-0.2672	0.6786	0.3938	0.7040
Current CO2	0.1759	0.9596	0.1833	0.8591
Phenotype	0.1235	0.9596	0.1287	0.9008
Current CO2 : Phenotype	0.4699	1.357	0.3462	0.7381

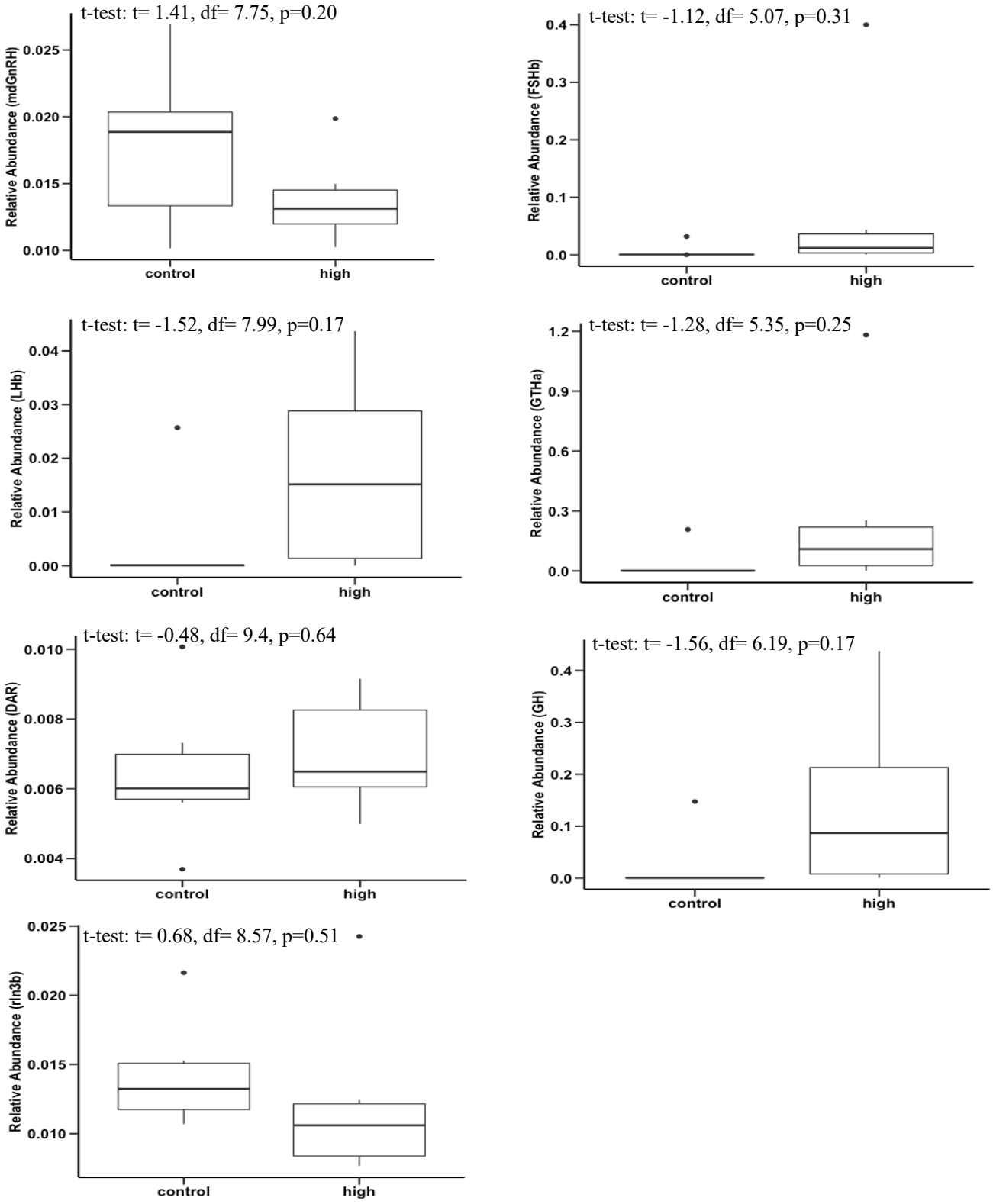


Figure 4.5 Relative abundance of principle component two genes for the parental (P) generation by current CO_2 exposure. Student's t-test results are included for these genes; *medaka type*

gonadotropin-releasing hormone (*mdgnrh*), *follicle stimulating hormone beta subunit* (*fshb*), *lutenizing hormone beta subunit* (*lhb), glycoprotein hormones alpha chain* (*gtha*), *dopamine receptor* (*dar*), *growth hormone* (*gh*), and *relaxin 3b* (*rln3b*).

4.3 Filial Generation One

Filial generation one fish were held in their respective CO_2 conditions for ~18 weeks, from egg and embryo development through to adulthood when growth and development measures (length, weight, size, sex ratio), behaviour (total distance moved, swimming direction, time spent in outer versus inner zone of arena), reproductive outputs (egg size, number of eggs per female per day, hatch success), and the relative abundance of various mRNA transcripts in whole brain tissue was measured.

Weight, Length, Size, and Sex Ratio

Growth was influenced by CO_2 exposure (Table 4.6). The length of F_1 fish significantly differed by the interaction of current CO_2 and parental CO_2 exposure ($F_{4,303} = 17.30$; $P < 0.0001$; Table 4.6; Figure 4.6). Fish who were exposed to elevated CO_2 but whose parents were not exposed to CO_2 (developmental exposure) were on average 7 % smaller. Fish whose parents had been exposed to elevated CO_2, but who were not currently exposed to elevated CO_2 (parental only exposure) were 10% shorter than control fish. Weight of F_1 fish differed significantly by the current CO_2 and parental CO_2 exposure interaction term ($F_{4,303} = 26.64$; $P < 0.0001$; Table 4.6; Figure 4.7). Developmental exposure fish, who were exposed to elevated CO_2 in F_1 but not P generation, were 16 % heavier than control CO_2 exposure fish. Fish whose parents were exposed to elevated CO_2 but who had not themselves been exposed to elevated CO_2 (parental only exposure) were 30 % heavier than control CO_2 exposure fish. Fish who were exposed to elevated

CO_2 throughout developmental and whose parents had also been exposed to elevated CO_2 (developmental and parental exposure) were 13 % heaver relative to control CO_2 exposure fish. Fish with non-responsive behavioural phenotype parents were on average 10 % heavier than fish with responsive behaviour phenotype parents (Figure 4.9). F_1 size (weight:length) significantly differed by both parent phenotype (Figure 4.10) and the current CO_2 and parental CO_2 exposure interaction term ($F_{4,303} = 24.61$; $P < 0.0001$; Table 4.6; Figure 4.8). Fish held in elevated CO_2 conditions through development but whose parents were not exposed to elevated CO_2 had a 14 % higher weight to length ratio, fish with parental only elevated CO_2 exposure resulted in a 31 % higher weight to length ratio, and fish whose parents had been exposed to elevated CO_2 and who had also been exposed to elevated CO_2 throughout developmental had a 12 % higher weight to length ratio relative to control CO_2 exposure fish. Fish with parents of non-responsive behaviour phenotype had an 9 % higher weight to length ratio relative to fish with parents of responsive behaviour phenotype (Figure 4.10).

The female to male ratio of F_1 fish was not significantly different by current CO_2 exposure, parental phenotype, parental CO_2 exposure, or current CO_2 and parent CO_2 exposure interaction term ($F_{4,3} = 1.030$; $P = 0.5108$; Table 4.6).

Behaviour

Percent time spent in outer zone and swimming directionality did not significantly differ by current CO_2 exposure, parent CO_2 exposure, parent phenotype or the interaction of parent CO_2 exposure and current CO_2 exposure for F_1 generation fish (Table 4.7). Total distance moved significantly differed by parental phenotype whereby F_1 fish with responder parents moved 22 % more by distance compared to non-responder fish (Figure 4.11). Total distance moved did not differ by current CO_2 exposure, parental CO_2 exposure, or the interaction term of current and

parent CO_2 exposure ($F_{4,151} = 2.813$; $P = 0.0274$; Table 4.7). The average distance moved for F_1 generation fish was 610.2 cm (S.E. ± 340.1). The average time spent in the outer zone of the behavioural arena was 77.5% (S.E. ± 22.3). The average direction a F_1 fish swam was -0.19° (S.E. ± 7.75).

Acute CO_2 exposure in a behaviour assay of F_1 fish did not result in any differences for any term (Table 4.8). In this assay, F_1 fish on average moved 572.2 cm (S.E. ± 274.8) during acute CO_2 exposure behaviour assay, and on average spent 81.8% of their time (S.E. ± 23.0) in the outer zone of the arena during their acute CO_2 exposure behaviour assays. F_1 fish on average swam in an -8.5 degree directionality (S.E. ± 99.9) during their acute CO_2 exposure behaviour assays.

Reproduction

Filial generation one (F_1) reproductive measures did not significantly differ for any measure by any term (Table 4.9). The average egg diameter for F_1 generation fish was 1.26 cm (S.E. ± 0.05). The average eggs produced per female per day for F_1 was 10.7 (S.E. ± 2.4). Hatching success was 62% (S.E. ± 23%).

Relative mRNA Abundance

Principal component analysis of relative mRNA abundance quantified in F_1 fish resulted in five PCs that contributed to 85 % of total explained variance, cumulating a total of 85.9 2% variance (Table 4.10). PC1 contributed a 39.25 % proportion of variance, PC2 19.81 %, PC3 12.88 %, 7.87 % and PC5 6.11 %. Current CO_2 influenced F_1 PC1 ($F_{4,19} = 10.54$; $P = 0.0001$; Table 4.11). Genes with loading factors ≥ ±0.5 in PC1 included reproductive associated genes, behaviour related genes, growth and feeding related genes, as well as *gra* and *mbd2* (Table 4.10;

Figure 4.12). PC1 genes in fish exposed to elevated CO_2 had reduced relative mRNA abundance for genes *lutenizing hormone beta subunit* (*lhb*), and *glycoprotein hormones alpha chain* (*gtha*), *growth hormone* (*gh*), and an elevated relative mRNA abundance for genes *mineralcorticotropin hormone receptor* (*mr*), *gamma-aminobutyic acid receptor subunit alpha-2* (*gabra2*), *glutamate decarboxylase 1* (*gad1*), *serine hydroxymethlytransferase* (*shmt2*), *growth hormone* (*gh*), *relaxin family peptide receptor 3-2a* (*rxfp-2a*), *sodium-hydrogen antiporter 1* (*nhe-1*), *glucocorticoid receptor alpha* (*gra*), and *methyl CpG binding protein 2* (*mbd2*). Individual t-tests were conducted for each of these genes, and all differences were significant for current CO_2 exposure, with exception of *gh, rxfp3-2a, lhb* and *gtha* (Figure 4.12).

Sex influenced PC4 ($F_{4,19}$=3.311; P=0.0322; Table 4.11). The sole gene in PC4 with a loading factor $\geq \pm 0.5$ was *insulin-like peptide 5b* (*insl5i*), which was higher in relative mRNA abundance for female fish (Table 4.10; Figure 4.13).

Table 4.6 Filial generation one (F_1) multiple linear regression model statistical outputs for length, weight, size, and sex ratio by current CO_2 exposure level, parent behavioural phenotype, parent CO_2 exposure, and a current CO_2 exposure level and parent CO_2 exposure interaction term.

Variable/Term	Estimate	Standard error	$\|t\|$	*P* value
Length (rank(cm))				
Intercept	**130.3**	**8.593**	**15.16**	**<0.0001**
Current CO_2 Exposure	**51.99**	**12.64**	**4.113**	**<0.0001**
Parent Phenotype	-16.29	9.352	1.741	0.0826
Parent CO_2 Exposure	**110.4**	**13.85**	**7.970**	**<0.0001**
Current CO_2 : Parent CO_2	**-137.3**	**19.44**	**7.064**	**<0.0001**
Weight (log(g))				
Intercept	**-0.6311**	**0.01110**	**56.87**	**<0.0001**
Current CO_2 Exposure	**0.07667**	**0.01633**	**4.696**	**<0.0001**
Parent Phenotype	**-0.05720**	**0.01208**	**4.736**	**<0.0001**
Parent CO_2 Exposure	**0.1685**	**0.01789**	**9.416**	**<0.0001**
Current CO_2 : Parent CO_2	**-0.1824**	**0.02511**	**7.266**	**<0.0001**
Size (log(g/cm))				
Intercept	**-1.050**	**0.008803**	**119.3**	**<0.0001**
Current CO_2 Exposure	**0.05663**	**0.01295**	**4.373**	**<0.0001**
Parent Phenotype	**-0.04948**	**0.009581**	**5.164**	**<0.0001**
Parent CO_2 Exposure	**0.1242**	**0.01419**	**8.753**	**<0.0001**
Current CO_2 : Parent CO_2	**-0.1265**	**0.01992**	**6.352**	**<0.0001**
Sex Ratio (%)				
Intercept	**65.00**	**12.08**	**5.383**	**0.0126**
Current CO_2 Exposure	-14.00	10.80	1.296	0.2856
Parent Phenotype	-7.000	15.28	0.4583	0.6779
Parent CO_2 Exposure	0.000	15.28	0.000	>0.9999
Current CO_2 : Parent CO_2	23.00	21.60	1.065	0.3651

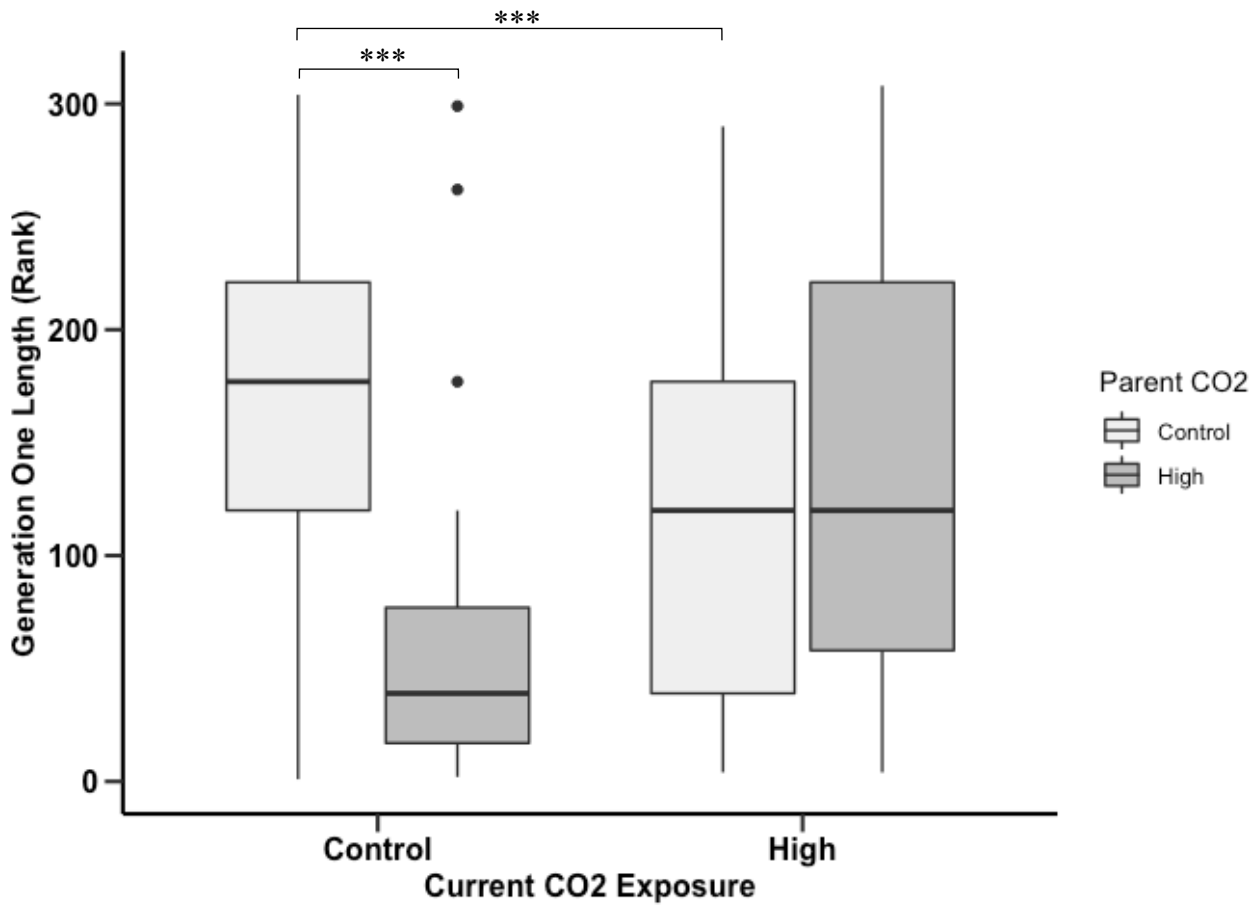


Figure 4.6 Filial generation one (F_1) length (rank (cm)) by current CO_2 exposure and parental CO_2 exposure interaction term ($F_{4,303} = 17.30$; $P < 0.0001$). Relative to control fish, length significantly differed in groups of interest: developmental exposure of fish exposed to elevated CO_2 who had parents who were not exposed to elevated CO_2 (TukeyHSD; $P = 0.0004$), and parental only elevated CO_2 exposure fish (TukeyHSD; $P = < 0.0001$). Asterisks and bars indicate statistical differences.

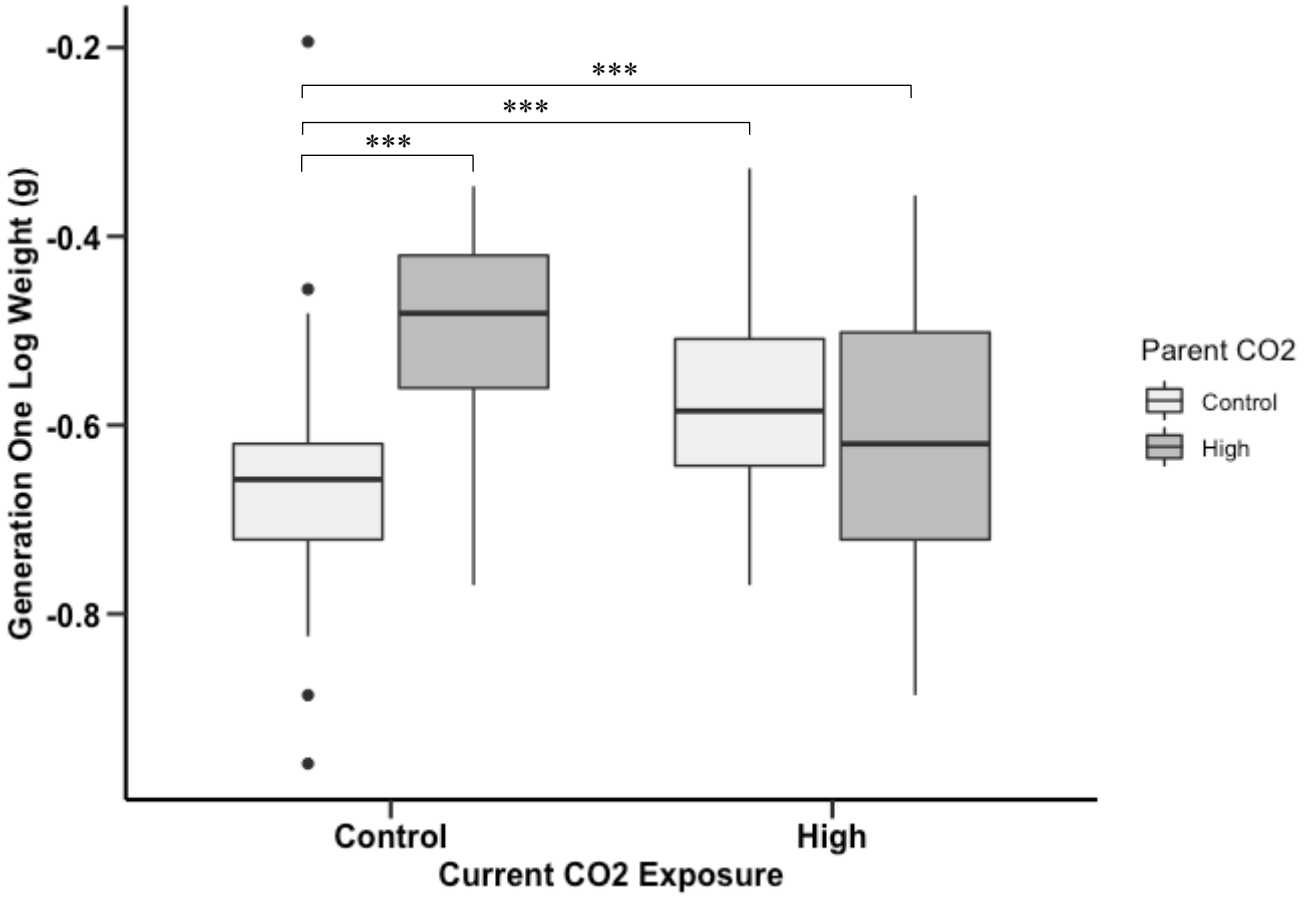


Figure 4.7 Filial generation one (F_1) log weight (g) by current CO_2 exposure and parental CO_2 exposure interaction term. Relative to control fish, weight significantly differed in groups of interest: developmental exposure of fish exposed to elevated CO_2 who had parents who were not exposed to elevated CO_2 (TukeyHSD: $P < 0.0001$), parental only elevated CO_2 exposure (TukeyHSD: $P < 0.0001$) and parental plus developmental elevated CO_2 exposure (TukeyHSD: $P = 0.0007$). Asterisks indicate statistical differences as determined by Tukey HSD test.

