An Examination of the Effects of Elevated CO_2 on Juvenile Salmonids

By

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Chapter 1: General Introduction

The level of carbon dioxide (CO_2) in Earth's atmosphere has reached > 400 ppm, a 68 % rise since pre-industrial levels (IPCC, 2014). Levels of CO_2 are projected to increase to 1429 ppm by 2150 under the worst predicted scenario (Meinshausen et al., 2011). Rising atmospheric levels of CO_2 eventually lead to higher levels of CO_2 within aquatic ecosystems, which induces weak acidification. The weak acidification occurs as a result of the formation of carbonic acid when CO_2 dissolves in water. Carbonic acid quickly disassociates into bicarbonate; however, as more CO_2 is added, more carbonic acid is created which reduces the pH of the water body. Ultimately, as climate change continues, and more CO_2 enters the atmosphere, aquatic ecosystems are expected to experience both a rise in CO_2 and weak acidification (Hasler et al., 2016a; Hasler et al., 2018a).

Atmospheric CO_2 is not the only source for CO_2 in freshwater ecosystems. For example, factors like run-off, groundwater upwelling, the residence time of CO_2 in water, and many other geological processes influence the levels of CO_2 in freshwater ecosystems (Cole et al., 1994; Wetzel, 2001). Biological processes are also responsible for changes in CO_2 levels due to the presence of dissolved organic carbon (DOC), and dissolve inorganic carbon (DIC). In freshwater environments, biotic processes like photosynthesis and respiration produce dissolved organic matter (DOM), which can act as a source of hydrogen ions that can be broken down into DOC (McNeil and Matsumoto, 2019). Furthermore, DIC within the freshwater environment comes from the respiration of microbes decomposing the organic matter resulting in the production of CO_2 (Sobek et al., 2003). These additional explanations of how CO_2 is deposited into freshwater ecosystems helps to explain why CO_2 in these systems can change by 1000 μatm within hours, as a rise in temperature and light increases the productivity in microbes as well as primary

producers (Xu et al., 2019). With wide variations in the amount of CO_2 within aquatic ecosystems it is difficult to predict how these levels of CO_2 will change in the future (Hasler et al., 2016a).

What is known, however, is that there is variation in CO_2 levels across freshwater environments, with some systems already being supersaturated with CO_2 (Cole et al., 2007; Raymond et al., 2013). There is a large range of ppm levels in freshwater ranging from 36 to 23000 ppm (converted to ~ 36 x 10^6 µatm – 23000 x 10^6 µatm; Abril et al., 2015). It is also not out of the realm of possibility in some areas of the world to range from 0 to 81000 µatm of pCO_2 depending on the water chemistry (i.e. the amounts of salts within the water; Lazzarino et al., 2009). Other studies looking at hourly changes in the US have seen drastic swings in pCO_2 close to 1000 µatm within a 12-hour period (Xu et al., 2019). Therefore, depending on the body of freshwater, levels of pCO_2 can be vastly different but generally, freshwater has high saturation levels of CO_2. Hence the main question in regards to freshwater is not if CO_2 is elevated, but how high it gets and at what rate it is increasing.

There is mounting evidence that rising CO_2 has positive and negative consequences for freshwater organisms. For example, increased CO_2 can cause phytoplankton to rapidly grow creating harmful algae blooms (Qiu and Gao, 2002; Verschoor et al., 2013; Verspagen et al., 2014). In zooplankton, high CO_2 can cause a decrease in growth rates, likely due to the poor nutritional quality of phytoplankton grown in the high CO_2 environments (Urabe and Waki, 2009; Urabe et al., 2003). Further, another study found evidence of behavioural and reproductive changes in daphnia under high CO_2 conditions (Weiss et al., 2018). Macrophytes generally increase growth rates but only in species that are able to use CO_2 directly in their carbon concentration mechanisms (Cao and Ruan, 2015; Titus and Andorfer, 1996). Larger invertebrates

such as zebra mussels (*Dreissena polymorpha*; McMahon et al, 1995), spring snails (*Physella johnsoni*; O'Brien and Blinn, 1999), freshwater unionid mussels (*Fusconaia flava*; Hasler et al., 2017; Jeffrey et al., 2017), and crayfish (*Procambarus clarkia*; Robertson et al., 2018) can experience mortality at high levels of CO_2 as well as behavioural changes. Overall, elevated CO_2 in freshwater environments has the potential to drastically alter the freshwater community.