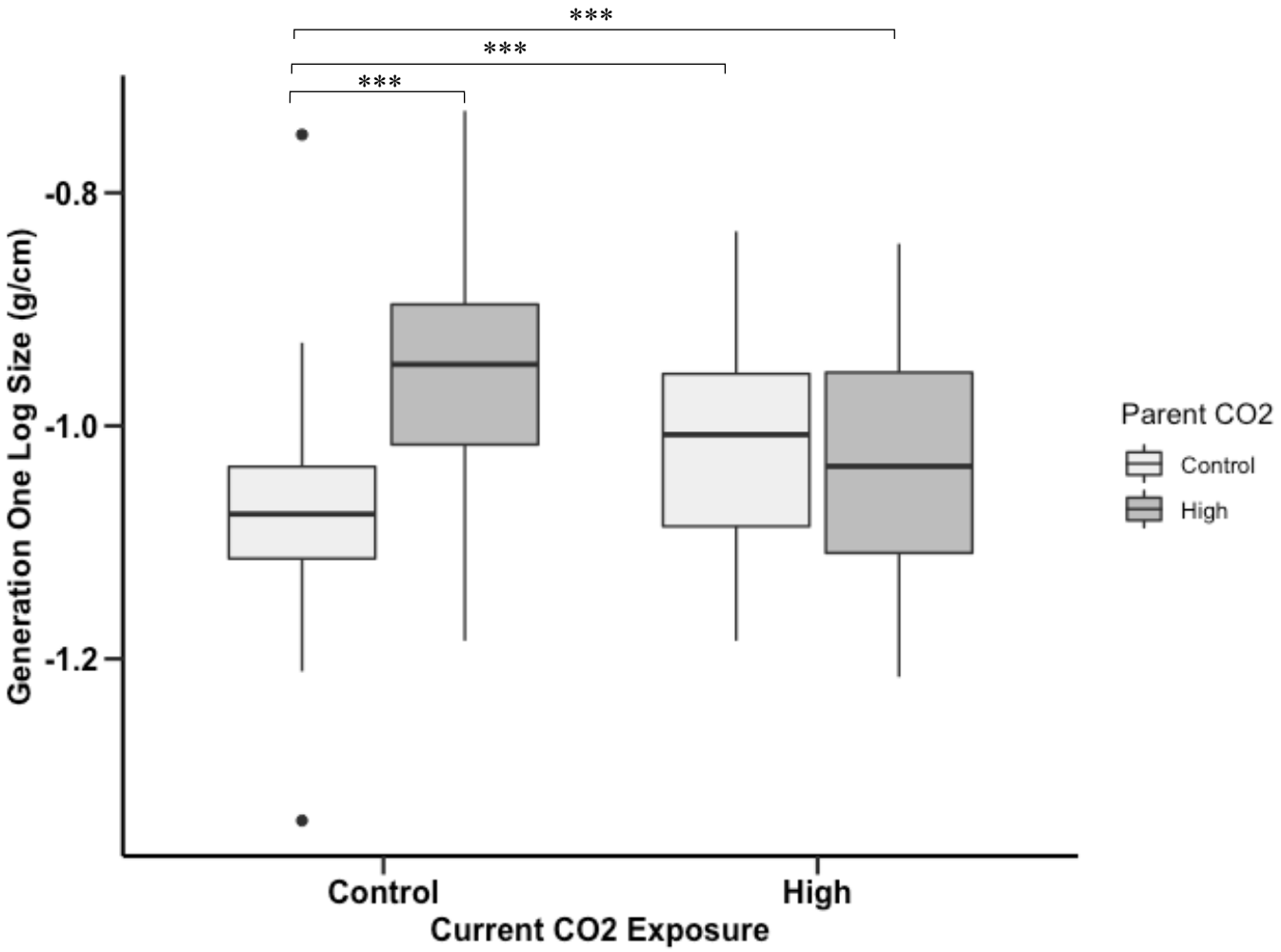


Figure 4.8 Filial generation one (F_1) log size (g/cm) by current CO_2 and parental exposure interaction term. Relative to control fish, size significantly differed in groups of interest: developmental exposure of fish exposed to elevated CO_2 who had parents who were not exposed to elevated CO_2 (TukeyHSD: $P = 0.0002$), parental only elevated CO_2 exposure (TukeyHSD: $P < 0.0001$), and parental plus developmental exposure (TukeyHSD: $P = 0.0002$). Asterisks indicate statistical differences as determined by Tukey HSD test.

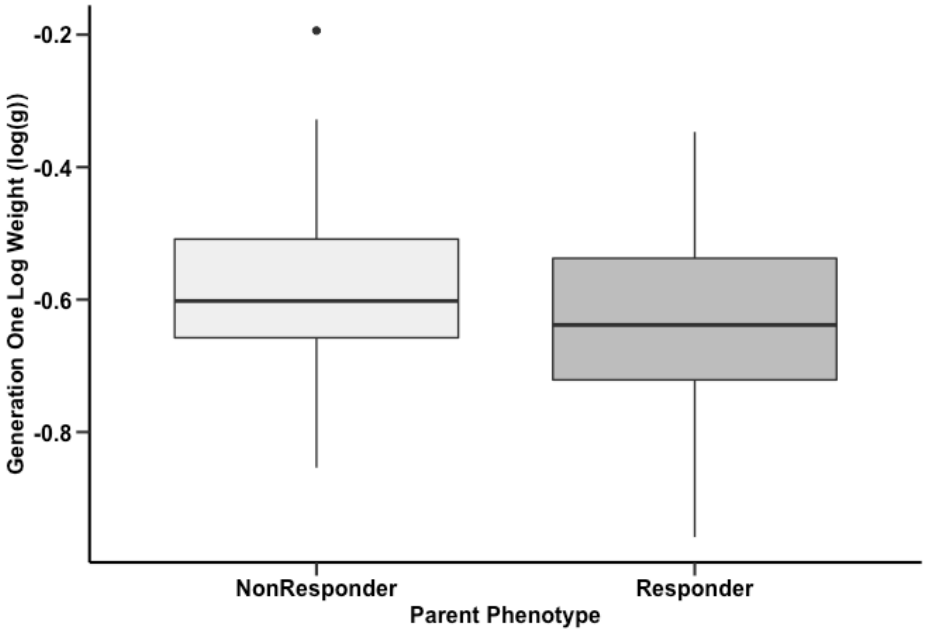


Figure 4.9 Filial generation one (F_1) log weight (g) by parent behavioural phenotype ($P <$ 0.0001).

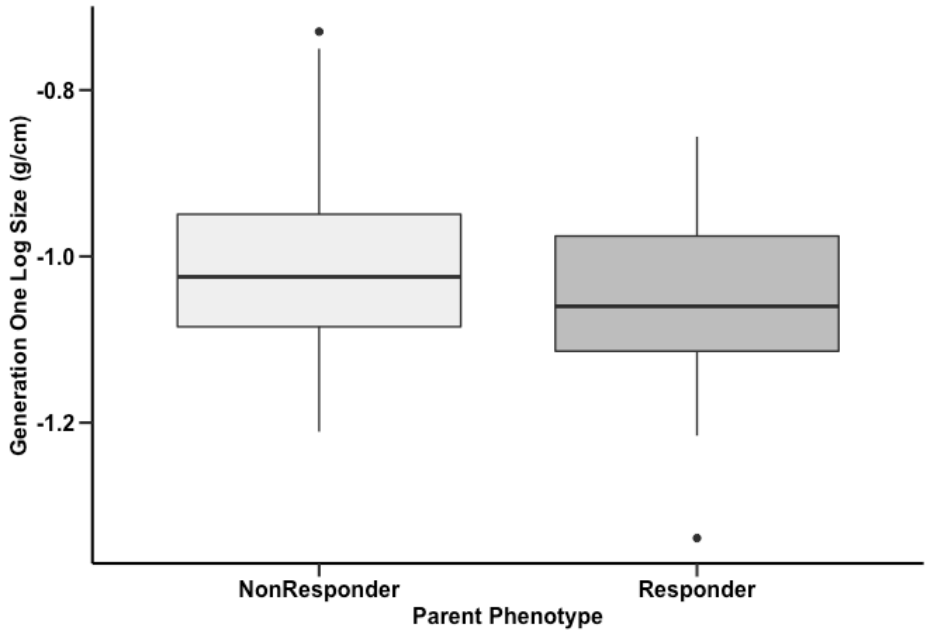


Figure 4.10 Filial generation one (F_1) log size (g/cm) by parent behavioural phenotype ($P <$ 0.0001).

Table 4.7 Filial generation one (F_1) multiple linear regression model statistical outputs for time spent in outer zone of arena, total distance moved, and swimming direction by current CO_2 exposure, parental CO_2 exposure, parent behavioural phenotype, and a current CO_2 exposure and parent CO_2 exposure interaction term.

Variable/Term	Estimate	Standard error	$\|t\|$	*P* value
Time Spent in Outer Zone (%)				
Intercept	**64.45**	**3.607**	**17.87**	**<0.0001**
Parental CO_2 exposure	-3.211	4.644	0.6915	0.4903
Current CO_2 exposure	-6.497	4.580	1.419	0.1581
Parent Phenotype	-3.091	3.262	0.9477	0.3448
Parental CO_2 : Current CO_2	-5.894	6.521	0.9039	0.3675
Distant Moved (sqrt(cm))				
Intercept	**23.80**	**1.139**	**20.89**	**<0.0001**
Parental CO_2	-0.7957	1.467	0.5425	0.5883
Current CO_2 Exposure	-2.112	1.447	1.460	0.1464
Parent Phenotype	**2.740**	**1.030**	**2.660**	**0.0087**
Parental CO_2 : Current CO_2	0.3490	2.060	0.1694	0.8657
Directionality (rank(°))				
Intercept	**79.93**	**7.969**	**10.03**	**<0.0001**
Parental CO_2 Exposure	4.427	10.26	0.4316	0.6667
Current CO_2 Exposure	6.649	10.12	0.6572	0.5120
Parent Phenotype	-0.6139	7.205	0.08520	0.9322
Parental CO_2 : Current CO_2	-26.03	14.41	1.807	0.0728

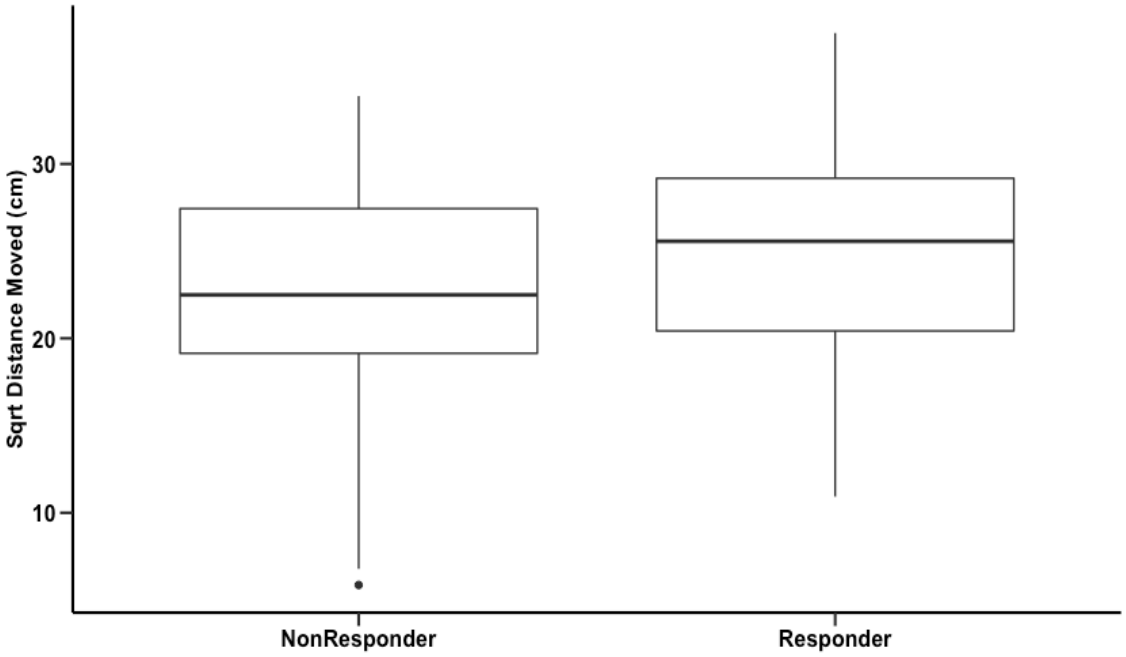


Figure 4.11 Filial generation one (F_1) square root distance moved (cm) by non-responder and responder parent behavioural phenotypes ($F_{4,151} = 2.813$; $P = 0.0274$).

Table 4.8 Filial generation one (F_1) multiple linear regression model statistical outputs for behaviour assay after exposure to acute elevated CO_2 for time spent in outer zone, total distance moved and swimming directionality by current CO_2 exposure, parent CO_2 exposure, parent behavioural phenotype, and current CO_2 and parent CO_2 exposure condition interaction term.

Variables/Term	Estimate	Standard error	$\|t\|$	*P* value
Time Spent in Outer Zone (%)				
Intercept	**58.05**	**5.607**	**10.35**	**<0.0001**
Parent Phenotype	7.627	5.844	1.305	0.1967
Parent CO_2 Exposure	5.320	8.578	0.6202	0.5374
Current CO_2 Exposure	-0.6464	6.728	0.09608	0.9238
Parent CO_2: Current CO_2	-3.500	11.07	0.3160	0.7531
Distance Moved (sqrt(cm))				
Intercept	**21.86**	**1.550**	**14.10**	**<0.0001**
Parent Phenotype	1.432	1.616	0.8863	0.3789
Parental CO_2 Exposure	0.6464	2.371	0.2726	0.7861
Current CO_2 Exposure	0.6006	1.860	0.3229	0.7479
Parental CO_2 : Current CO_2	1.005	3.062	0.3284	0.7437
Directionality (rank)				
Intercept	**27.79**	**5.140**	**5.407**	**<0.0001**
Parent Phenotype	3.767	5.357	0.7032	0.4846
Parental CO_2 Exposure	4.847	7.862	0.6165	0.5398
Current CO_2 Exposure	7.878	6.167	1.277	0.2062
Parental CO_2 : Current CO_2	-5.570	10.15	0.5487	0.5852

Table 4.9 Filial generation one (F_1) multiple linear regression model statistical outputs for egg size, the average eggs produced per female per day, and hatching success of incubated eggs by current CO_2 exposure, parent behavioural phenotype, parent CO_2 exposure and current and parent CO_2 exposure condition interaction term. Significant values bolded.

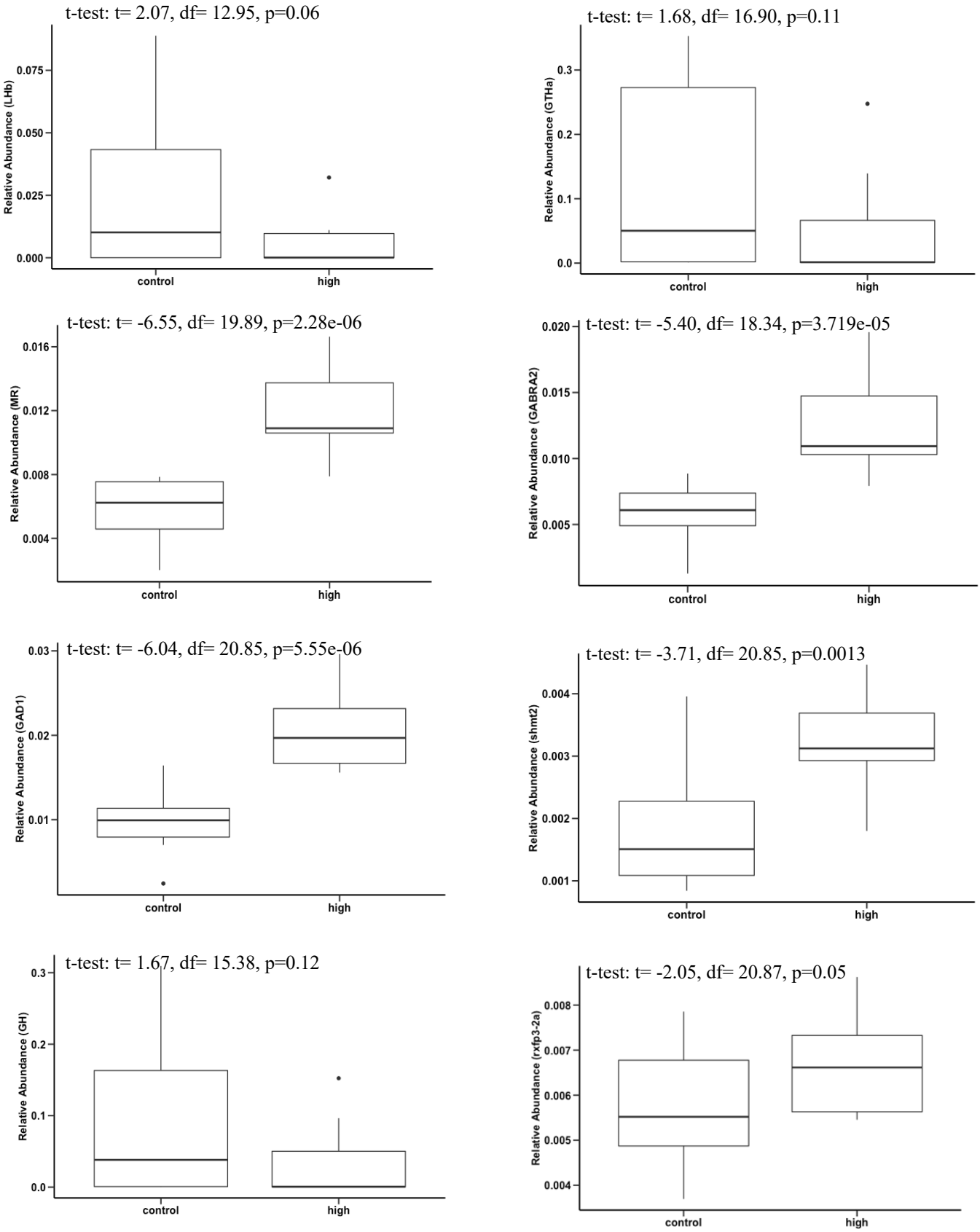
Variable/Term	Estimate	Standard error	$\|t\|$	*P* value
Egg Size (rank)				
Intercept	**464.2**	**21.66**	**21.43**	**<0.0001**
Current CO_2 Exposure	45.26	27.89	1.623	0.1049
Parent Phenotype	-30.94	18.09	1.711	0.0875
Parent CO_2 Exposure	42.43	25.60	1.657	0.0978
Current CO_2 : Parent CO_2	37.18	36.52	1.018	0.3090
Average Eggs per Female				
Intercept	**9.257**	**0.9796**	**9.449**	**<0.0001**
Current CO_2 Exposure	1.079	1.221	0.8842	0.3822
Parent Phenotype	-1.608	1.327	1.211	0.2333
Parent CO_2 Exposure	1.770	0.9270	1.909	0.0638
Current CO_2 : Parent CO_2	3.176	1.839	1.728	0.0922
Hatch Success				
Intercept	**51.13**	**14.49**	**3.527**	**0.0387**
Current CO_2 Exposure	39.00	18.33	2.127	0.1233
Parent Phenotype	3.500	18.33	0.1909	0.8608
Parent CO_2 Exposure	-15.25	12.96	1.176	0.3243
Current CO_2 : Parent CO_2	-10.50	25.93	0.4050	0.7126

Table 4.10 Filial generation one (F_1) loading factor identified by a principal component analysis for relative abundance of genes in whole brain tissue of adult medaka fish based on 85% of total explained variance. Genes with factor loadings ≥ ±0.5 are shown in bold.

Gene	PC1	PC2	PC3	PC4	PC5
cyp19b	-0.300	0.173	-0.470	0.518	-0.008
npy	0.115	**-0.616**	**-0.598**	0.297	0.104
fshb	0.455	**0.730**	-0.318	-0.138	-0.003
gtha	**0.564**	**0.756**	-0.224	-0.082	0.191
dar	-0.451	**-0.625**	-0.400	-0.319	0.162
shmt2	**-0.809**	0.128	-0.337	-0.259	-0.280
gra	**-0.867**	0.031	-0.297	-0.159	-0.173
ccka	0.256	-0.031	**-0.516**	0.305	-0.184
insl5b	0.231	0.071	**-0.605**	**-0.508**	-0.347
rln3b	0.143	-0.334	-0.030	-0.277	**0.791**
rxfp32a	**-0.709**	-0.243	-0.314	-0.493	0.138
gh	**0.518**	**0.733**	-0.321	-0.068	0.141
lhb	**0.565**	**0.654**	-0.043	-0.254	0.132
mdgnrh	0.344	-0.153	**-0.708**	0.344	0.244
gad1	**-0.856**	0.381	0.108	0.060	0.156
mbd2	**-0.898**	0.348	-0.030	0.132	0.066
gabra2	**-0.861**	0.366	-0.074	0.144	0.172
mr	**-0.892**	0.378	-0.004	0.096	0.119
nhe1	**-0.893**	0.366	0.029	0.105	0.108

Table 4.11 Filial generation one (F_1) multiple linear regression model statistical outputs for principal components contributing to 85% total explained variance of relative gene abundance by current CO_2 exposure condition, parent CO_2 exposure, sex, and current CO_2 exposure and parent CO_2 exposure interaction term.

Variable/Term	Estimate	Standard error	$\|t\|$	*P* value
PC1 Variables				
Intercept	**2.328**	**0.7643**	**3.046**	**0.0066**
Current CO2	**-4.921**	**0.9668**	**5.090**	**<0.0001**
Parent CO2	-0.08623	0.9668	0.08919	0.9299
Sex	-0.1923	0.6836	0.2813	0.7815
Current CO2 : parent CO2	1.087	1.367	0.7949	0.4365
PC2 Variables				
Intercept	0.7728	0.8540	0.9049	0.3769
Current CO2	-0.07338	1.080	0.06792	0.9466
Parental CO2	-1.269	1.080	1.175	0.2545
Sex	-1.196	0.7639	1.566	0.1339
Current CO2 : parental CO2	1.986	1.528	1.300	0.2091
PC3 Variables				
Intercept	-1.197	0.7030	1.702	0.1050
Current CO2	1.563	0.8892	1.758	0.0948
Parental CO2	0.7438	0.8892	0.8365	0.4133
Sex	0.7993	0.6288	1.271	0.2190
Current CO2 : parental CO2	-1.426	1.258	1.134	0.2709
PC4 Variables				
Intercept	0.5157	0.4714	1.094	0.2877
Current CO2	-0.02393	0.5963	0.04013	0.9684
Parental CO2	0.3674	0.5963	0.6161	0.5451
Sex	**-1.462**	**0.4217**	**3.467**	**0.0026**
Current CO2 : parental CO_2	0.1738	0.8433	0.2061	0.8389
PC5 Variable				
Intercept	-0.2746	0.5257	0.5224	0.6074
Current CO2	0.2869	0.6650	0.4314	0.6710
Parental CO2	0.6484	0.6650	0.9750	0.3418
Sex	0.03824	0.4702	0.08132	0.9360
Current CO2 : parental CO2	-0.8484	0.9404	0.9022	0.3783



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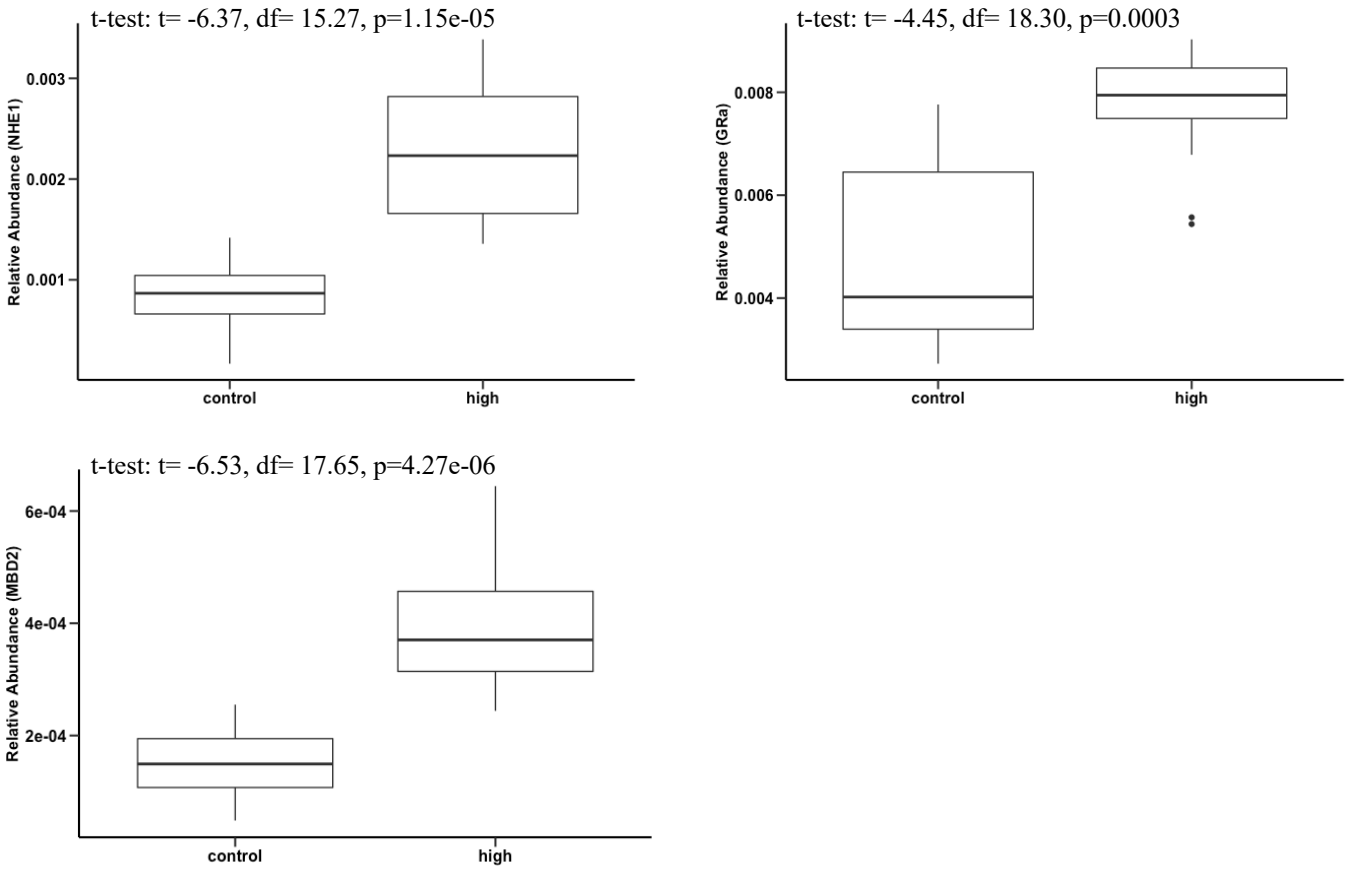


Figure 4.12 Relative abundance of mRNA for genes contributing to filial generation one (F_1) principal component one by current CO_2 exposure. Student's t-test results are included for genes: *lutenizing hormone beta subunit* (*lhb*), *glycoprotein hormones alpha chain* (*gtha*), *mineralcorticotropin hormone receptor* (*mr*), *gamma-aminobutyic acid receptor subunit alpha-2* (*gabra2*), *glutamate decarboxylase 1* (*gad1*), *serine hydroxymethlytransferase* (*shmt2*), *growth hormone* (*gh*), *relaxin family peptide receptor 3-2a (rxfp-2a),sodium-hydrogen antiporter 1* (*nhe-1*), *glucocorticoid receptor alpha* (*gra*), and *methyl CpG binding protein 2* (*mbd2*).

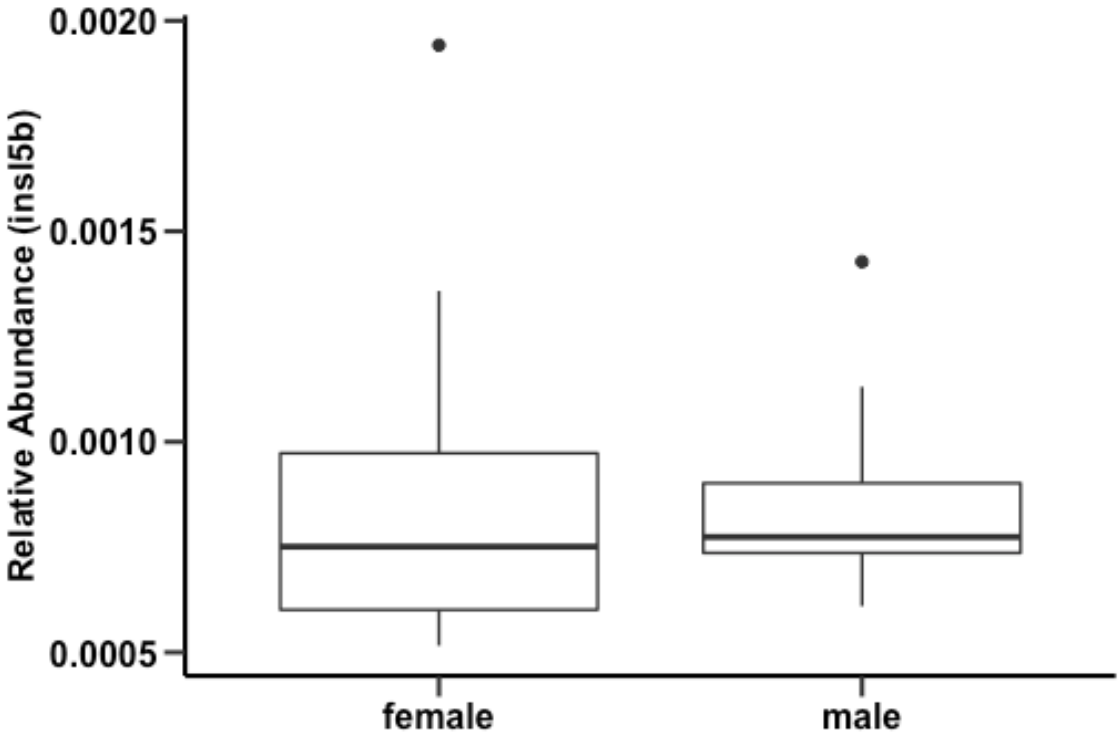


Figure 4.13 Filial generation one (F_1) PC4 gene *insulin-like peptide 5b* (*insl5b*), which significantly differed by sex ($P = 0.0026$)

4.4 Filial Generation Two

Filial generation two (F_2) fish were held in the same CO_2 conditions as their parents (F_1) and growth and development (length, weight, size, sex ratio), behaviour (total distance moved, swimming direction, time spent in outer versus inner zone of arena), reproductive outputs (egg size, number of eggs per female per day), and the relative abundance of various mRNA transcripts in whole brain tissue were measured 24 weeks after egg collection.

Weight, Length, Size, and Sex Ratio

Length significantly differed by grandparent phenotype but did not differ by current CO_2 exposure, grandparent CO_2 exposure or the interaction term of current CO_2 and grandparent CO_2 exposure (rank transformed, $F_{4,499} = 3.665$; $P = 0.0059$; Table 4.12). Fish with grandparents of non-responder phenotypes were on average 2 % longer than fish of responder phenotypes (Figure 4.17). Weight significantly differed by current CO_2 exposure and grandparent phenotype but did not differ by grandparent CO_2 exposure or the interaction term of current CO_2 and grandparent CO_2 exposure (log transformed, $F_{4,499} = 17.06$; $P = < 0.0001$; Table 4.12). Fish with grandparents that demonstrated the non-responder behaviour phenotype were on average 8 % heavier than fish with responder grandparents (Figure 4.18). Fish in elevated CO_2 conditions were 24 % heavier than fish in control CO_2 conditions (Figure 4.14). Size significantly differed by current CO_2 exposure, grandparent phenotype, and grandparent CO_2 exposure, but did not differ by grandparent and parent CO_2 exposure condition interaction term ($F_{4,499} = 33.80$; P <0.0001; Table 4.12). Fish held in elevated CO_2 conditions had a weight to length ratio 21 % higher than fish in control CO_2 conditions (Figure 4.15). Fish whose grandparents were exposed to elevated CO_2 had a weight to length ratio 5 % higher than fish in control CO_2 conditions

(Figure 4.16). Fish whose grandparents were of non-responsive behavioural phenotype had a weight to length ratio 7 % higher relative to responsive grandparent phenotypes (Figure 4.19).

Sex ratio of adult fish by percentage differed significantly by current CO_2 exposure but not grandparent phenotype, grandparent CO_2 exposure, or grandparent CO_2 and current CO_2 exposure condition interaction term ($F_{4,3} = 13.91$; $P = 0.0281$). Control CO_2 exposure condition fish were 23% female, and high CO_2 exposure condition fish were 75% female. The number of female fish in elevated CO_2 conditions was 69 % higher than control CO_2 condition fish. The average length for F_2 fish was 3.19 cm (S.E. ± 0.27). The average weight of F_2 fish was 0.40 g (S.E. ± 0.13). The average size for all F_2 fish was 0.12 g/cm (S.E. ± 0.03).

Behaviour

Behaviour in the novel tank assay and acute CO_2 novel tank assay for F_2 fish did not differ signfiicantly by current CO_2 exposure, grandparent CO_2 exposure, grandparent phenotype or the grandparent and parent CO_2 exposure interaction term (Table 4.13; Table 4.14). The average distance moved by F_2 fish during the novel tank behavioural assay was 884.3 cm (S.E. ± 371.6). The average percent of time F_2 fish spent in the outer zone of the behavioural arena was 89.3% (S.E. ± 13.8). The average direction F_2 fish swam during their behaviour assay was 7.97° (S.E. ± 98.1). The average distance moved by F_2 fish during the acute CO_2 exposure assay was 686.3 cm (S.E. ± 312.4). The average percent of time F_2 fish spent in the outer zone of the arena was 87.1% (S.E. ± 17.5). The average direction during the acute CO_2 exposure assay was 0.25° (S.E. ± 114.4) for all fish.

Reproduction

Egg size significantly differed by grandparent behavioural phenotype and the interaction term of current and grandparent CO_2 exposure (rank transformed, $F_{4,1801} = 220.1$; $P = <0.0001$; Table 4.15). Transgenerational exposure, whereby a fish's grandparents (P) had been exposed to elevated CO_2 but were not themselves exposed to elevated CO_2 nor were their parents (F_1), resulted in egg diameters 4 % larger relative to control eggs (Figure 4.20). Three generational CO_2 exposure, where P, F_1, and current F_2 exposures were all in elevated CO_2 conditions, resulted in eggs that were on average 1% larger relative to control fish (Figure 4.20). No significant difference was observed between two generation exposure and control fish. Fish whose grandparents were of non-responder phenotype had eggs that were on average 4 % smaller relative to grandparent responder phenotype fish (Figure 4.22). The average egg diameter for F_2 generation was 1.2 mm (S.E. ± 0.05). Fish whose grandparents were of non-responsive behaviour phenotype had 3 % more eggs per day per female on average relative to fish whose grandparents were of responsive phenotype (Figure 4.21). The average number of eggs collected per female per day for F_2 generation was 12.3 (S.E. ± 3.9).

Relative mRNA Abundance

Five principle components were identified to explain 85 % percent of total explained variance for F_2 relative mRNA abundance (Table 4.16). The proportion of variance explained by PC1 was 39.66 %, PC2 22.23 %, PC3 13.73 %, PC4 7.15 % and PC5 4.82 %. The interaction term that included current CO_2 and generation CO_2 had a significant effect on PC1 and PC2 mRNA abundances ($F_{4,18} = 6.680$; $P = 0.0018$; Table 4.17). No variables had a significant effect on PC3, PC4, or PC5 genes.

For PC1, three generation exposure effects ($P = 0.0044$) were the only comparison of interest found to significantly differ. PC1 relevant genes included *follicle stimulating hormone beta subunit* (*fshb*), *dopamine receptor* (*dar*), *cytochrome P450 19b* (*cyp19b*), *mineralcorticotropin hormone receptor* (*mr*), *relaxin family peptide receptor 3-2a* (*rxfp3-2a*),*glutamate decarboxylase 1* (*gad1*), *gamma-aminobutyic acid receptor subunit alpha-2* (*gabra2*), *sodium-hydrogen antiporter 1* (*nhe-1*), *neuropeptide Y* (*npy*), *cholecystokinin* (*ccka*), *methyl CpG binding protein 2* (*mbd2*), and *glucocorticoid receptor alpha* (*gra*) (Figure 4.23). Three generation elevated CO2 exposure resulted in a reduced relative mRNA abundance for all of PC1 genes (Figure 4.16). For genes within PC1 exposure, the genes which were found to significantly differ by elevated CO_2 over three generations within individual anova tests was *dopamine receptor* (*dar*), *relaxin family peptide receptor 3-2a* (*rxfp3-2a*) and *cholecystokinin* (*ccka*) all of which resulted in reduced relative mRNA abundances of (Figure 4.23).

For PC2 genes, relative mRNA abundances significantly differed by transgenerational exposure, which are F_2 fish who were not exposed to elevated CO_2 and whose parent weren't exposed to elevated CO_2, but whose grandparents were exposed to CO_2 (TukeyHSD; $P = 0.0469$; Table 4.17). PC2 genes with favor loadings $\geq \pm 0.5$ was *follicle stimulating hormone beta subunit (fshb), lutenizing hormone beta subunit (lhb), glycoprotein hormones alpha chain (gtha), relaxin 3b (rln3b), growth hormone (gh), gamma-aminobutyic acid receptor subunit alpha-2 (gabra2)* and *glutamate decarboxylase 1 (gad1)* (Table 4.16; Figure 4.24). By transgenerational exposure, *fshb, lhb, gtha, rln3b,* and *gh* decreased in relative mRNA abundance, and increased for *gabra2* and *gad.* Within PC2 genes, only three generation elevated CO_2 expression resulted in a reduced relative mRNA abundance for *gth*a.