At the organismal-level, the major processes affected by elevated CO_2 includes growth rates, metabolism, reproduction, and behaviour (sensory systems and locomotion). Interestingly, it seems that increases or decreases in growth may occur depending on if the species is a primary producer or a consumer (Hasler et al., 2018b). Primary producers tend to see an increase in growth when exposed to inorganic carbon (Verschoor et al., 2013; Verspagen et al., 2014), this is thought to be due to CO_2 assimilation being more efficient in high CO_2 environments (Verspagen et al., 2014). However, the opposite is true for large organisms, such as fish, as they tend to experience a decrease in growth rate in high CO_2 environments (Khan et al., 2018; Ou et al., 2015). This drop-in growth rate may be due to reallocation of energy towards ion and acid-base regulatory mechanisms (Hannan et al., 2016; Perry, 1982). It is important to note that there have been some studies that have observed no effects on growth rate (Hosfeld et al., 2008) and some that displayed an increase in growth rate (Fivelstad et al., 1999). Similarly, the maximum metabolic rate (MMR) was seen to decrease in fishes exposed to elevated CO_2 conditions (Khan et al., 2018; Ou et al., 2015). The effects of CO_2 on reproduction have shown mixed results. In one case looking at coral reef fish, there was an increase in reproductive activity in the presence of high CO_2 (Miller et al., 2012). However, another study looking at spotted gobies (*Gobiusculus flavescens*), showed that even though there was no effect of CO_2 on clutch size, parental mating was affected (Forsgren et al., 2013). It might be more insightful to first look at how inorganic

carbon affects the behaviours of these fishes, not just reproduction. Depending on the trophic level responses may change as, behavioural responses to inorganic carbons vary greatly between and even within tropic levels. Studies observing behavioural changes in fish exposed to elevated CO_2 include the altering of olfaction (Tix *et al.*, 2017b), alarm cues responses (Leduc et al., 2013; Tix et al., 2017a), and even changes in migration patterns (Ikuta et al., 2003; Munday et al., 2009). As previously mentioned, species experience a varying degree of behavioural changes as a result of exposure to elevated CO_2 with some species experiencing minimal to no effect at all (Tix et al., 2017b). Moreover, it seems that once these individuals are placed back into baseline levels of CO_2 the behavioural responses can disappear, indicating reversible effects (Hasler et al., 2016b; Tix et al., 2017b). Species' response to high CO_2 in terms of its possible effects on growth rates, metabolism, reproduction, and behaviour is complex which requires further research to aid in prediction or response after an organism is exposed.

The widespread effects of elevated CO_2 on freshwater biota are not surprising as organisms are susceptible to fluctuations within their internal environment when external conditions change. When CO_2 enters the freshwater system, it can remain as dissolved CO_2 or it can be transformed into a weak acid. The presence of CO_2 in the environment can result in several physiological changes within fish. The first includes an alteration in energy and metabolism where the presence of high CO_2 (or weak acidification) resulted in a reduction in the yolk to tissue conversion contributing to a greater energetic cost associated with acid-base regulation (Ou et al., 2015). Within a similar study, growth rates were also reduced and MMR decreased in high CO_2 treatments (Khan et al., 2018). It was theorized that this decrease in MMR could be due to a reduction in ventilation and cardiac muscle activity (Kugino et al., 2016).

Moreover, the physiological effects of high CO_2 can cause impairment to a number of energy and metabolic related processes.

Behaviourally, there are multiple processes that can be observed as it relates to the effects of changing levels of CO_2. One of the most commonly evaluated topics is how high CO_2 could affect olfactory cues (Porteus et al., 2018). This topic is particularly important due to the implications that high CO_2 can alter the ability of a fish to detect its' predators. Some studies have shown that high CO_2 and low pH cause fish chemosensory abilities to be altered, leading to the inability of individuals to avoid predators (Elvidge and Brown, 2014). However, other studies have shown that there is no effect of CO_2 on olfaction (Tix et al., 2017a). One of the underlying mechanisms being affected is the interference of the neurotransmitters by altering GABA-A receptors in larval fish (Nilsson et al., 2012). An additional explanation might be that the chemical cues are experiencing structural and functional changes (Leduc et al., 2013; Roggatz et al., 2016). Moreover, another explanation could be related to the quality of the odorants used and the sensitivity of the odorants being affected (Porteus et al., 2018). These are just a few of the possible mechanisms that could explain how CO_2 may be affecting olfaction. The factors listed above are all tests that can be done through external observation. Conversely, looking internally, using histology, may aid in explaining how the elevated CO_2 might be impacting fish at a tissue level. Though limited literature is available on the effects of CO_2 on histology a study found tissue death within the livers of Atlantic cod that were exposed to increase CO_2 levels (Frommel et al., 2012). If there are any common themes seen among research done on the effects of high CO_2 it is that outcomes are highly variability depending on the environmental conditions and species tested.