Table 4.12 Filial generation two (F_2) multiple linear regression model statistical outputs for length, weight, size, and sex ratio by current CO_2 exposure, grandparent behavioural phenotype, grandparent CO_2 and current CO_2 and grandparent CO_2 exposure interaction term.

Variable/Term	Estimate	Standard error	$\|t\|$	*P* value
Length (rank)				
Intercept	**277.0**	**13.75**	**20.15**	**<0.0001**
Current CO_2 exposure	-18.14	17.48	1.038	0.2998
Grandparent Phenotype	**-40.97**	**12.92**	**3.171**	**0.0016**
Grandparent CO_2 Exposure	14.29	16.66	0.8577	0.3915
Grandparent CO_2 : Parent CO_2	-17.73	26.28	0.6747	0.5002
Weight (log(g))				
Intercept	**-0.4493**	**0.01229**	**36.56**	**<0.0001**
Current CO_2 exposure	**0.08795**	**0.01562**	**5.631**	**<0.0001**
Grandparent Phenotype	**-0.03135**	**0.01155**	**2.715**	**0.0069**
Grandparent CO_2 Exposure	0.02527	0.01490	1.696	0.0905
Grandparent CO_2 : Parent CO_2	-0.003216	0.02349	0.1369	0.8912
Size (log(g/cm))				
Intercept	**-0.9599**	**0.009433**	**101.8**	**<0.0001**
Current CO_2 exposure	**0.09450**	**0.01199**	**7.881**	**<0.0001**
Grandparent Phenotype	**-0.02269**	**0.008864**	**2.560**	**0.0108**
Grandparent CO_2 Exposure	**0.02284**	**0.01143**	**1.998**	**0.0462**
Grandparent CO_2 : Parent CO_2	0.007135	0.01803	0.3956	0.6925
Sex Ratio (%)				
Intercept	**35.88**	**7.963**	**4.505**	**0.0204**
Current CO_2 exposure	**41.50**	**10.07**	**4.120**	**0.0259**
Grandparent Phenotype	-11.75	7.122	1.650	0.1976
Grandparent CO_2 Exposure	-14.00	10.07	1.390	0.2587
Grandparent CO_2 : Current CO_2	18.50	14.24	1.299	0.2849

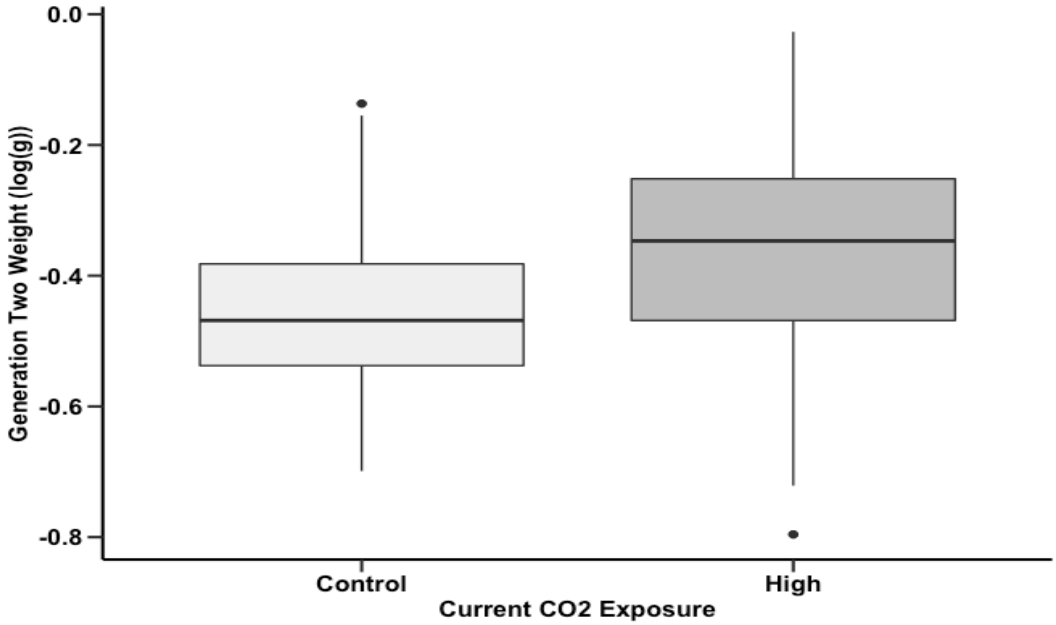


Figure 4.14 Filial generation two (F_2) log weight (g) by current CO_2 exposure condition ($P <$ 0.0001).

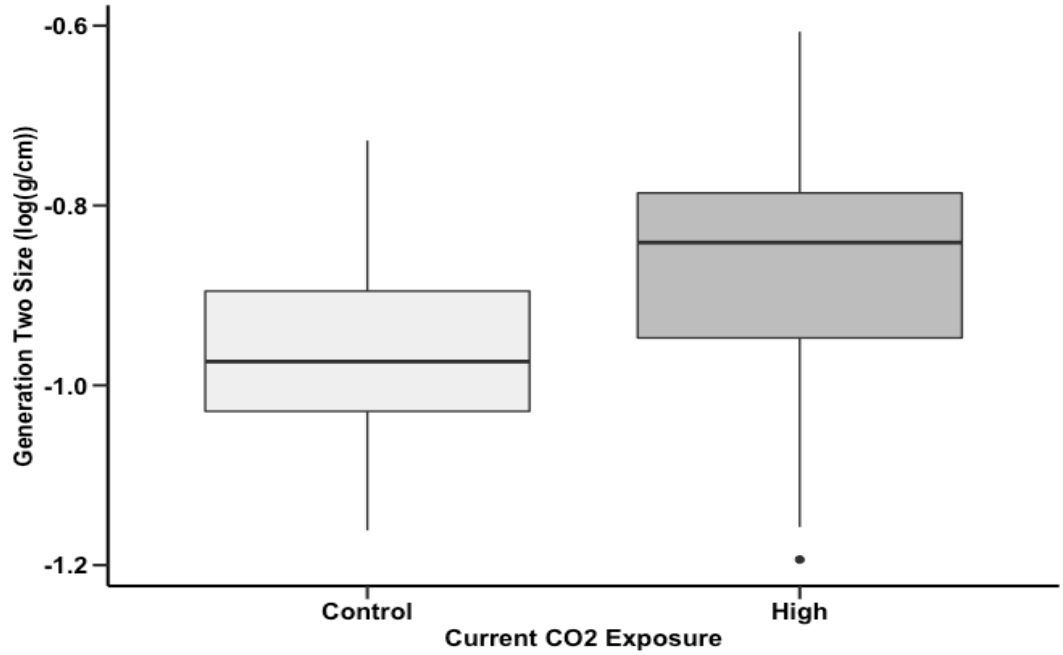


Figure 4.15 Filial generation two (F_2) log size (g/cm) by current CO_2 exposure condition ($P <$ 0.0001).

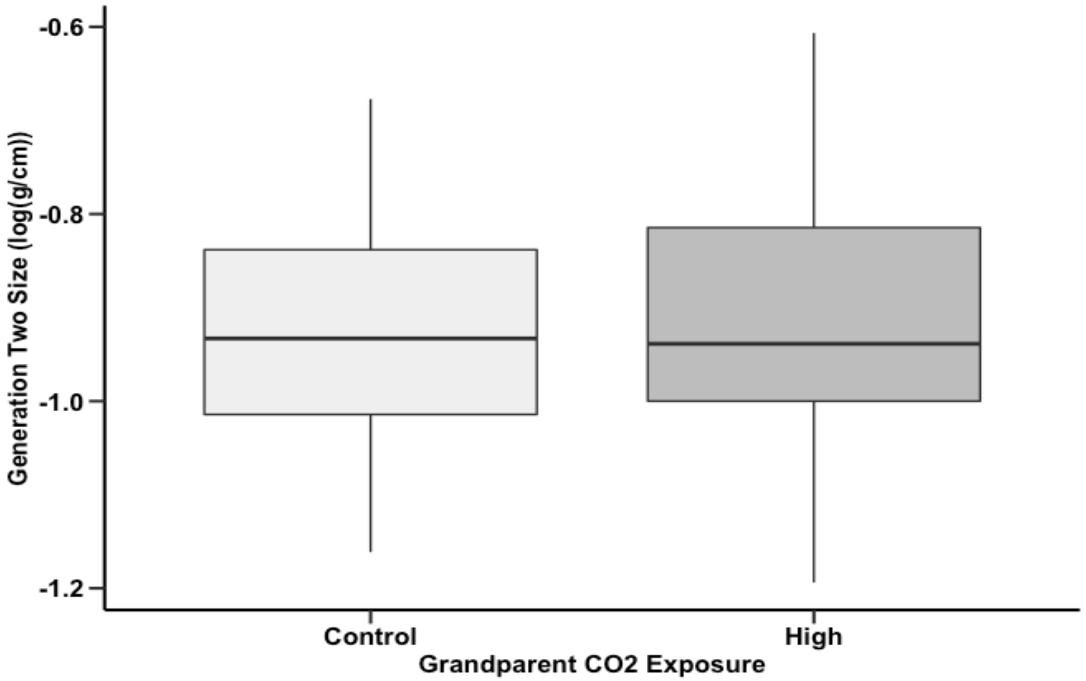


Figure 4.16 Filial generation two (F_2) log size (g/cm) by grandparent CO_2 exposure condition (*P* = 0.0462)

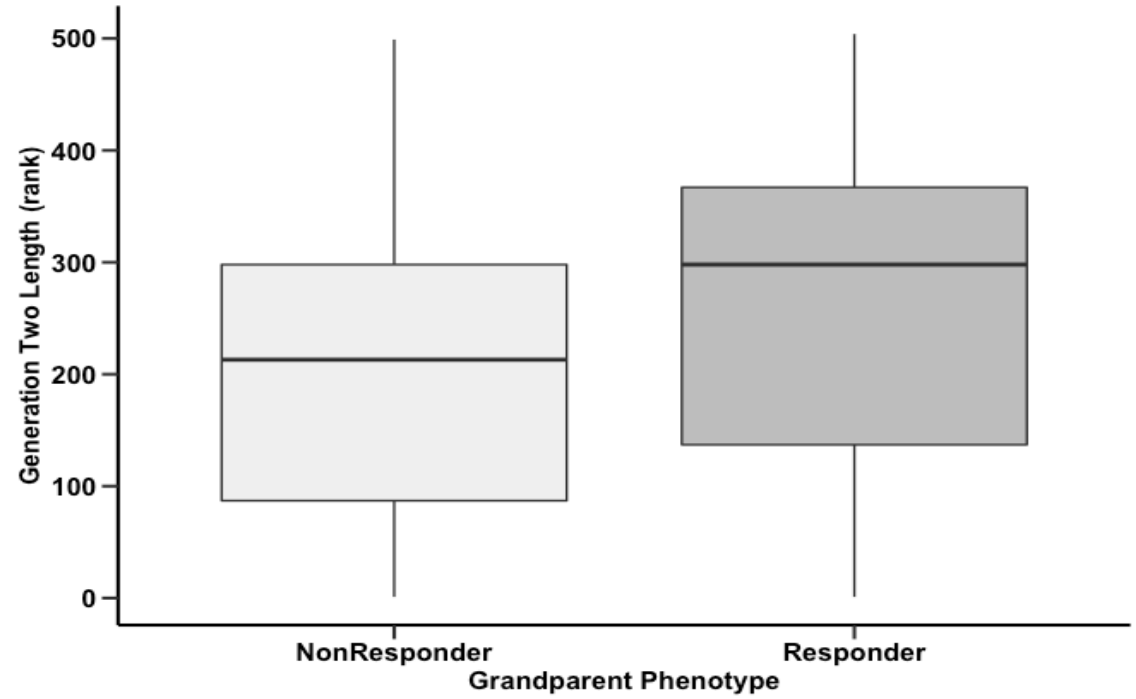


Figure 4.17 Filial generation two (F_2) length (cm rank) by grandparent behavioural phenotype (*P* = 0.0016).

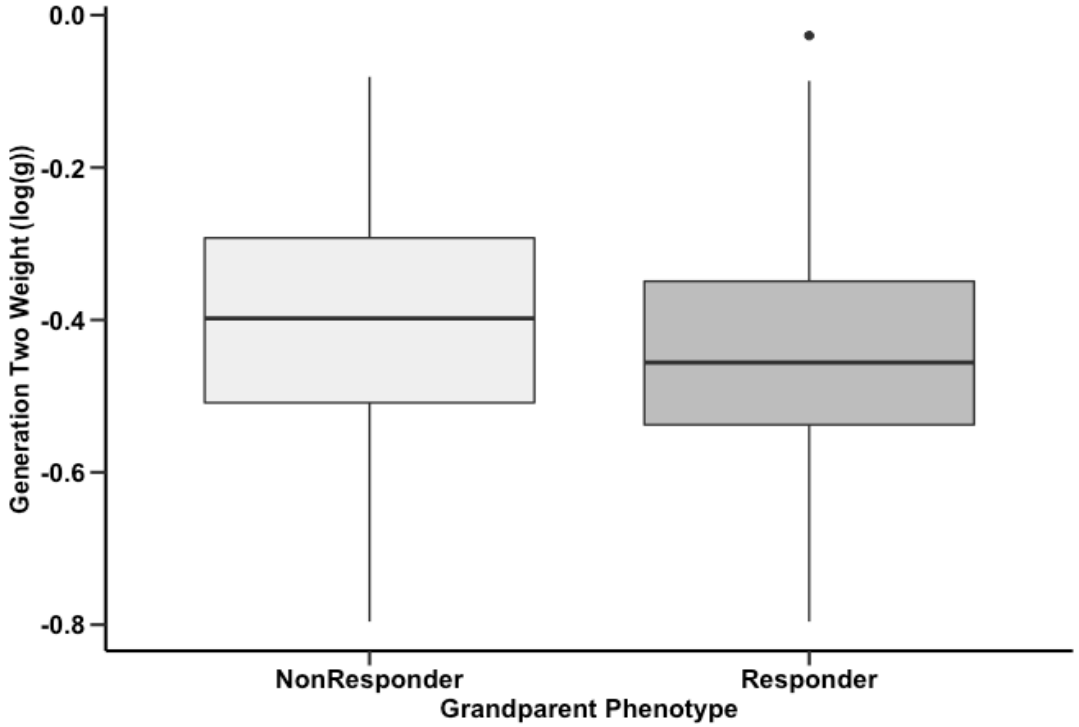


Figure 4.18 Filial generation two (F_2) weight (log (g)) by grandparent behavioural phenotype (*P* = 0.0069)

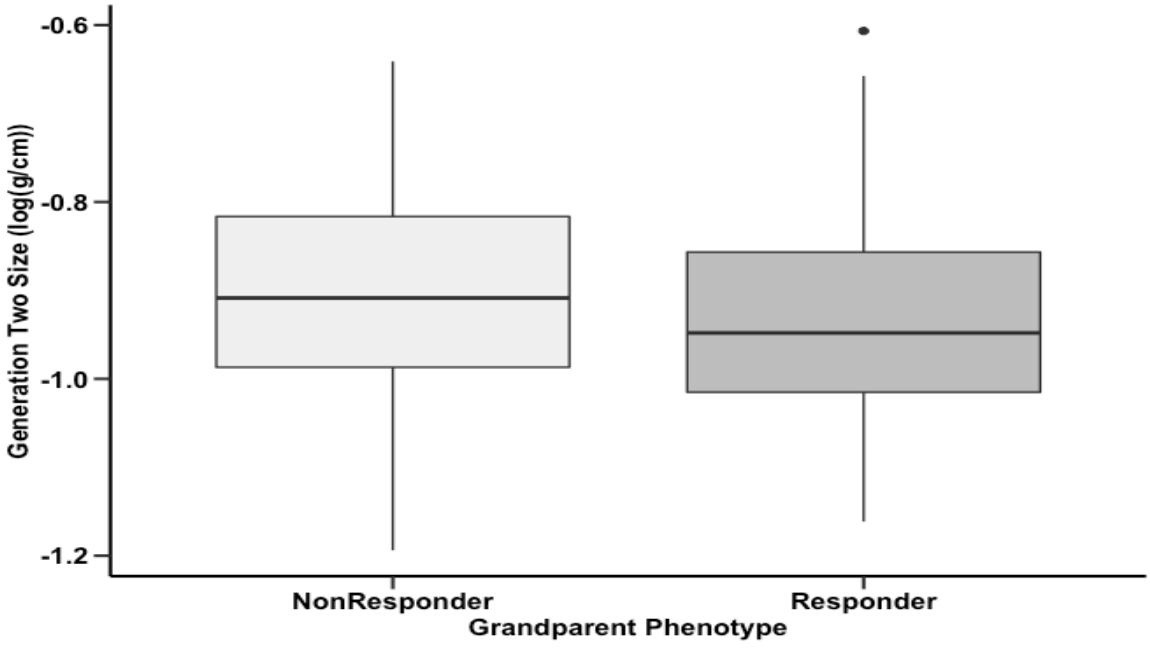


Figure 4.19 Filial generation two (F_2) size (log (g/cm)) by grandparent behavioural phenotype (*P* = 0.0108).

Table 4.13 Filial generation two (F_2) multiple linear regression model statistical outputs for time spent in outer zone, total distance moved, and swimming directionality during behaviour assay by current CO_2 exposure, grandparent CO_2 exposure, grandparent behavioural phenotype, and parent CO_2 and grandparent CO_2 exposure condition interaction term.

Variables/Term	Estimate	Standard error	$\|t\|$	*p* value
Time Spent in Outer Zone (%)				
Intercept	**60.77**	**4.017**	**15.13**	**<0.0001**
Grandparent CO_2 Exposure	2.481	5.081	0.4883	0.6268
Current CO_2 Exposure	-0.1055	5.081	0.02077	0.9835
Grandparent Phenotype	-1.279	3.593	0.3560	0.7228
Grandparent CO_2 : Current CO_2	8.253	7.186	1.148	0.2544
Distance Moved (sqrt(cm))				
Intercept	**29.35**	**1.637**	**17.93**	**<0.0001**
Grandparent CO_2 Exposure	-0.9280	2.071	0.4481	0.6554
Current CO_2 Exposure	-1.329	2.071	0.6416	0.5231
Grandparent Phenotype	0.8296	1.465	0.5665	0.5728
Grandparent CO_2 : Current CO_2	1.637	2.929	0.5590	0.5778
Directionality (rank)				
Intercept	**36.38**	**5.790**	**6.282**	**<0.0001**
Grandparent CO_2 Exposure	5.600	7.324	0.7646	0.4469
Current CO_2 Exposure	11.45	7.324	1.563	0.1222
Grandparent Phenotype	1.150	5.179	0.2221	0.8249
Grandparent CO_2 : Current CO_2	-19.90	10.36	1.921	0.0585

Table 4.14 Filial generation two (F_2) multiple linear regression model statistical outputs for acute CO_2 behaviour assay measures time spent in outer zone, total distance moved, and swimming directionality during acute CO_2 exposure behaviour assay by current CO_2 exposure, grandparent CO_2 exposure, grandparent behavioural phenotype, and parent CO_2 and grandparent CO_2 exposure condition interaction term.

Variables/Term	Estimate	Standard error	$\|t\|$	p value
Time Spent in Outer Zone (%)				
Intercept	**60.24**	**7.662**	**7.862**	**<0.0001**
Grandparent CO_2	4.110	9.560	0.4299	0.6694
Current CO_2	-5.851	9.728	0.6015	0.5507
Grandparent Phenotype	-3.396	6.736	0.5041	0.6167
Grandparent CO_2 : Current CO_2	-14.13	13.48	1.048	0.3006
Distance Moved (sqrt(cm))				
Intercept	**27.36**	**2.093**	**13.07**	**<0.0001**
Grandparent CO_2	-3.105	2.611	1.189	0.2410
Current CO_2	-2.191	2.657	0.8244	0.4142
Grandparent Phenotype	0.04867	1.840	0.02645	0.9790
Grandparent CO_2 : Current CO_2	3.183	3.683	0.8642	0.3923
Directionality (Rank)				
Intercept	**27.19**	**4.679**	**5.811**	**<0.0001**
Grandparent CO_2	1.389	5.838	0.2380	0.8130
Current CO_2	-2.914	5.941	0.4905	0.6263
Grandparent Phenotype	-1.225	4.114	0.2977	0.7674
Grandparent CO_2 : Current CO_2	-5.389	8.235	0.6544	0.5163

Table 4.15 Filial generation two (F_2) multiple linear regression model statistical outputs for egg diameter and the average eggs per female per day by current CO_2 exposure condition, grandparent CO_2 exposure condition, grandparent behavioural phenotype, and current CO_2 and grandparent CO_2 exposure condition interaction term.

Variable/Term	Estimate	Standard error	$\|t\|$	p value
Egg Size (rank cm)				
Intercept	**1107**	**23.09**	**47.93**	**<0.0001**
Current CO_2 Exposure	**-104.3**	**26.14**	**3.989**	**<0.0001**
Grandparent Phenotype	**-421.9**	**21.16**	**19.94**	**<0.0001**
Grandparent CO_2 Exposure	**-120.2**	**30.96**	**3.881**	**<0.0001**
Current CO_2 : Grandparent CO_2	**552.4**	**41.56**	**13.29**	**<0.0001**
Average Eggs Per Female				
Intercept	**12.59**	**1.831**	**6.873**	**<0.0001**
Current CO_2 Exposure	1.197	2.316	0.5167	0.6096
Grandparent Phenotype	**-5.843**	**2.316**	**2.522**	**0.0179**
Grandparent CO_2 Exposure	0.5763	1.638	0.3518	0.7277
Current CO_2 : Grandparent CO_2	6.721	3.276	2.052	0.0500

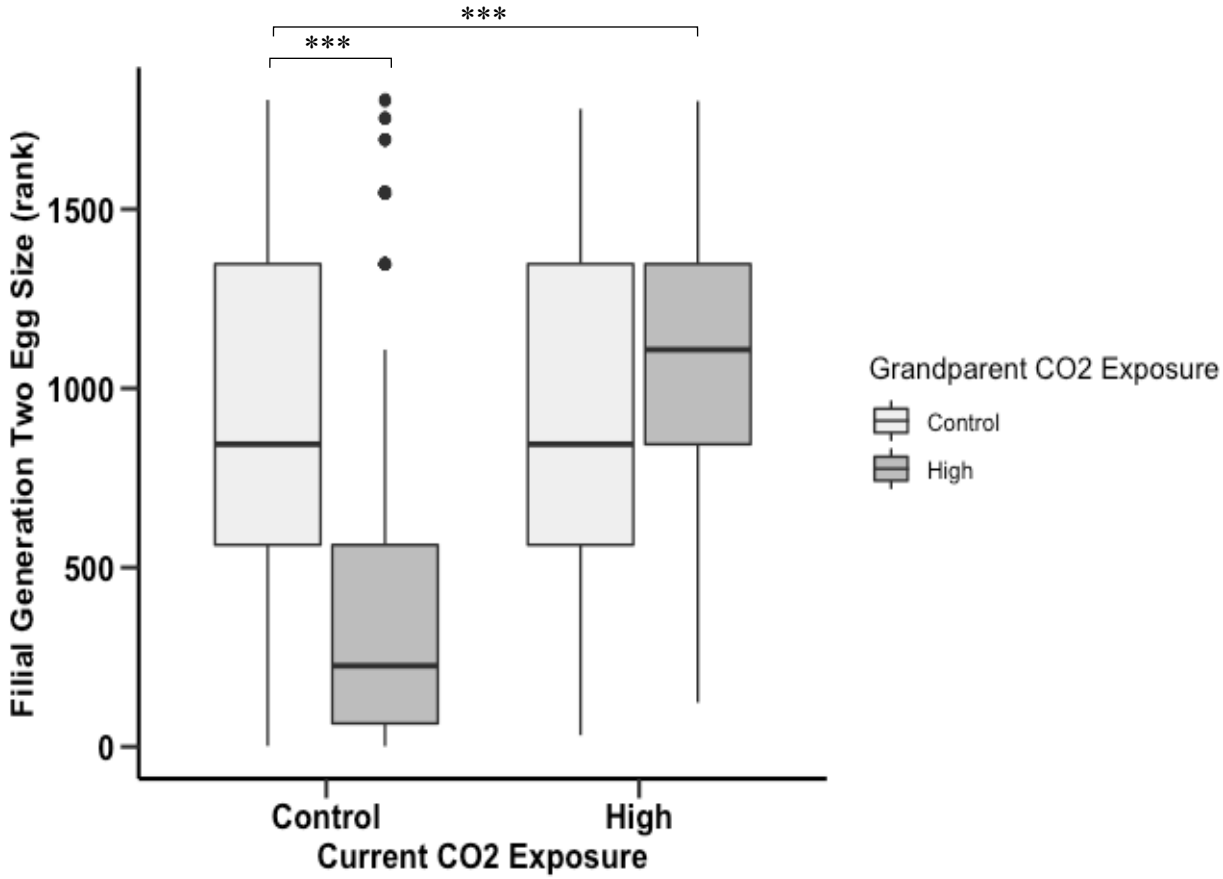


Figure 4.20 Filial generation two (F_2) egg diameter (mm) ranked by current CO_2 exposure condition and grandparent CO_2 exposure condition interaction term. Groups of interest significantly differing by interaction effect included: Transgenerational exposure, where parental generation exposure fish were exposed to elevated CO_2 but filial generation one and two were not, relative to three generational control CO_2 exposure fish (TukeyHSD: P <0.0001), and three generational elevated CO_2 exposure relative to three generational control CO_2 exposure (TukeyHSD: P <0.0001). Asterisks indicate statistical differences as determined by Tukey HSD test.

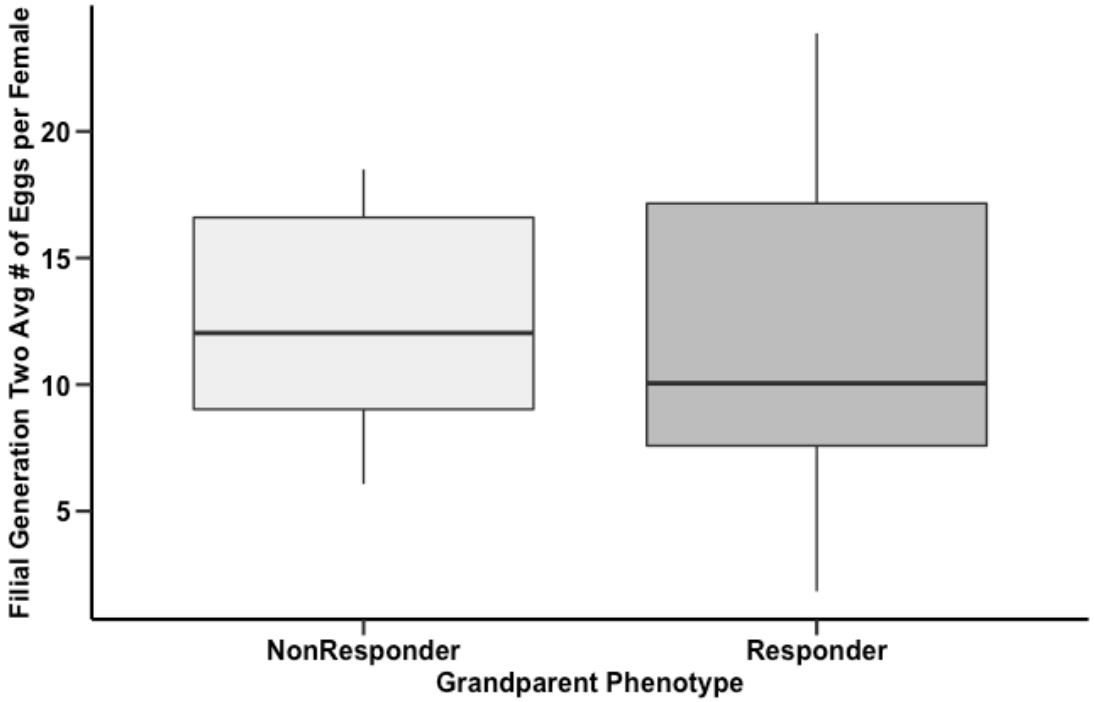


Figure 4.21 Filial generation two (F_2) number of eggs per female per day by grandparent behavioural phenotype (P = <0.0001).

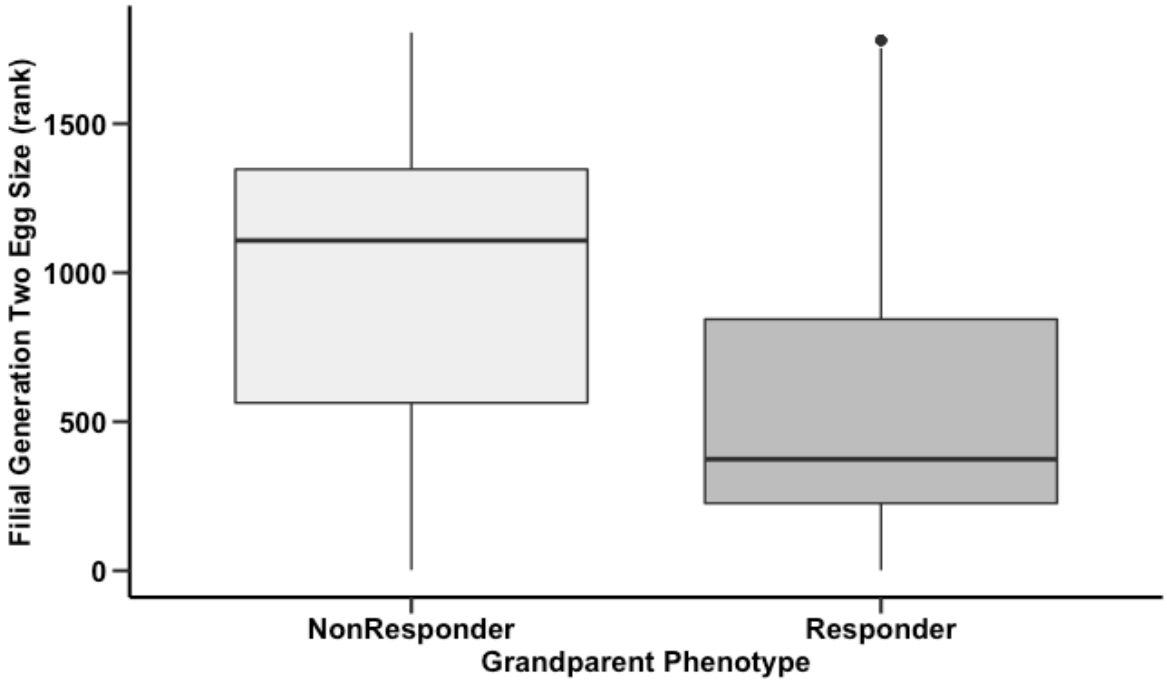


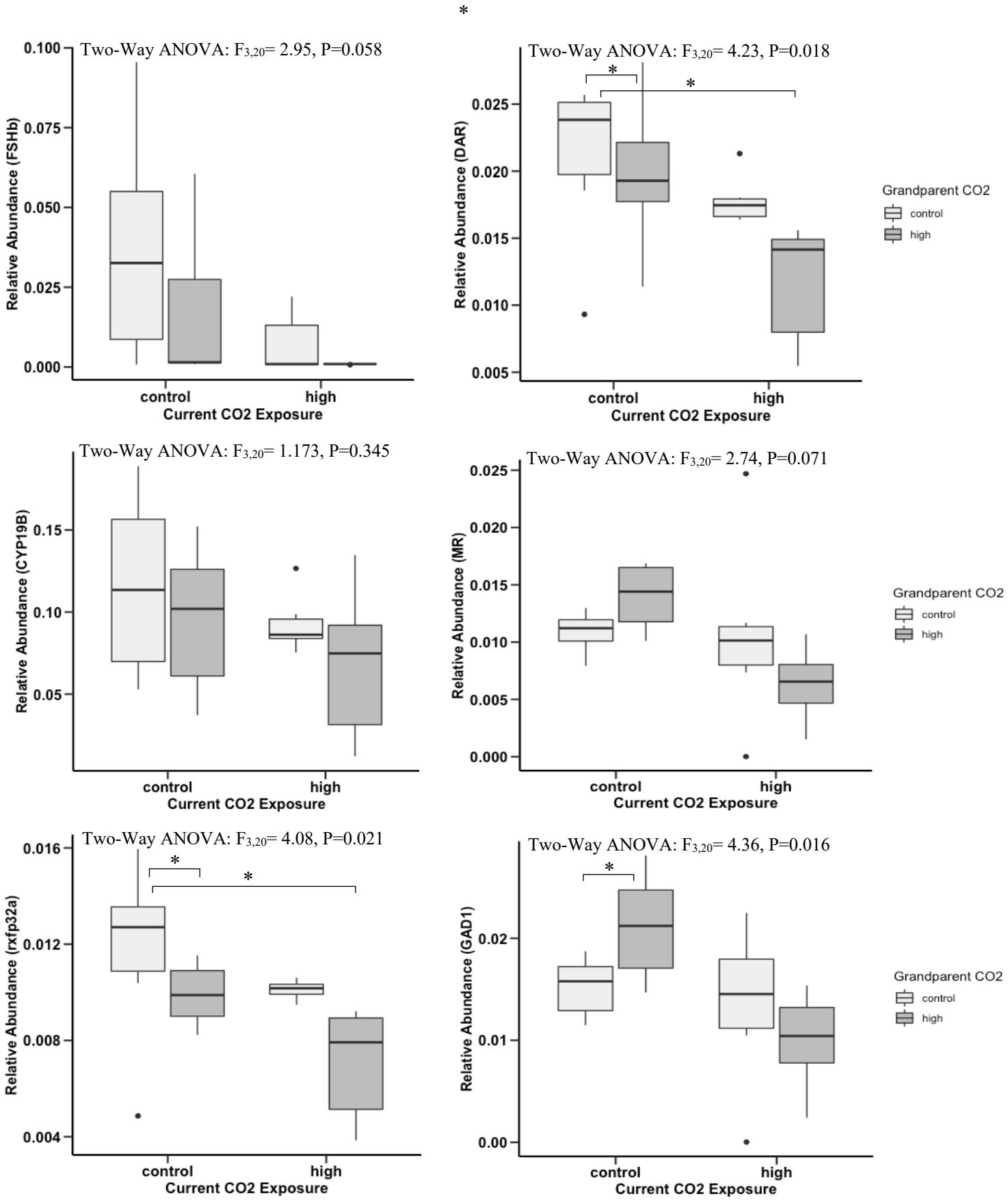
Figure 4.22 Filial generation two (F_2) egg diamter (mm) rank by grandparent behavioural phenotype (P = 0.0179).

Table 4.16 Loading factors for relative abundance of genes in whole brain tissue of filial generation two (F_2) adult medaka selected based on 85% percent of total explained variance. Variables with factor loadings ≥ ±0.5 are shown in bold.

Gene	PC1	PC2	PC3	PC4	PC5
cyp19b	**-0.606**	0.417	-0.364	0.109	-0.405
npy	**-0.632**	-0.286	**0.564**	0.162	-0.295
fshb	**-0.517**	**0.647**	-0.177	-0.442	0.0287
gtha	-0.431	**0.608**	-0.015	-0.001	**0.546**
dar	**-0.840**	0.321	-0.038	0.335	-0.118
shmt2	-0.471	-0.254	**-0.747**	0.109	-0.050
gr$_a$	**-0.529**	-0.140	**-0.658**	0.299	-0.136
ccka	**-0.762**	0.137	0.400	0.001	-0.187
insl5b	-0.291	0.305	**0.500**	0.194	0.267
rln3b	-0.287	**0.589**	0.247	**0.535**	0.183
rxfp32a	**-0.735**	0.453	-0.134	0.364	0.061
gh	-0.479	**0.686**	2.754e-004	-0.472	-0.043
lhb	-0.488	**0.670**	-0.017	-0.400	-0.171
mdgnrh	-0.497	-0.207	0.772	-0.050	-0.220
gad1	**-0.794**	**-0.559**	-0.011	-0.138	0.098
mbd2	**-0.842**	-0.434	-0.056	-0.110	0.182
gabra2	**-0.736**	**-0.599**	0.126	-0.122	0.085
mr	**-0.760**	-0.487	-0.175	-0.109	0.147
nhe1	**-0.806**	-0.480	-0.011	-0.108	0.190

Table 4.17 Filial generation two (F_2) multiple linear regression model statistical outputs for principle components selected based on 85% total variance by current CO_2 exposure condition, grandparent CO_2 exposure condition, grandparent behavioural phenotype, and current CO_2 and grandparent CO_2 exposure condition interaction term.

Variable/Term	Estimate	Standard error	$\|t\|$	p value
PC1 Variables				
Intercept	-1.392	0.8834	1.576	0.1325
CurrentCO2	**5.101**	**1.112**	**4.589**	**0.0002**
Grandparent CO2	0.6729	1.112	0.6053	0.5525
Phenotype	-1.098	0.8065	1.362	0.1901
currentCO2: Grandparent CO2	**-3.663**	**1.613**	**2.271**	**0.0357**
PC2 Variables				
Intercept	-1.703	0.8266	2.060	0.0542
Current CO2	1.622	1.040	1.559	0.1363
Grandparent CO2	**2.938**	**1.040**	**2.825**	**0.0112**
Phenotype	0.8345	0.7545	1.106	0.2833
currentCO2 : Grandparent CO2	**-4.202**	**1.509**	**2.785**	**0.0122**
PC3 Variables				
Intercept	0.7884	0.7170	1.100	0.2860
Current CO2	-0.4527	0.9022	0.5018	0.6219
Grandparent CO2	-0.9097	0.9022	1.008	0.3267
Phenotype	0.05790	0.6545	0.08846	0.9305
currentCO2 : Grandparent CO2	-0.7683	1.309	0.5869	0.5645
PC4 Variables				
Intercept	-0.2395	0.5583	0.4291	0.6730
Current CO2	0.5742	0.7025	0.8174	0.4244
Grandparent CO2	0.08229	0.7025	0.1171	0.9081
Phenotype	-0.2486	0.5097	0.4877	0.6317
Current CO2 : Grandparent CO2	0.2542	1.019	0.2493	0.8059
PC5 Variables				
Intercept	-0.6162	0.4298	1.434	0.1687
Current CO2	0.2914	0.5408	0.5389	0.5965
Grandparent CO2	0.4102	0.5408	0.7586	0.4579
Phenotype	0.8121	0.3923	2.070	0.0531
currentCO2 : Grandparent CO2	-0.6579	0.7846	0.8385	0.4127