Studies examining how multiple species respond to various CO_2 levels are limited. Therefore, in this thesis, my first major goal was to examine the effects of elevated CO_2 on growth rate, metabolic rate, feeding rate, and behaviour of juvenile freshwater Arctic charr (*Salvenlinus alpinus*), rainbow trout (*Oncorhynchus mykiss*), and brook charr (*Salvelinus fontinalis*) to determine basic survival. My hypothesis was that fish exposed to these elevated levels of CO_2 will show negative responses to elevated CO_2. From this I predicted that salmonids exposed to elevated CO_2 will show reduced growth, metabolism, and feeding in comparison to controls individuals (Khan et al., 2018; Ou et al., 2015). Behaviourally, escape responses maybe slower and poor swimming performance may be observed (Schneider et al., 2019). The second major goal of this thesis was to observe and to quantify tissue level responses within these same salmonids under elevated CO_2. I hypothesized that there would be a wide array of possible tissue changes occurring within the CO_2 treatment compared to the control fish. This led me to predict that the changes seen within the tissues will have a negative effect on the fish (Frommel et al., 2012; Noor et al., 2019). Further, I also expected there to be degrees of variation among the responses to elevated CO_2 related to species level differences within all experiments.

One of the strengths of this thesis is the use of multiple species. Many studies using elevated CO_2 only look at one, or two species. By using a multiple species approach, all the species being used are exposed to the exact same conditions allowing for easy comparison (Manley et al., 2004). This is a large advantage as generally studies looking at elevated CO_2 select their exposure levels based off of three categories of CO_2 research: future climate levels, aquaculture facility levels, and levels associated with invasive species barriers. With all the different concentrations of CO_2 being used as well as factors associated with the number of ions within the testing water, it makes comparing the results from different studies complex (McNeil

and Matsumoto, 2019). Thus, using multiple species within a study allows for a consistent use of methods and concentration of CO_2 to be used for each species. In this study we took the approach of using two species from the same genus and one outgroup from another species but all located within the family Salmonidae. This approach allows for possible patterns to be observed between species but also within a genus (Dentinger and Woods, 2018). This design adds insight into how individuals within the same family may exhibit different sensitivities to elevated CO_2.

A secondary strength of this study is the multiple biological scale approach implemented. Many studies focus on growth of a fish because growth is a large factor in determining the survival rates of juvenile fish (Nunn et al., 2012). Measuring growth is also an important factor for studies looking at the effects of aquaculture, as the goal for many facilities is the rapid growth of these fish to market sizes. Apart from growth rate, metabolic rate is also a fairly common experiment for fish exposed to elevated CO_2. This is generally done to determine if there is a change in metabolic rate that can be translated to a change in the energy expenditure of the fish. A multitude of CO_2 studies also focus on different behavioural aspects to determine how these fish may act within their ecosystems as a result of future climate change projections. These three metrics are commonly found within the limited literature about the effects of CO_2 on fish, however, a more uncommon metric looked at is the tissue levels response. This is usually done with histology and specific tissues such as the gills (Noor et al., 2019). However, in this study all the above metrics will be used. This wide array of approaches including ecological relevant behaviour, and physiology allows for a comprehensive view of how elevated CO_2 may have an effect. These results will help to determine which species may fair better (or worse) in the future in regards to elevated CO_2.

Chapter 2: An Energetic and Behavioural Investigation of the Effects of CO_2 on Juvenile Salmonids.

Abstract

In many freshwater ecosystems carbon dioxide (CO_2) is increasing. Unknown, however, are the risks that high CO_2 poses for freshwater organisms, especially of fish. The objective of this study was to determine how CO_2 may influence the growth rate, metabolic rate, feeding rate, and volitional behaviour of juvenile Arctic charr (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*), and brook charr (*Salvelinus fontinalis*). For this study, fish were held at control CO_2 levels (1100 µatm) or elevated CO_2 (5236 µatm) for 15 d. During which time metabolic rate and behavioural tests were conducted on alternating days for each treatment. Weight and length of each fish was taken on day 0, 7 and 15. There was no evidence that elevated CO_2 affected the growth rate, feeding rate, or behaviour in any of these species. The standard metabolic rate in Arctic charr did differ based on CO_2 exposure but not for the other species. By using multiple related species, the information learned will be more ecologically relevant and will also help industry quantify the effects of high CO_2 on juvenile salmonids.