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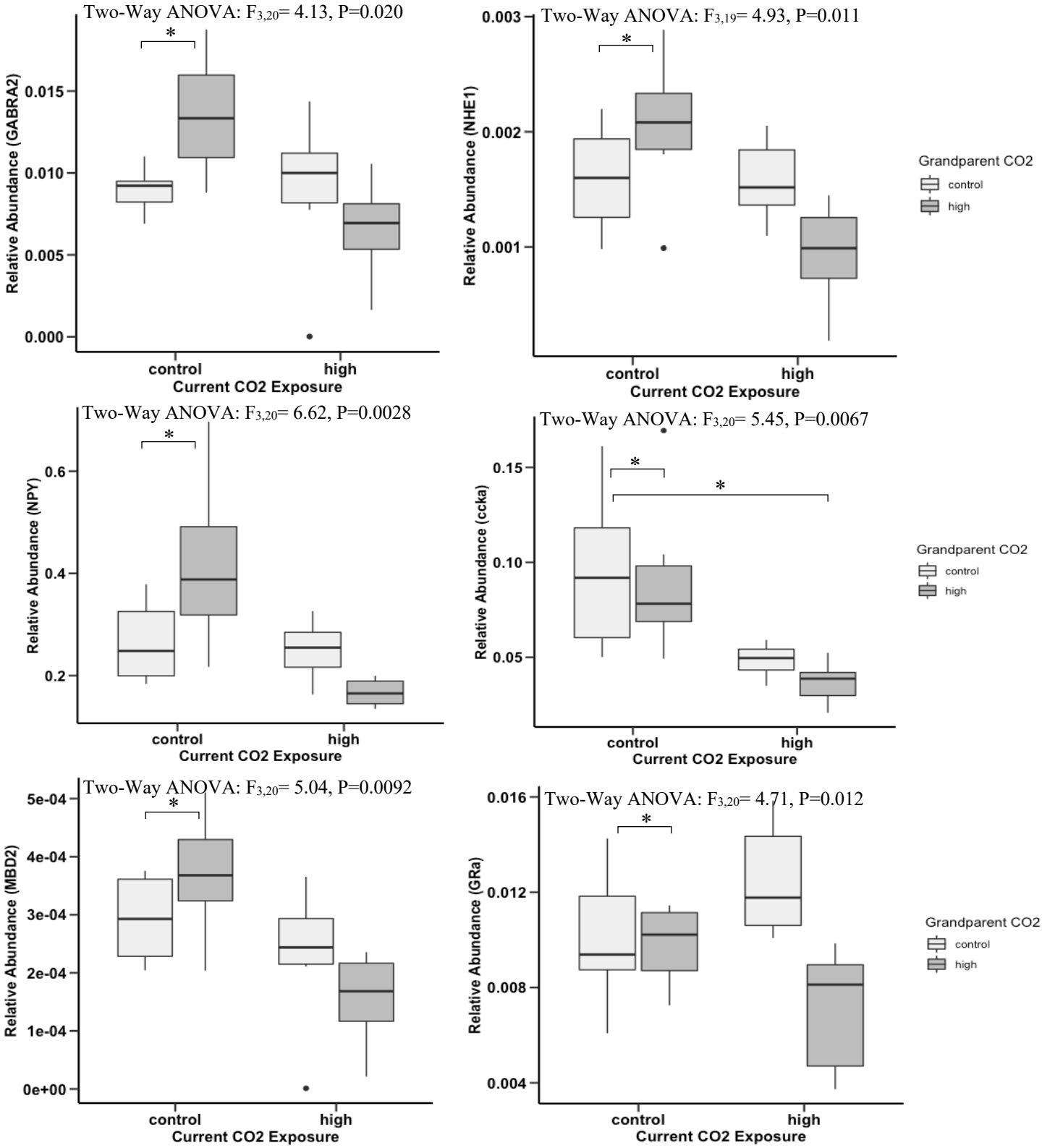
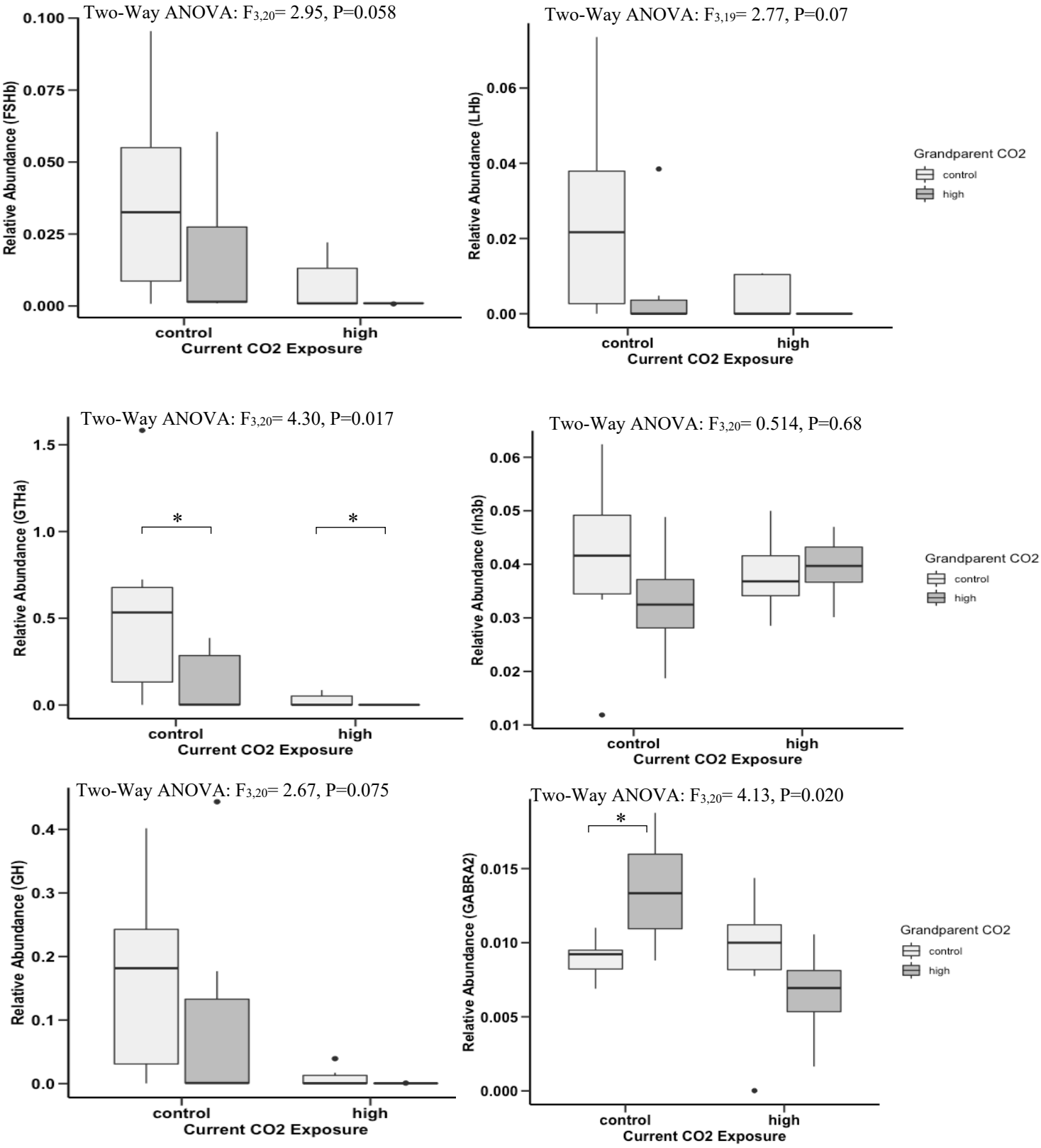


Figure 4.23 Filial generation two (F_2) principal component one (PC1) relative abundance of whole brain tissue genes with factor loadings ≥ ±0.5 by current CO_2 exposure and grandparent

CO_2 exposure condition interaction term. Three generation exposure (P, F_1, F_2) was significant for PC1. Two-way anova results are included for the following genes: *follicle stimulating hormone beta subunit (fshb), dopamine receptor (da r), cytochrome p450 19b (cyp19b), mineralcorticotropin hormone receptor (mr), relaxin family peptide receptor 3-2a (rxfp3-2a),glutamate decarboxylase 1 (gad1), gamma-aminobutyic acid receptor subunit alpha-2 (gabra2), sodium-hydrogen antiporter 1 (nhe-1), neuropeptide y (npy), cholecystokinin (ccka), methyl cpg binding protein 2 (mbd2),* and *glucocorticoid receptor alpha (gra).* Of these anovas, three generational elevated CO_2 exposure resulted in significant differences relative to three generation control CO_2 exposure for genes *dar* (TukeyHSD: $p = 0.017$*), rxfp3-2a* (TukeyHSD: $p = 0.013$), and *ccka* (TukeyHSD: $p = 0.02$). Three generation elevated CO_2 exposure and transgenerational exposure (P, but not F_1 or F_2 elevated CO_2 exposure) resulted in significant differences in relative mRNA abundance for *dar* (TukeyHSD: $p = 0.017$), *gad1* (TukeyHSD: $p = 0.010$), *gabra2* (TukeyHSD: $p = 0.013$), *nhe1* (TukeyHSD: $p = 0.006$), *npy* (TukeyHSD: $p = 0.002$), *ccka* (TukeyHSD: $p = 0.032$), *mbd2* (TukeyHSD: $p = 0.007$), and *gra* (TukeyHSD: $p = 0.006$).



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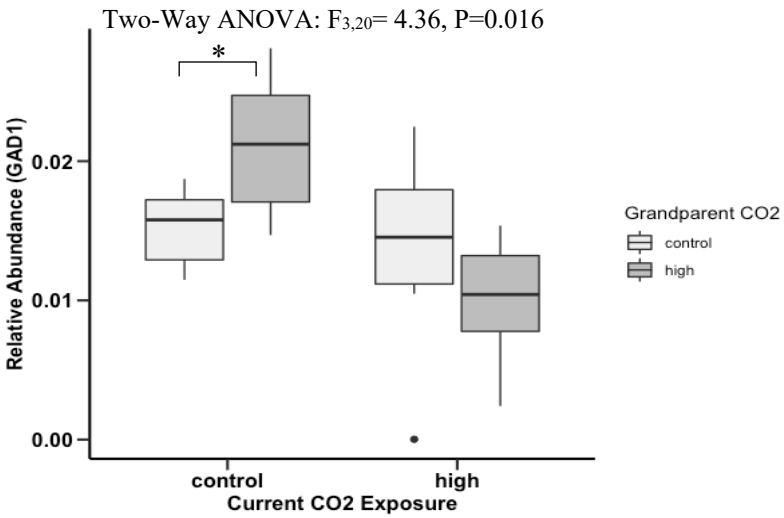

Figure 4.24 Filial generation two (F_2) principal component two (PC2) relative abundance of whole brain tissue genes with factor loadings ≥ ±0.5 by current CO_2 exposure and grandparent CO_2 exposure condition interaction term. PC2 was significant for transgenerational exposure, in which fish were exposed to elevated CO_2 in grandparent generation but not in the current or parental generation. PC2 genes with significant loading factors include: *follicle stimulating hormone beta subunit (fshb), lutenizing hormone beta subunit (lhb), glycoprotein hormones alpha chain (gtha), relaxin 3b (rln3b), growth hormone (gh), gamma-aminobutyic acid receptor subunit alpha-2 (gabra2)* and *glutamate decarboxylase 1 (gad1).* Two-way anovas were performed for each individual gene, of which *gtha* differed significantly for three generational CO_2 exposure versus three generational control CO_2 exposure (TukeyHSD: $p = 0.02$), and for two-generational CO_2 exposure (F_1 and F_2) relative to three generational CO_2 exposure (TukeyHSD: $p = 0.03$). Three generational elevated CO_2 versus transgenerational CO_2 exposure (P but not F_1 or F_2 exposure) resulted in significant relative mRNA abundance for genes *gabra2* (TukeyHSD: $p = 0.013$) and *gad1* (TukeyHSD: $p = 0.010$).

CHAPTER 5

Discussion

Chapter 5: Discussion and Conclusions

5.1 Discussion

The primary objectives of this thesis were to 1) examine if behavioural phenotype in response to acute elevated CO_2 resulted in within and across generational differences in growth, behaviour, reproduction, and brain mRNA abundances following exposure to chronic elevated CO_2; and 2) investigate whether exposure to elevated CO_2 conditions across multiple generations influenced growth, behaviour, reproduction, and mRNA abundances. I hypothesized that 1) behavioural phenotypes would influence how a fish responds to elevated CO_2 because repeatable behavioural variation has previously been linked to differences in an animal's physiology and life history traits which can impact how an organism responds to environmental stressors, and 2) that elevated CO_2 exposure would affect a fish's growth, reproduction, and behaviour due to differential energy allocation strategies which would prioritize acid-base acclimatization efforts.

Behavioural phenotypes did not have a significant effect on mRNA abundance as was expected but did influence reproduction for P and F_2 generation fish, and growth for F_1 and F_2 fish; suggesting that intraspecific variation in behavioural phenotypes may partly influence how medaka respond to elevated CO_2. Multigenerational exposure to elevated CO_2 in this study were shown to improve the performance of offspring in some measures, however transgenerational exposure, where a parent or grandparent was exposed to elevated CO_2, but the offspring were not exposed to elevated CO_2, resulted in some deleterious effects suggesting that environmental changes may put fish especially at risk. In this study, current CO_2 exposure appeared to be the best predictor of overall condition, where fish exposed to elevated CO_2 were worse off than fish exposed to control CO_2 conditions. A summary of generational CO_2 exposure effects can be seen in Figure 5.1.

Behavioural phenotype had some effects on responses to CO_2

In this study, the goal of behavioural phenotyping was to capture consistent behavioural tendencies of fish who were, relative to the population mean, either highly responsive to changes in elevated CO_2 or were non-responsive and behaving rigidly to change in CO_2. Within the parental (P) generation, the behavioural phenotype of the fish and its interaction with exposure to CO_2 was found to affect reproductive measures after a 6-week elevated CO_2 exposure. Specifically, under elevated CO_2 conditions, responder behavioural phenotype fish had smaller eggs compared to non-responder fish who had larger eggs but 72% fewer eggs per female per day. The high reduction in number of eggs per day for non-responder behavioural phenotype fishes was expected, particularly on a short-term scale, where fish who are less behaviourally flexible are more likely to be less adapted to acute changes in the environment (Coppens et al., 2010; Archard et al., 2012; Baker et al., 2018). Additionally, the increased egg size but reduced number of eggs may reflect a difference in reproductive investment and thus life history strategy or risk mitigation 'decisions' as would be expected for differences between responder and non-responder phenotypes (Wolf et al., 2008).

The negative affects of non-responder phenotype reproduction observed in the parental generation were seemingly eliminated in the F_1 and F_2 generations. For example, F_2 generation fish who had grandparents with the non-responder behavioural phenotype had more eggs per day per female relative to fish who had grandparents of responder behavioural phenotype. This finding of elimination of deleterious effects for reproductive measures is important because it may indicate that some individuals in a population are more immediately at risk to changes in environmental CO_2 but that long-term acclimation to elevated CO_2 may be possible (Munday, 2014). A similar finding to mine was observed by Welch and Munday (2017) who found that

behavioural phenotypes can significantly affect a fish's responses to elevated CO_2 conditions, but that long term exposure to elevated CO_2 may eliminate these effects. That is, benefits of being a behaviourally flexible responder fish will only benefit said fish compared to a non-responder fish when the environment has changing parameters. Additionally, this finding may relate to the hypothesis that animals with low acute reactivity to environmental change may be more likely to make decisions that rely on more detailed information and that may benefit the organism in the long-term if change in the environment is stable (Coppens et al., 2010).

The observed differences in reproductive and morphological measures of fish with differing behavioural phenotype lineages are consistent with studies that have found that behavioural phenotypes can result in differences in other physiological measures such as relative mRNA abundance and measures of stress (Baker et al., 2018; Archard et al., 2012). An explanation for why such behavioural phenotypes correlate with other physiology measures is that these animals may have underlying variation in brain circuitry in the prefrontal cortex or in neurochemical signalling pathways such as the dopaminergic pathway, that link a suite of biological functions (Coppens et al., 2010). In my study, underlying differences in the relative abundance of mRNA in the brain for genes of interest were not found to be different by behavioural phenotypes and therefore my data do not support a connection between behavioural phenotypes and my genes of interest. Repeatable behavioural phenotypes are expected to have a heritable component (Dingemanse, et al., 2002) and thus I had expected to find significant variation in the relative mRNA abundance of the genes measured. Phenotypes are plastic in response to environmental change, but it is genetic diversity that ultimately determines the plasticity possible for a population and species (Crozier and Hutchings, 2013). Schunter et al. (2018) in spiny damselfish (*Acanthochromis polyacanthus*), for example, found that the offspring of parents behaviorally more 'tolerant' or 'sensitive' to CO_2 had significantly different variations

in their transcriptomes (i.e., mRNA) and proteins which was not found in the present study. Notably, my behavioural phenotyping methods study differed from Schunter et al., (2018) methods in several ways. For example, behaviour for conspecific chemical alarm cue responses in a two-way flume test was used by Schunter et al. (2018) to measure phenotypes, while responses to elevated CO_2 in a novel tank diving and shuttle box behaviour assay was used in my study. However, due to the lack of significant and consistent variation between phenotypes, as was expected, especially in parental generation fish whose behavioural phenotypes were captured, it is possible that a repeatable behaviour phenotype was not captured, or that the behaviour phenotype captured was not significantly impacted by elevated CO_2 as intended. This is consistent with other research that has been unable to identify repeatable behavioural responses to CO_2 (Clark et al., 2020).

Additionally, the sample size for CO_2 and phenotype interaction effects were small, especially in the parental generation (n = 9–10 per group), and therefore repeating a similar study with greater samples sizes and that measures repeatability and heritability of behavioural phenotypes would be important. For example, parental generational phenotype and CO_2 exposure was significant for directionality, although a discernable trend was not found and the model itself was not significant (Table 4.2). Because lateralization has been shown to be non-repeatable in several fish species (Roche et al., 2020), that there was a low sample size in interaction effect groups, that no other behaviours were affected for this generation, and that behaviours tend to be most non-repeatable when environmental variations are involved (Bell et al., 2009), it is thought that the significant effect on directionally by phenotype and current CO_2 exposure in parental generation may be a false positive.

Phenotypic variance in a population is important because it can allow a population and species to be more resilient to environmental change (Crozier and Hutchings, 2013) and has been

found to affect how individuals respond to elevated CO_2 (Schunter et al., 2018). To determine how repeatable behaviour phenotype effect a populations more accurately, an in-depth study on how repeatable behavioural phenotypes affect responses to elevated CO_2 and other corresponding variations of physiology and gene expression is recommended for future research.

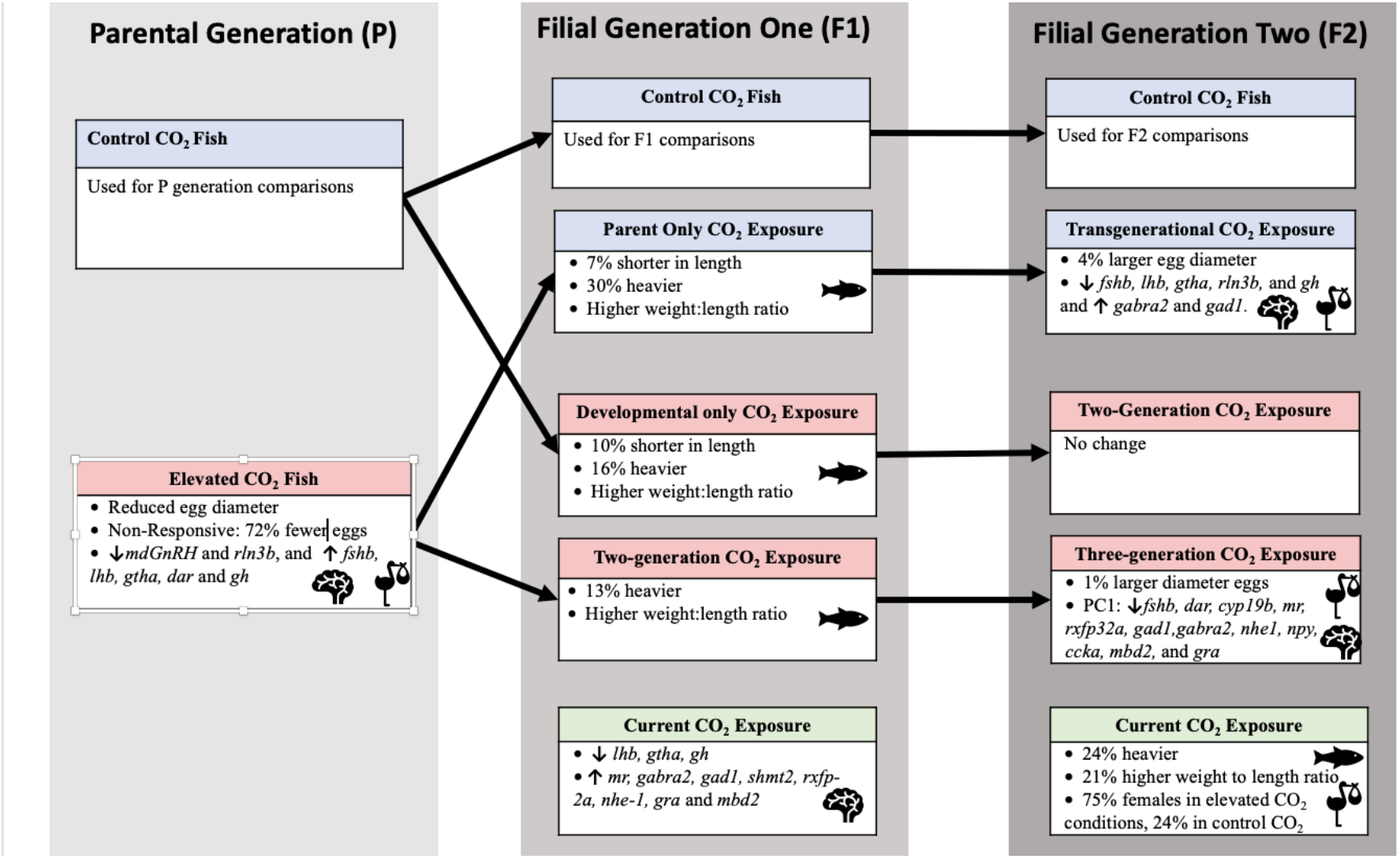

Figure 5.1 Summary of results for generational CO_2 exposure effects. Red title backgrounds represent fishes exposed to elevated CO_2 as a current generation CO_2 exposure, while blue title backgrounds represent control CO_2 exposures. Arrows indicate the groups of

which eggs were randomly selected into. Green title background blocks are effects observed in all fishes currently exposed to elevated CO_2 compared with fishes exposed to control CO_2 within each generation. Black image icons are quick visual references of effects seen in general grouping of effects. Brain icon represents significant changes found in the relative abundance of mRNA of some genes, stork with bag represents significant effects on reproduction, and fish icon represents changes in body condition.

Multigenerational acclimation may occur for some measures

For clarity, epigenetics refers to any heritable effect on an offspring's phenotype that is due to factors other than DNA sequences, such as DNA modification, and nutritional provisioning (Bhandari, 2016). Multigenerational exposures are those where a fish is currently in an elevated CO_2 exposure condition, and whose parents and/or grandparents have also been exposed to elevated CO_2. The three groups in this study where effects of multigenerational exposures were applicable are, 1) fish exposed to elevated CO_2 during both development and adulthood, but whose parents were not exposed to elevated CO_2 (I have referred to this group as "F_1 two-generation exposure"); 2) fish exposed to elevated CO_2 in F_1 and F_2 generation, but not P generation (I have referred to this group was F_2 two-generation exposure); and, 3) fish that were exposed to elevated CO_2 across P, F_1, and F_2 generations (I have referred to this group as "three generation elevated CO_2 exposure group").

In the F_1 generation, fish that experienced a developmental exposure to CO_2 were shorter and fatter relative to control fish, while F_1 fish who experienced two-generation exposure to CO_2 were less overweight and did not significantly differ from control conditions. This finding suggests that parental exposure to elevated CO_2 reduced effects on length and weight of fish exposed to elevated CO_2 conditions. This result is the best example in my study that suggests that multigenerational acclimation may have occurred. Previous studies have found similar multigenerational acclimatization effects after parental exposure to elevated CO_2. For example, in a study on Trinidadian guppies (*Poecilia reticulata*), offspring growth was not affected in fish maternally exposed and acclimatized to elevated CO_2 but was affected in fish who did not have parental exposure to elevated CO_2 (George et al., 2019). Additionally, reduced growth and weight of cinnamon anemonefish (*Amphiprion melanopus*) was observed after exposure to elevated CO_2

but was not observed when parents had also experienced elevated CO_2 exposure (Miller et al., 2012). My finding and these studies demonstrate that the parental environment of a fish may mediate effects of exposure to elevated CO_2 on growth and condition.

Three generation exposure to elevated CO_2 conditions resulted in larger average egg size and a significant variance of the relative mRNA abundance of some genes of interest, relative to control fish. Genes of interest that changed in relation to CO_2 exposure were *follicle stimulating hormone beta subunit* (*fshb*)*, dopamine receptor* (*dar*)*, cytochrome P450 19b* (*cyp19b*)*, mineralcorticotropin hormone receptor* (*mr*)*, relaxin family peptide receptor 3-2a* (*rxfp3-2a*)*, glutamate decarboxylase 1* (*gad1*)*, gamma-aminobutyic acid receptor subunit alpha-2* (*gabra2*)*, sodium-hydrogen antiporter 1* (*nhe-1*)*, neuropeptide Y* (*npy*)*, cholecystokinin* (*ccka*)*, methyl CpG binding protein 2* (*mbd2*)*,* and *glucocorticoid receptor alpha* (*gra*), all of which reduced in relative mRNA abundance for CO_2 fish relative to control fish. Those that were found to significantly differ for three-generation exposure in individual anovas, and thus are thought to especially contribute to this finding, were *dar, rxfp3-2a,* and *ccka* which were all lower in relative mRNA abundance of high CO_2 fish, relative to control fish. In teleost fish, dopamine has an inhibitory effect on GnRH and other hormones of the HPG axis, and thus a reduction of *dar* receptors suggests reduced inhibition of the HPG axis, consistent with an increase of egg size (Dufour et al., 2010). *Ccka* is an anorexigenic (appetite-reducing) agent, and *rln3b/rxfp3-2a* has been implicated in stress modulation among other functions such as feeding metabolism and reward (Dalmolin et al., 2015; Alnafea et al., 2019; Ganella et al., 2013). A reduction in the relative mRNA abundance of these genes, suggests it is possible there was reduction of appetite-reducing agent effects and may also implicate reduced anxiety and stress related feeding, which suggests some reduced effects of stress on the feeding axis for fish with long-term acclimation to elevated CO_2. Notably, F_2 three-generational exposure and two generational exposure fish

(current CO_2 exposure) both had an increased weight relative to control elevated CO_2 condition fish demonstrating that increased eating behaviours were not eliminated due to multigenerational exposure.

Reduced *gr* expression and changes in methylation have been correlated with early-life stress (Cunliffe et al., 2016), and reduction of *gr* and *mr*, which are thought to coregulate the stress axis in fish, can lead to excess circulating cortisol (Faught and Vijatan, 2018). An observed reduction of *mr* and *gr* therefore suggests that three generational exposures resulted in chronic effects on the HPI axis. Chronic elevated cortisol can result in reduced reactivity to this hormone in some tissues and may explain why a reduction of *ccka* and *rxfp3-2a* was observed. Changes in *mbd2* also suggests that long term changes in epigenetic expression of genes may have occurred since mbd2 is a methylation factor that tends to bind preferentially to promotor regions of genes (Fan and Hutnick, 2005) and has been found to significantly influence multigenerational effects of stress and is implicated in persisting through the germ-line (Crudo et al., 2012). These changes appear to be in the benefit of the offspring, since no apparent deleterious effects were observed for this group. However, as noted above, fish held in elevated CO_2 for F_2 generation were found to have significantly higher weights, and a significantly larger female to male ratio. Since current elevated CO_2 exposure for F_2 generation includes both three-generation and two-generation CO_2 exposure effects, effects on fish that can affect populations occurred, regardless of multigenerational acclimation. In future studies, comparing multigenerational acclimatized CO_2 fish to multigenerational control fish following an acute elevated CO_2 exposure would be recommended to look at short-term acclimation differences observed during a high CO_2 fluctuation event such as that expect with a pollution event.

Transgenerational exposure and changing environmental conditions affect fish

Transgenerational exposure group are F_2 fish whose grandparents were exposed to elevated CO_2 exposure but who were not exposed to elevated CO_2 themselves and neither were their parents. Transgenerational exposure did not have a significant effect on growth measures or behavioural measures in this study, but reproductive and relative mRNA expression was affected. Transgenerational exposure resulted in significantly larger egg diameter relative to control fish. Additionally, mRNA abundances differed based on transgenerational exposure, where *gabra*2 and *gad*1 had higher relative abundance, and *fshb, lhb, gth*a, *rln3b*, and *gh* had lower relative abundance, relative to control fish. A lower relative abundance of *fshb, lhb,* and *gth*a are inconsistent with the observed increased egg size, since a reduction of these hormones suggests downregulation of the HPG axis (Wootton and Smith, 2015). However, GABA can inhibit dopamine (DA) which typically has downregulatory effects on the HPG axis; thus, GABA can result in indirect upregulation of the HPG axis (Wootton and Smith, 2015). Overall, transgenerational effects were observed in this group, particularly in mRNA abundance, although no clear deleterious effects or benefits from these changes were apparent.

F_1 parental only exposure fish, who were not exposed to elevated CO_2, but whose parents were exposed to elevated CO_2 exposure, were 10% shorter in length and 30% heavier than control fish. A possible mechanism for this finding will be explained in further detail in following paragraphs, but briefly, both shortened length and increased weight is thought to be a possible deleterious effect and suggest the stress axis has been chronically activated. Reduced body condition is indication that exposure to environmental conditions that differ from those of their parents may increase stress. This implies that although grandparent or parental exposure to elevated CO_2 can benefit the offspring when the offspring are exposed to the same elevated CO_2 conditions, when the offspring are exposed to an environment that differs from those of its

parents, even if it is control CO_2 conditions, transgenerational effects can be detrimental to the offspring.

Elevated CO_2 correlates with shorter lengths and heavier fish

Weight, length, and size were not significantly impacted by elevated CO_2 exposures for parental generation fish; however, F_1 and F_2 fish were both significantly impacted and were smaller, but heavier. A non-significant body condition effect was expected for parental generation because adult fish are thought to have a better ability to metabolically acclimatize to the elevated CO_2 conditions relative to juvenile fish, and changes in size are less likely to be observed, especially since the rate of growth in adults is lower (Tseng et al., 2013). For F_1 and F_2 generation, a reduction in length as well as growth, was expected with the hypothesis that energy required to meet the metabolic demands required for acid-base regulation and acclimatization to elevated CO_2 would be allocated away from growth. A study examining the effects of CO_2 found reduced embryo survival in inland silverside (*Menidia beryllina*) exposed to elevated CO_2, and a reduced larval length (Baumann et al., 2012). Another study has found that growth rate and whole-body condition were reduced and a higher HCO_3^- concentration and energy expenditure was detected in sea bass exposed to elevated CO_2 (*Dicentrarchus labrax*) (Alves et al., 2020). Alternatively, other studies have found no effect on growth in response to elevated CO_2 (Sundin et al., 2019; Hurst et al., 2021) while others have found that growth increases in response to elevated CO_2 (Chambers et al., 2014).

For F_1 fish, both elevated parental CO_2 only exposure and elevated developmental CO_2 exposure (i.e., current CO_2) resulted in shortened overall length of adult F_1 fish. A decrease in length in parent only CO_2 exposure fish suggests that epigenetic inheritance occurred but did not benefit offspring for this measure. Within generation developmental effects on body condition

suggest that developmental exposures are more susceptible to body condition effects of elevated CO_2. The relative mRNA abundance of *gh* was found to decrease significantly with F_1 PC1 and may explain this reduction of size. This finding is consistent with other studies that have found early development to be an especially vulnerable stage (Tseng et al., 2013). Two possible reasons for the finding of reduced length is: uncompensated acidosis in the body is thought to lead to metabolic depression that can result in reduced length (Tseng et al., 2013); and energy allocation towards increased energy expenditure for elevated CO_2 acclimation (Baumann et al., 2012; Alves et al., 2020; Miller et al., 2012; Jutfelt et al., 2013).

Parental only, developmental, and parental-and-developmental (F_1 two-generation exposure) elevated CO_2 exposure all resulted in an increased total weight of the adult F_1 fish, and higher weight to length ratios relative to control fish. This was an unexpected finding because previous literature has found a reduction in body weight after exposure to elevated CO_2 (1,184 ppm) (Tseng et al., 2013). A suggested mechanism for this finding is the chronic stress response. Chronic stress conditions have been found to increase stress hormone CRF and orexigenic rln3 in rats which corresponded to an increase in eating behaviours and a gain of body fat weight (Lenglos et al., 2013). The stress hormone norepinephrine (NE) is also known to have inhibitory effects on GH, GnRH, and DA (Peng and Peter, 1997). This hypothesis is reflected by the PC2 relevant loading factors that significantly differed by CO_2 exposure of F_1 generation in which *rxfp3-2a, gabra2, mr* and *gra* relative abundance of mRNA increased in expression, and *gh* decreased in expression. An increase in *mr* and *gra* are consistent with CO_2 influencing the HPI axis (Faught and Vijayan, 2018). The Rln-3/RXFP3 system is also related to stress, as well as growth, as it has been linked to stress-induced binge eating behaviours in rats (Calvez et al., 2016) and a fat weight gain (Lenglos et al., 2013). Finally, GABA has been observed to reduce GH and is thought to do so by inhibiting DA's effect on GH, which normally elicits GH secretion

(Canosa et al., 2007). Obesity in humans is typically correlated with reduced expression of GH, and reduced GH expression is thought to act to conserve lipid deposit stores when energy is readily available, as was possibly true for the medaka in this study (Company et al., 2001). Overfeeding is another strong implication for these findings. Overfeeding of fish can result in tissue resistance to lipolytic effects of GH and a high degree of adiposity (Company et al., 2001).

F_2 fish exposed to elevated CO_2 had higher weights, higher size ratios, and a significantly higher female:male ratio relative to control CO_2 exposure fish. The higher female to male ratio contrasts with prior literature which has found that under elevated stress conditions during development, the number of intersex males (XX) increased resulting in a higher male to female ratio (Nada et al., 2003). However, in a study on medaka, male to female sex reversal in response to environmental estrogens and stress was observed (Kuhl et al., 2005), which may explain the findings in my study if the increase weight (i.e., adipose tissue) resulted in a higher concentration of estrogen in fish exposed to elevated CO_2. Adipose tissue is where a significant proportion of estrogen is converted from testosterone or androstenedione via the aromatase enzyme. In human cases, increases in adipose tissue can be associated with increased aromatase and estrogen (Rubinow, 2017). In my study three-generation exposure fish had reduced *cyp19b* which conflicts with this hypothesis, although it is possible the function of this gene is most important during developmental stages (Kinoshita et al., 2009; Kuhl et al., 2005). Measuring this mRNA abundance during larval stages is therefore recommended in future studies that measure sex ratios.

Future studies observing larval growth and size is also recommended, since although size was not measured in larvae fish in this study, a noticeable difference in the rate of growth between high and control groups was obvious, particularly in F_2 larvae in which larvae from high CO_2 conditions were unable to be transferred from a sieved larvae tube into a normal tank until

two weeks after the control CO_2 larvae, which met the required ~1cm length earlier than high CO_2 fish. Thus, although adult medaka lengths were not significantly affected by elevated CO_2 outside generation one parental only and developmental exposure, since larvae are thought to be most susceptible to stunted and delayed growth, future studies measuring larvae length is recommended (Tseng et al., 2013).

Additionally, mold growth on some fish's gills was found on fish in parental generation and F_1 elevated CO_2 fish, but not control CO_2 fish. Water mold is a mold that exists in the air and tends to form on unhatched eggs in aquarium systems. While it is unlikely for this mold to impact fish when mold is promptly removed, fish in poor physical condition can be infected (Kinoshita et al., 2009).

Overall, current CO_2 exposure was the biggest determination of effects on body condition, where fish exposed to elevated CO_2 were more likely to be shorter in length and have an increased body fat which is most consistent with a chronic stress response.

Reproduction is negatively affected by elevated CO_2 exposure

Parental generation egg diameter was smaller on average for fish in elevated CO_2 conditions relative to control CO_2 conditions. Fish in elevated CO_2 conditions also had a reduced number of eggs collected per female per day relative to control groups. Reproduction is energetically expensive, and the demand for ATP (energy) for facilitating acid-base regulation required for long term exposure to elevated CO_2, may be averted away from reproduction which may be observed in low egg number and nutrient allocation as measured by reduced egg size (Heuer and Grosell, 2014). My findings in P generation were consistent with the hypothesis that assumed both elevated stress and energy allocation to acclimatize to elevated CO_2 conditions

would result in a suppression of the reproductive axis in fish (Heuer and Grosell, 2014; Miller et al., 2012; Kinoshita et al., 2009).

Reproductive relevant genes were included in P generation PC1 significant loading factors that were deemed to be related to CO_2 exposure. Specifically, *mdGnRH*, was lower in relative mRNA abundance in elevated CO_2 fish and *da* was higher in relative abundance, both of which suggest downregulation of the HPG axis. However, *fshb*, *lhb*, and *gtha* genes all increased in expression, which is contrary to what would be expected with HPG axis downregulation. To the best of my knowledge, there has not been literature on fish that reports this combination of findings. However, literature on human subjects suggests that higher-than-normal levels of LH and FSH in conjunction with reduced success in egg size and number of eggs can indicate ovarian/testes problems (Jankowska, 2017). Specifically, in females, this can indicate premature ovarian failure which can be caused for a suite of reasons including environmental toxins, or problems in receptor and negative feedback cycles for estrogen which typically causes negative feedback on the pituitary for release of FSH (Jankiwska, 2017). Notably, reproductive cycles and hormone concentration can vary by cycles and age, and thus it is also possible that fish in elevated CO_2 were not as developmentally mature (Kinoshita et al., 2009).

Hatch success did not differ by CO_2 exposure in the parental or F_1 generation (hatch success was not monitored in the F_2 generation). This finding is consistent with previous literature in which survival has only been affected by CO_2 in a small number of studies (Heuer and Grosell, 2014). Of these studies, survival seems to be most affected when individuals are exposed during the egg stage, less so in larvae stages and seldom affected post-larvae stages (Heuer and Grosell, 2014). Although the eggs in generation one was exposed to elevated CO_2 in this study, the concentration of this exposure may have not been high enough to cause a significant effect in hatch success. Additionally, hatch success was measured using eggs that

were deemed "viable" that is: eggs that were moldy and/or brown were not added to an incubator tube. While unfortunately not quantified, a disproportionally large amounts of "dead" eggs were found for fish in elevated CO_2 conditions in comparison to control conditions. In one study, elevated CO_2 did not affect clutch size, but did increase the occurrence of egg loss and embryotic abnormalities in two-spotted goby fish (*Gobiusculus favescens*) (Forsgren et al., 2013). This suggests that had hatch success been determined by all eggs, and not only those pre-selected, that this measure may have been significant. Future research practicing this method is highly recommended.

Lack of behavioural responses to elevated CO_2

Behaviour was the least significant measure in this study, where P, F_1, and F_2 all did not result in any significant differences in behaviour by terms related to elevated CO_2 exposure. However, for F_1 current elevated CO_2 exposure, and F_2 three generation exposure and transgenerational exposure comparisons, the relative abundance of *gad1* (glutamate → GABA enzyme gene) and *gabra2* (receptor gene) increased. This suggests it is possible GABA pathways are affected by elevated CO_2 conditions and that acclimation occurred so that behaviours were not significantly affected. One suggestion for how this could occur is through downregulation of $Na^+/K/2Cl^-$ cotransporter and/or upregulation of $K^+/2Cl^-$ which would restore a more normal chemoelectrical gradient concentrations of Cl^- which would reduce GABA receptor reversal (Tresguerres and Hamilton, 2017). Many post-transcriptional modifications also exist, including phosphorylation and protein kinase activity which can change the channel opening frequency of GABA receptors (Mele et al., 2016). The affinity of GABA, the interaction of regulatory proteins, and distribution on a synaptic cleft are also all factors that could affect the function of $GABA_A$Rs and may explain why no effect on behaviour was observed by the higher relative

mRNA abundance of *gad1* and *gabra2* (Mele et al., 2016). In this study, effect on *gaba* and *gad1* may have been more influential on other measures, such as reproduction.

A study by Hamilton et al., (2021) found that zebra fish (*Danio rerio*) behavioural responses to elevated CO_2 were non-linear with dosage, in that at weak levels (900 μatm) resulted in increased anxiety-like behaviour, moderate levels (2200 μatm) had no difference in behaviour and high levels (4200 μatm) resulted in a decrease in anxiety like behaviours. It is therefore also possible that similar non-linear effects on behaviour in the medaka in this study occurred, and that the elevated CO_2 conditions were at a concentration which elicited normal behavioural responses. A study that measures dose dependent effects on medaka is suggested to assess this possible influence.