Introduction

Freshwater acidification caused by rising CO_2 has been a noted stressor for freshwater biota (Hasler et al., 2016; Hasler et al., 2018). High CO_2 and acidification, is caused by several interconnected ecosystem processes including, but not limited to, lake respiration, terrestrial primary productivity, soil microbe respiration, and substrate type (Wetzel, 2001). Some of these processes will be altered as a result of rising temperatures and increased atmospheric CO_2 levels, leading to even higher levels of CO_2 in freshwater ecosystems (Hasler et al., 2016). Elevated CO_2 can have consequences for many freshwater biota, including phytoplankton, plants, invertebrates, and fish (Drake et al., 1997; Jeffery et al., 2017; Munday et al., 2019; Schippers et al., 2004).

Elevated levels of CO_2 within freshwater have several consequences for fish especially in regards to metabolism, growth, and behaviour. For example, the maximum metabolic rate (MMR) decreased in freshwater pink salmon (*Oncorhynchus gorbuscha*) and Atlantic salmon (*Salmo salar*) exposed to elevated CO_2 conditions (Khan et al., 2018; Ou et al., 2015). These studies may indicate that metabolic performance is reduced in physically active freshwater salmonids. This may be due to the reallocation of energy from growth towards ion and acid-base regulatory mechanisms (Hannan et al., 2016; Perry, 1982). Additionally, elevated CO_2 has also been observed to shift behaviour. For example, changes within the migration patterns of some fish (Ikuta et al., 2003; Munday et al., 2009). The changes in migration maybe due to the interaction of CO_2 and the sensory system of these fish (Diamond, 1968). Studies looking at the same species have contradictory outcomes (Fivelstad et al., 1999; Fivelstad et al., 2003), which makes predicting responses to CO_2 complex and outcomes hard to predict.

Even though consequences of elevated CO_2 have been observed, variation in how freshwater fish respond, both within and across species, has also been noted. This variation has been demonstrated in a study observing the reactions of both bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*) displaying avoidance and narcosis behaviours to elevated CO_2 (Tix et al., 2018). However, there was a difference between the amount of CO_2 needed to observe similar behavioural effects, with one level of CO_2 at approximately 1000 µatm to the other being just under 10000 µatm CO_2 (Tix et al., 2018). Additionally, some species (*Lepomis macrochirus*), experience minimal to no effect after being exposed to elevated CO_2 (Tix et al., 2017b). Moreover, it seems that some species (*Lepomis macrochirus* and *Micropterus salmoides*) that are placed back into baseline levels of CO_2 can reverse their responses (Hasler et al., 2016b; Tix et al., 2017b). It is also important to note that even within species there can be variation as one study in Atlantic salmon found an affect of CO_2 on growth rate (Khan et al., 2018) well another study found no affect (Fivelstad et al., 2003). Both studies used similar CO_2 concentrations but differed in fish size and housing temperature. This variation makes comparing results to the literature and predicting other species responses to elevated CO_2 difficult.

The variation in response to CO_2 observed in freshwater fishes has implications for predicting how biodiversity might shift under future climate change scenarios, and thus, should be studied. For example, within families, species differences might help to predict which populations will be more or less effected by rising levels of CO_2. Salmonids are a great family to helps understand this concept as they are a popular sports fish, a diverse family, and are sensitive to environmental change. Within Canada the trout and charr are some of the highest caught and harvested species (Brownscombe et al., 2014). The province of Manitoba also puts resources into

a stocking program to increase the number of these fish in their lakes. Additionally, salmonids are found all over the world in both freshwater and marine environments. This separation and specialization within salmonids have given rise to different species with many diverse morphs associated with them. These different species adapted to their environment which may translate to varied tolerances to environmental change. Furthermore, some salmonids, such as rainbow trout (*Oncorhynchus mykiss*), are used in ecotoxicology studies as they are a sensitive to environmental change and pollutants (Van der Oost et al., 2003). Determining which species might be more affected by elevated CO_2 in the future may help the management of these species in order to maintain the large economic sports fishery and their status in the wild.

Studies on why fishes vary in their response to high CO_2 are lacking, which limits our understanding of how climate change will impact freshwater biodiversity. A more comprehensive study involving multiple related species might be able to help explain why there is so much variation in responses to elevated CO_2. Therefore, the goal of my study is to determine if salmonids are similarly affected by a 15-day CO_2 exposure during the rearing stage (free-swimming stage, age-0). My first objective will be to examine growth rate, metabolism, and feeding rate in juvenile Arctic charr *(Salvelinus alpinus*), brook charr (*Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*) exposed to elevated CO_2. I predict that salmonids exposed to elevated CO_2 will show reduced growth rate, metabolism, and feeding rate in comparison to the control individuals (Khan et al., 2018; Ou et al., 2015). My second objective is to examine ecologically-relevant behavioural responses to elevated CO_2. To do this, I will expose fish to elevated CO_2 then measure escape responses, and volitional activity. I predict fish exposed to CO_2 will have a slower escape response and poor swimming performance (Schneider et al., 2019). Ultimately, I predict that there will be variation among the responses to CO_2

between the three species, which may relate to CO_2 levels experienced during their unique environmental exposures during their evolutionary histories.

Methods

Study animals and husbandry

Experiments were completed between June 4 and July 28, 2021 and divided into three 15-day CO_2 exposure periods. Exposure periods were completed one species at a time, starting with juvenile Arctic charr *(Salvelinus alpinus*; mass = 2.9 ± 0.7 g, total length = 6.1 ± 0.2 cm; Nauvuk Lake strain); then juvenile rainbow trout (*Oncorhynchus mykiss*; mass = 2.3 ± 0.4 g, total length = 5.9 ± 0.4 cm; Nipigon River strain); and, finally juvenile brook charr (*Salvelinus fontinalis*; mass = 4.7 ± 1.3 g, total length = 7.8 ± 0.8 cm; Nipigon River strain). These fish were from the 2020 fall spawning season. All the fish used in the study were reared and experimented on at the provincial Whiteshell Hatchery, Whiteshell, Manitoba, Canada. Over the course of the study, fish were fed Skretting number 1 crumble (Skretting Canada, Vancouver, British Columbia) at two percent of their body weight per day. A day before a CO_2 exposure period began, fish were netted from outdoor holding raceways and assigned to one of four indoor raceways (4.3 x 0.30 x 0.30 m) with flow through water from West Hawk Lake, Manitoba. Lake water first passed through UV light and bio-filters to remove microbes and reduce nutrient loads before entering the raceways.

Half of the fish (n = 100) were treated with ambient lake CO_2 (n = 2 raceways), and half with elevated CO_2 conditions (n = 2 raceways). Elevated CO_2 was maintained at 5237 ± 1532 µatm using compressed CO_2 gas bubbled into the water using air stones (38 x 8 cm, a max flow rate of 9 L/min, Point Four Micro bubble diffusers, Cary, North Carolina, USA) controlled by pH pinpoint meters set to a low point of 6.2 and the high of 6.4 with a solenoid valve (American

Marine Inc., Ridgefield, Connecticut, USA) (Table 1; Figure 1). The level of CO_2 used was 5 times higher than the current lake levels and was intended to represent future lake levels if dissolved CO_2 rises in the watershed (Figure 1). The partial pressure of CO_2 was monitored in the raceways using a modified infrared CO_2 probe (GM70, Vaisala, Helsinki, Finland; Table 1; Johnson et al., 2010). Water samples were titrated twice daily using a digital titration kit to determine total dissolved CO_2 (mg/L)(Hach model CA-DT; 10-1000 mg/L, Hach Company, Loveland, Colorado, USA). Additionally, pH, ammonia, and alkalinity were also monitored. Temperature and dissolved oxygen (DO % saturation) were also measured twice daily using YSI Pro 1020 dissolved oxygen and pH meter (Xylem Inc, Yellow Springs, Ohio, USA).