5.2 Limitations

A laboratory and in-bred strain of medaka were used in this study. In zebrafish (*Danio rerio*), another commonly used model organism, one study found that laboratory zebrafish have significant genomic variation from their wild counterparts (Whiteley et al., 2011). Likely, laboratory-bred medaka also show some variation in their genomes and gene expression in comparison to their wild counterparts, and thus findings in our study cannot be assumed to represent a true wild population. Because CO_2 fluctuates normally in ecosystems due to diurnal and seasonal effects, the exposure of CO_2 in this study which did not account for these differences, also cannot be directly compared to CO2 exposure as would be expected in situ. Additionally, because changes in CO_2 are directly related to pH changes, CO_2 cannot be concluded as the single manipulated variable in my study (i.e., both CO_2 and pH are manipulated variables). Another notable limitation is that medaka were fed to satiation throughout my study.

Previous research has found that fish who are fed in excess can compensate for increased energy demands of stress and acclimation which reduce the negative effects that might otherwise be seen in ecologically equivalent food conditions (Miller et al., 2012). Thus, constraining a fish's diet to reflect normal environmental food limitations may reveal stronger differences between control and experimental conditions in future studies (Miller et al., 2012).

In this study, whole brain tissue was used to measure the relative abundance of genes of interest. Because genes can be differentially affected in different brain regions, measuring these genes in whole brain tissue rather than specific brain areas (i.e., the pituitary) may have masked region-specific changes.

Due to limited access to the vivarium and laboratories during COVID-19, fish in P, F_1, and F_2 generation significantly differed in age at the time that measurements were captured from the fish and from their date of dissections. Due to expected difference between ages in the measures captured, comparisons could not accurately be made between generations as originally expected, and only within generation differences were considered.

Finally, because female fish in the parental generations bear their entirety of non-fertilized eggs in their ovaries, unfertilized F_1 fish would have also been exposed to elevated CO_2 if their maternal parent had, as would the eggs have carried within F_1 fish (Lacal and Ventura, 2018). This means that F_3, which was not observed in this study, would be the first true generation of which transgenerational effects could be observed, thus true transgenerational effects cannot be concluded.

5.3 Broad Implications

One way to protect aquatic ecosystems from negative effects caused by elevated CO_2 is to reduce greenhouse emissions. Decreasing greenhouse gas emissions may be done through several ways. For one, restrictions on industry emissions of greenhouse gas could be more strictly regulated, which may look like taxations or penalties if an industry produces more emissions than allocated, or rewards if they produce less than allocated. Additionally, citizens should be included in the responsibility to decreased emissions. Two examples could be by increasing public education efforts or implementing programs to support and reward individuals who attempt to reduce their own footprint. Education on one hand will provide a reason why individuals should make an effort to change their lifestyles, while free programs will help individuals gain easy access to emission decreasing strategies and resources. These strategies can be implemented at any level of government.

Freshwater fish are important members to ecosystems and contribute to and support several ecosystem services including provisional services, regulatory services, cultural services, and supporting services (TEEB, 2011; McIntyre et al., 2016). Provisional services are those that provide direct use products such as food and water, regulatory services are services which manage larger systematics or services such as regulating regional and global climate or water quality, cultural services are indirect use services such as recreation and tourism, and supporting services include soil formation, and photosynthesis (TEEB, 2011).

Food security is one major form of provisional service offered by freshwater fishes (McIntyre et al., 2016). Globally, freshwater fishes provide all dietary animal protein for an estimated 158 million people (McIntyre et al., 2016). In 1995 fishery biomass included over 101 million tonnes of fish from capture freshwater fisheries and 11 million tonnes from aquaculture fisheries (Holmlund and Hammer, 1999). Cultural services are also provided by freshwater fishes

largely in the form of recreation activities (i.e. angling) (Holmlund and Hammer, 1999). Regulatory services by freshwater fishes include regulating freshwater population dynamics and nutrient availability, bioturbation in or near sediments and carbon exchange (Holmlund and Hammer, 1999). For population dynamics, this includes fish being consumed and consuming other organisms, but also includes impacts of fish waste products, eggs, young, and carcasses. Bioturbation is physical disturbance and reworking of sediment/soils by various activities such as burrowing or foraging (Holmlund and Hammer, 1999). Bioturbation has major impacts on ecosystems and biodiversity, in part, by making invertebrates and nutrients from the sediment more available in the water column. Changing physical elements in sediments also is a major benefit of bioturbation because it creates diversity in the topography of the bottom of freshwater ecosystems, which enables more diverse inhabitants (Holmlund and Hammer, 1999). Other trickledown effects of fish presence in freshwater ecosystems include regulatory influences on water flow, temperature, upwellings, storms, seasonal variability, nutrient content, diseases, and water depth (Holmlund and Hammer, 1999). Effects on reproduction and growth were both observed in my study, which can be acted upon by natural selection and can reduce recruitment success, ultimately resulting in reduced population size. A decline in freshwater fishes from elevated CO_2 would threaten human health, food accessibility, and would significantly limit the array of ecosystems services provided by fresh water (Dudgeon et al. 2006).

5.4 Conclusions

The results in this study suggest that while behaviour phenotypes and transgenerational or multigeneration effects may have changes in specific responses to elevated CO_2, elevated CO_2

exposure generally, is the best predictor of effects on fish including reproductive measures, morphological measures, and expression of mRNA in the brain.

By measuring several metrics of physiological and whole-organism responses, this project can contribute to building a more comprehensive understanding of how a freshwater fish species will respond to future elevated CO_2. This understanding will help us decipher the relationship between molecular and genetic differences with differences in phenotypes that can be acted on by natural selection. Understanding this relationship is especially important because it provides a mechanism by which biodiversity will change in response to climate change (Bonduriansky and Day, 2009). Non-genetic inheritance fills the gap between short term acclimation strategies of an individual and long-term adaptations and can have significant effects on the evolution of a species (Jablonka et al. 1995; Bonduriansky and Day, 2009).

Acute and developmental effects measured in my project help provide an understanding of immediate elevated CO_2 effects on growth, behaviour, and reproduction. This information may be relevant for application in aquaculture and global fisheries where elevated CO_2 conditions as high as 10,000–24,000ppm have been observed in some intensive aquaculture settings (Brauner et al*.,* 2019). Additionally, my data may also be relevant for application in developing invasive species management strategies because induced elevated-CO_2 in natural systems is being considered to deter invasive species movement and thus their spread (Treanor et al., 2017). Most importantly, because this study explored the effects of elevated CO_2 across generations at time scales more representative of what will happen in natural ecosystems, this study helps provide a better understanding about the potential threat of rising CO_2 in freshwater ecosystems on freshwater fish. It also gives an indication that exposure across multiple generations can mediate some negative consequences of exposure to elevated CO_2 and that epigenetic factors may be a source of some heritable effects.

Ultimately, this is important because our ability to preserve aquatic ecosystems is related to our ability to understand and effectively communicate the challenges they face. Freshwater ecosystems are important for food and water security, recreational activity, and are vital components of climate and the health of our ecosystems, making this research relevant to our broader society (Dudgeon et al*.,* 2006). Global fish stocks are projected to collapse by 2048 if significant changes in aquatic ecosystem management are not put into effect, and as of 2006 an estimated 29% of edible fish stocks had declined by 90% (TEEB, 2011). Carbon dioxide is one threat to aquatic ecosystem, and since some fresh-water ecosystems have already reached predicted end-of-century CO_2 concentrations, there is a sense of urgency to understand the implications of elevated CO_2 on freshwater organisms (Weiss et al*.,* 2018).

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APPENDIX

Table A.1 Fish identification number for brain tissue pooling for biological replicates to attain 2µ of RNA.

Parental Generation (P)						
	Responder Phenotype			**Non-Responder Phenotype**		
CO_2 Exposure	**Bio Rep 1**	**Bio Rep 2**	**Bio Rep 3**	**Bio Rep 1**	**Bio Rep 2**	**Bio Rep 3**
Control CO_2	1,2,4	3,5,8	6,7,9	1,2,4	5,6,7	8,9,10
High CO_2	1,2,3	4,5,6	7,8,9	1,2,3	4,5,6	7,8,9
Filial Generation One (F_1)						
Parental phenotype (R= responder, N= non-responder)						
	Female			**Male**		
CO2 Exposure	**Bio Rep 1**	**Bio Rep 2**	**Bio Rep 3**	**Bio Rep 1**	**Bio Rep 2**	**Bio Rep 3**
Control CO_2 current High CO_2 Parents	N 1,3 R 1	N 6 R 5,6	N 8,9 R 7	N 3 R 1,3	N 4, 5 R 4	N 7 R 9,10
Control CO_2 current Low CO_2 Parents	N 1,3 R 1	N 5 R 2,5	N 6,7 R 8	N 4 R 3,4	N 4,8 R 6	N 10 R 7,9
High CO_2 High CO_2 Parents	N 2,5 R 1	N 8 R 4,6	N 9,10 R 7	N 1 R 2,3	N 3,5 R 5	N 6 R 8,10
High CO_2 current Low CO_2 Parents	N 1 R 2,3	N 3,8 R 4	N 5,7 R 8	N 6 R 1,5	N 9 R 6,7	N 10 R 10,15
Filial Generation Two (F_2)						
	Responder Phenotype			**Non-Responder Phenotype**		
CO_2 Exposure	**Bio Rep 1**	**Bio Rep 2**	**Bio Rep 3**	**Bio Rep 1**	**Bio Rep 2**	**Bio Rep 3**
Control CO_2 current High CO_2 Parents	1,2,3	4,5,6	7,8,9	1,2,3	4,5,7	6,8,9
Control CO_2 current Low CO_2 Parents	1,2,3	4,5,6	7,8,9	1,2,3	4,5,6	7,8,9
High CO_2 High CO_2 Parents	1,2,3	4,5,6	7,8,9	1,2,3	4,5,6	7,8,9
High CO_2 current Low CO_2 Parents	1,2,3	4,5,6	7,8,9	1,2,3	4,5,6	7,8,9

Table A.2 Extracted ribonucleic acid concentrations and absorbance values from brain biological replicates. Impurities for all samples were "Guanidine ITC".

Parental Generation						
CO_2 Exposure		**Biological Replicate**	**Nucleic Acid Concentration (ng/µL)**	**A260/A280**	**A260/A230**	**Impurity mM**
Control		1	241.77	2.126	1.387	
Control		2	242.79	2.128	1.343	4.234
Control		3	251.82	2.126	1.429	4.031
Control		4	250.22	2.121	1.201	4.613
Control		5	185.39	2.124	0.987	4.843
Control		6	174.24	2.128	1.715	
High		1	254.96	2.133	1.695	
High		2	242.21	2.123	1.114	5.329
High		3	243.96	2.125	1.972	
High		4	269.51	2.133	1.591	
High		5	211.68	2.128	1.864	
High		6	222.46	2.073	1.490	
Filial Generation One						
CO_2 Exposure	**Parental CO_2 Exposure**	**Biological Replicate**	**Nucleic Acid Concentration (ng/µL)**	**A260/A280**	**A260/A230**	**Impurity mM**
Control	High	1	183.984	2.113	2.041	
Control	High	2	136.72	2.091	1.58	
Control	High	3	128.427	2.073	1.886	
Control	High	4	113.463	2.099	1.067	2.542
Control	High	5	135.686	2.099	0.872	4.871
Control	High	6	198.94	2.127	2.16	
Control	Control	1	99.785	2.076	2.29	
Control	Control	2	115.941	2.091	1.602	
Control	Control	3	105.496	2.09	1.472	
Control	Control	4	146.8	2.102	1.614	
Control	Control	5	130.406	2.105	1.267	
Control	Control	6	145.189	2.084	1.969	
High	Control	1	144.412	2.111	2.06	
High	Control	2	167.792	2.106	1.999	
High	Control	3	175.265	2.118	2.138	
High	Control	4	228.48	2.125	1.886	
High	Control	5	209.028	2.119	1.426	
High	Control	6	172.336	2.1	1.111	3.849
High	High	1	233.474	2.135	2.011	
High	High	2	207.695	2.117	1.568	

High	High	3	214.598	2.12	2.073	
High	High	4	237.123	2.128	1.372	3.612
High	High	5	196.237	2.116	2.27	
High	High	6	175.994	2.119	1.915	
Filial Generation Two						
CO_2 Exposure	**Grandparent CO_2 Exposure**	**Biological Replicate**	**Nucleic Acid Concentration (ng/µL)**	**A260/A280**	**A260/A230**	**Impurity mM**
Control	High	1	384.743	2.148	2.322	
Control	High	2	158.752	2.125	1.545	
Control	High	3	206.652	2.118	2.135	
Control	High	4	183.606	2.128	1.499	
Control	High	5	181.835	2.126	2.24	
Control	High	6	150.213	2.113	2.081	
Control	Control	1	249.598	2.135	2.076	
Control	Control	2	255.112	2.143	1.995	
Control	Control	3	197.523	2.133	1.993	
Control	Control	4	192.226	2.121	1.415	
Control	Control	5	185.994	2.123	1.821	
Control	Control	6	178.233	2.124	1.822	
High	Control	1	210.688	2.119	0.921	6.585
High	Control	2	223.912	2.127	2.112	
High	Control	3	266.445	2.122	1.223	5.002
High	Control	4	291.043	2.125	1.535	
High	Control	5	296.08	1.954	1.116	3.816
High	Control	6	239.949	2.003	1.181	3.553
High	High	1	238.156	2.139	1.966	
High	High	2	194.371	2.123	0.777	7.199
High	High	3	166.011	2.14	1.296	
High	High	4	138.081	2.083	1.136	2.85
High	High	5	171.624	2.123	0.991	4.277
High	High	6	246.041	2.136	1.656	

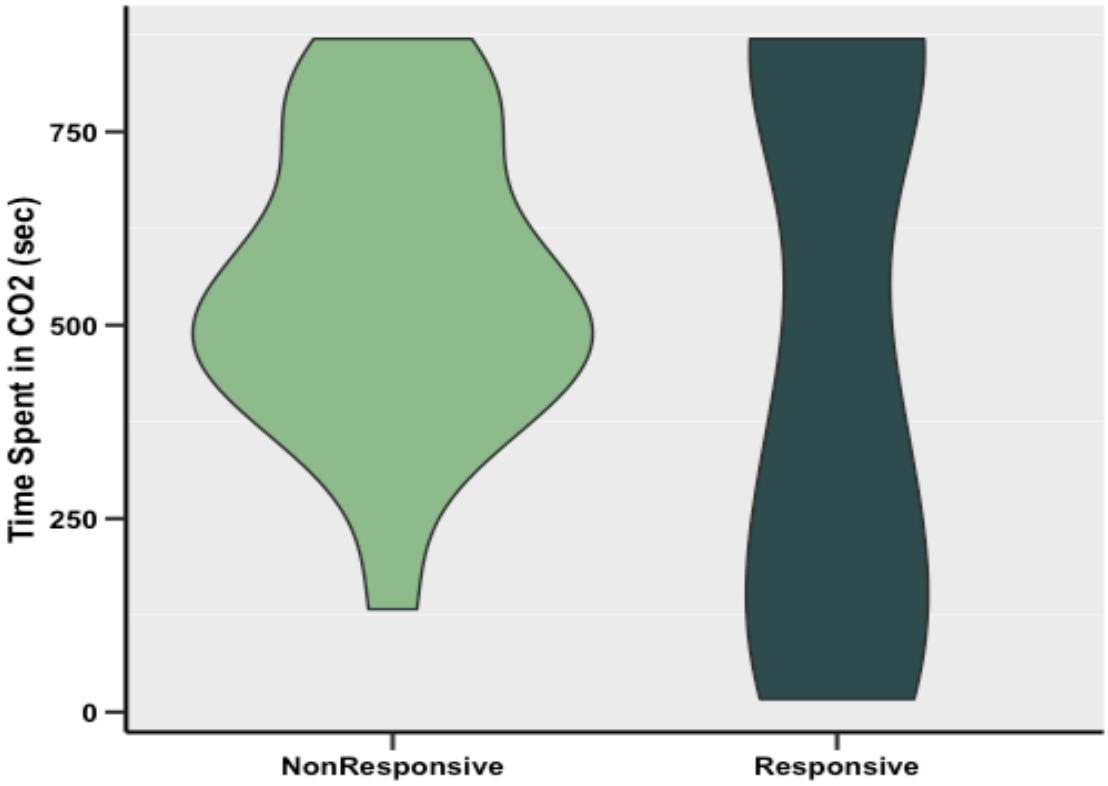

Figure A1. Visualization of time spent in CO_2 during phenotyping shuttle assay for fishes identified with non-responsive or responsive behavioural phenotypes.

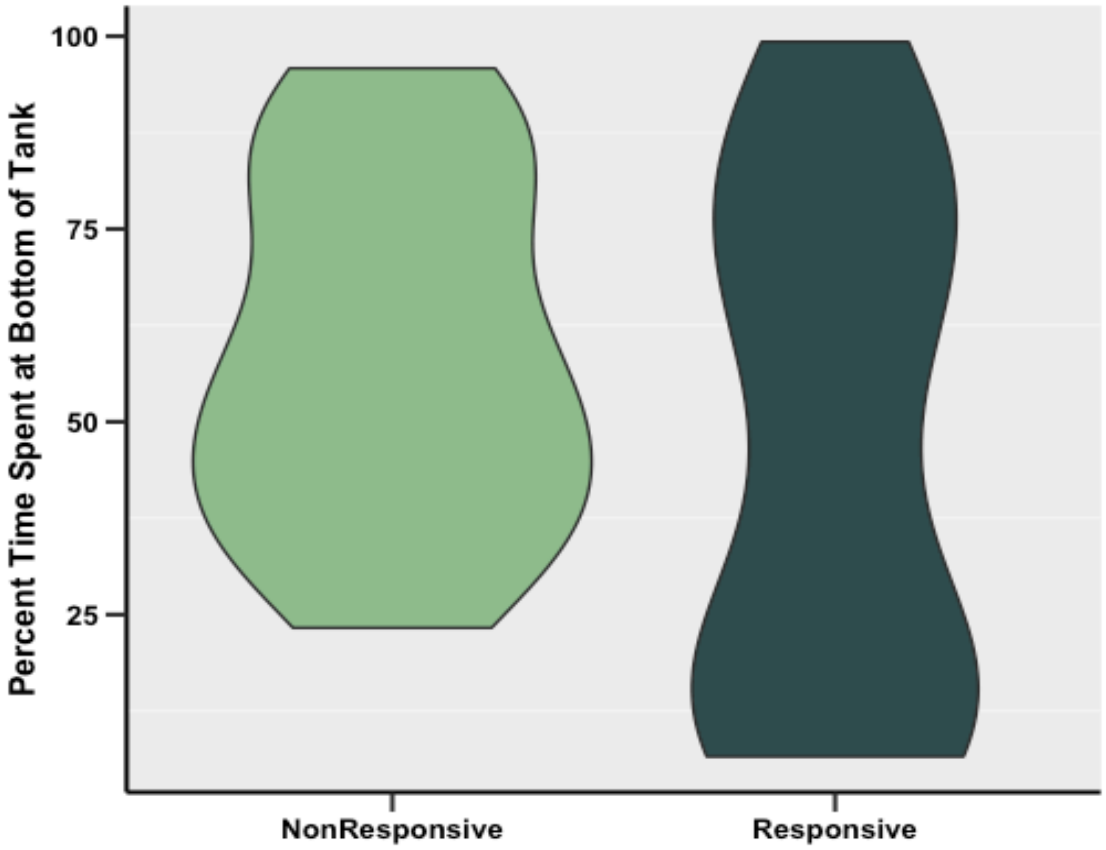

Figure A2. Visualization of time spent in the bottom portion of the tank during phenotyping novel tank diving assay for fishes identified with non-responsive or responsive behavioural phenotypes.