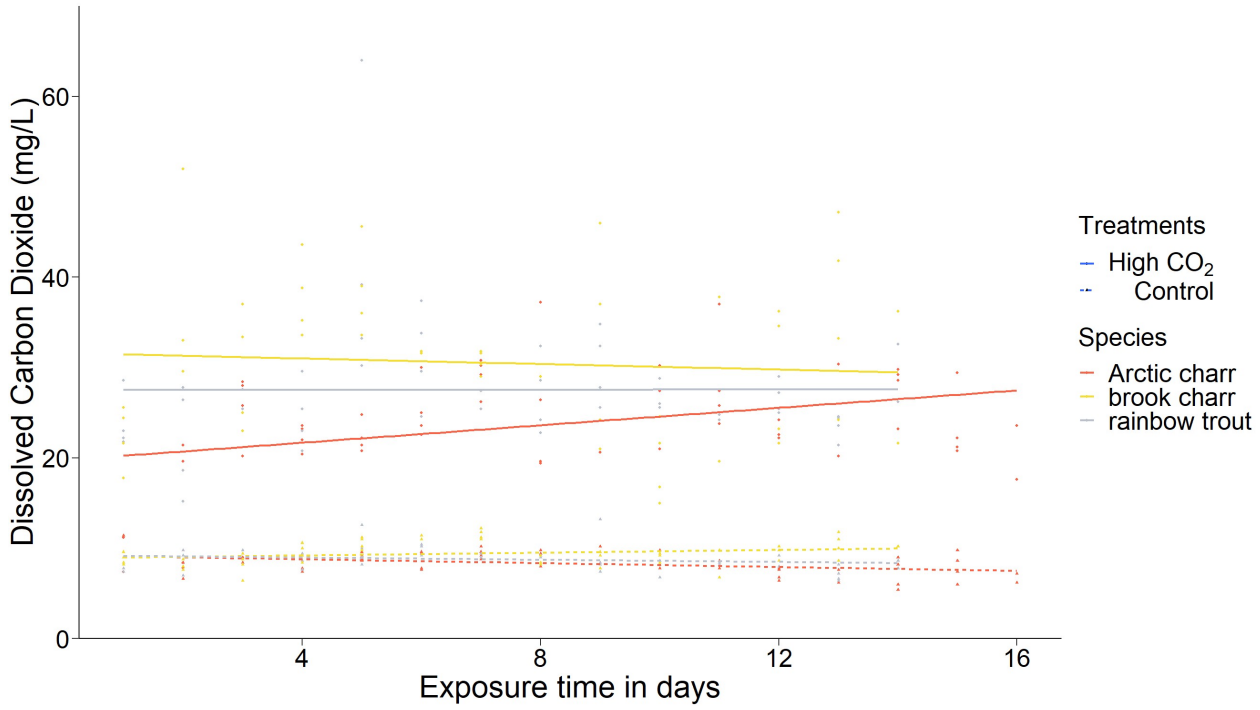

Figure 2.1. Amount of dissolved carbon dioxide in milligrams per liter used per Arctic charr experiments in red (*Salvelinus alpinus*), brook charr experiments in yellow (*Salvelinus fontinalis*), and rainbow trout experiments in grey (*Oncorhynchus mykiss*). Measurements were taken twice daily for the entire length of the 15-day exposure period. The triangle shape with the dotted line representing the Control treatment and the circles with the solid lines representing the CO_2 treatment. The coloured lines represent smoothers using a linear model method.

Table 2.1. Water quality parameters of the raceways recorded during the experiment. Water parameters were sampled twice daily, once in the morning and evening for each raceway. Values are presented as mean ± standard deviations.

Raceway	pH [ǂ]	Temperature (˚C) [*]	Ammonia [§]	Total alkalinity (ppm) [§§]	Dissolved oxygen (% saturation) [Ψ]	pCO_2 (µatm) [¶]	CO_2 titration (mg L^{-1}) [≠]
1st CO_2 treated	6.1 ± 0.5	10.4 ± 0.7	< 0.1	53.7	78 ± 4.5	5081 ± 1411	26.1 ± 8.5
1st Ambient	6.6 ± 0.2	10.4 ± 0.7	< 0.1	53.7	81 ± 2.8	1131 ± 304	8.9 ± 1.6
2nd CO_2 treated	6.2 ± 0.3	10.5 ± 0.8	< 0.1	53.7	79 ± 3.8	5392 ± 1652	27.1 ± 8.2
2nd Ambient	6.6 ± 0.2	10.6 ± 0.7	< 0.1	53.7	80 ± 4	1122 ± 252	8.8 ± 1.6

[ǂ] pH Probe, American Marine Inc., Ridgefield, Connecticut, USA

[*] Digital thermometer, Cooper-Atkins, Middlefield, Connecticut, USA

[§] Ammonia test kit: AQUASPIN, Mars incorporated, McLean, Virginia, USA

[§§] Total Alkalinity test kit: AQUASPIN, Mars incorporated, McLean, Virginia, USA

[Ψ] Professional Plus Multiparameter Instrument, YSI incorporated, Yellow Springs, Ohio, USA

[¶] Altered partial pressure CO_2 probe, GM70, Vaisala, Helsinki, Finland

[≠] Digital titrator CO_2 test kit, Hach Company, Loveland, Colorado, USA

Growth, Metabolism, and feeding

The weight (g) and total length (mm) of each fish was measured on days 0,7, and 15 of each exposure period to calculate the body mass. A container with water was placed on top of a balance scale (Ohaus Scout, Parsippany, New Jersey, USA) in which fish were placed to weigh them. Total length was recorded in mm by placing the fish in a fish viewer (Fish viewer 150 mm, Dynamic Aqua Supply Ltd, Surrey, British Columbia, Canada) that contained treatment water. Note, on day 0 of the Arctic charr collection, only a group of 200 Arctic charr, and not individual fish were weighed on a balance scale in a large container with the average weight and length recorded as day 0.

Metabolic rate was determined using intermittent flow respirometry (Clark, 2022). On alternating days of the entire 15-day exposure period, 4 fasted fish (25 hr) from either the CO_2 treatment or control treatment were placed within the respirometry chambers (Figure 2.2). The four custom-built plastic (polypropylene plastic, 1.9L, 17.5 x 11.5 x 15 cm) respirometers chambers (Table S1) were placed in the testing raceway and filled with either control water or elevated CO_2 water controlled using the apparatus described above to match the treatment raceways for the fish being used on that day. Water within the chambers was circulated using aquaria pumps (EHEIM Universal 300, Berlin, Germany). Oxygen saturation was measured every 3 s for 24 h using a fiberoptic probe (FireSting, PyroScience, Aachen Germany) placed within the recirculating water line of each chamber. Probes were connected to a recording unit (FSPRO-4, FireSting, PyroScience, Aachen Germany). Oxygen saturation levels were measured throughout an 18 min repeated cycle (3 min flush and 15 min closed). In total 52 Arctic charr were tested in the respirometer (CO_2 n = 28, Control n = 24), 55 rainbow trout (CO_2 n = 29, Control n = 26), and 55 brook charr (CO_2 n = 29, Control n = 26).

Standard metabolic rate (SMR), maximum metabolic rate (MMR), and aerobic scope (AS) were calculated using the dissolved oxygen data (Clark, 2022). SMR was equated to the MO_2 of the lowest 10 percentile (Rosewarne et al., 2014). MMR was induced before fish were placed into the chambers by using a standard chase test in which the fish was placed into a bucket of the treatment water and then chased for 3-min which has been shown to be a suitable method to elicit an MMR response (Norin and Clark, 2016). The upper 99 percentile equaled the MMR (Svendsen et al., 2016). The difference between MMR and SMR was used as an estimate of AS (Bennett and Rube, 1979).

Dried stomach weights were used as a coarse proxy for feeding. The entire fish was weighted using a balance scale (Ohaus Scout, Parsippany, New Jersey, USA) to obtain whole-body wet weight. Fish were then transferred to a -20˚C freezer where they were held for 7 months. Upon thawing, fish were dissected and the stomach removed. The stomach was weighed using the balance scale to obtain a wet weight. Stomachs were then placed in a metal dish alongside their whole body before being placed in a mechanical convection laboratory oven for drying (Quincy Lab, Burr Ridge, Illinois, USA). Tissues were left in the ovens for 48-h at 60˚C (Berg, 1979; da Silveira et al., 2020; Hyslop, 1980). During the 48-h period, stomachs were weighed periodically to ensure they had been fully dried. Immediately after removal from the oven the stomachs and whole bodies were weighed for a final time.

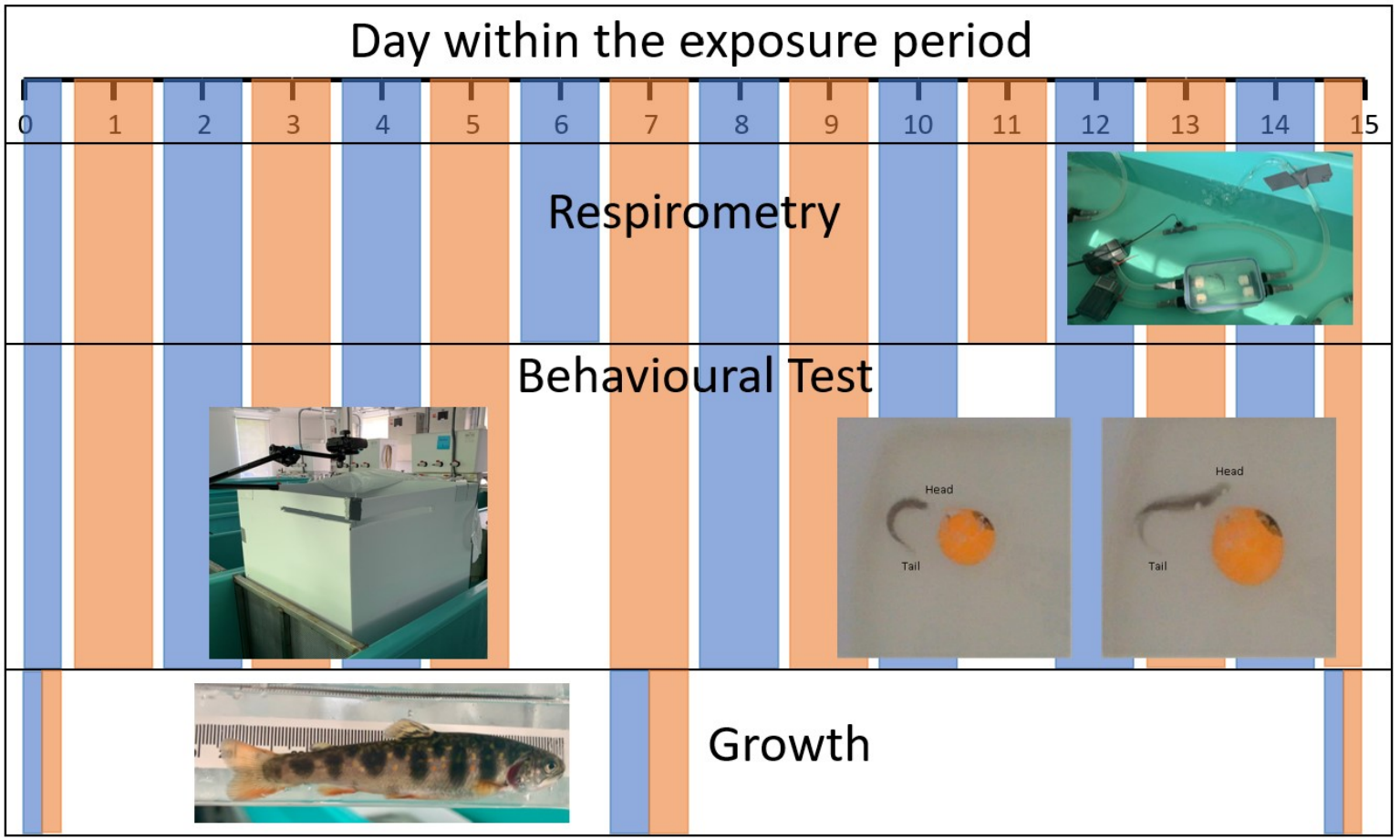

Figure 2.2. Timeline of the study showing days within the exposure period with the blue bars representing the control and the orange bars representing the CO_2 treatment groups. The bars crossing into each of the sections indicates that the test were completed.

Behaviour

To record C-start escape responses, individual fish were first placed into a plastic basin (40 x 31.8 x 15.2 cm) containing treatment water and allowed 2 h to habituate. Fish were selected from either the CO_2 treatment tank or control treatment tank via haphazardly netting in which the first fish that entered the net was used (Figure2.2). The escape response assay consisted of dropping a ping pong ball into the aquarium behind the fish to prompt a C-start response (Eaton et al., 1988; Johnson et al., 1996; Johnson et al., 1998). The procedure was recorded using a digital camera (Sony Rx100, Sony Canada Ltd, Toronto, Ontario, Canada) set to 1000 fps mounted over the testing arena. Five C-starts were filmed for each fish with a 10-min rest period in between elicited C-starts (Eaton et al., 1988; Johnson et al., 1996; Johnson et al., 1998). Videos were then downloaded and analyzed using Microsoft video editor version 2021.21110.8005.0. Once in the program, the time in which it took for the individual fish to go through both stage 1 and stage 2 of the C-start was recorded. Videos where fish did not go through both stages of a C-start were excluded from the analysis. From the accepted videos the time it takes for the fish to go through both stages of a C-Start was averaged.

Novel Tank

A novel tank assay was used to quantify a fish's response to being placed in a new environment (Cachat et al., 2010; Hong and Zha, 2019). Prior to the assay, an individual fish was haphazardly netted (the first fish that entered the net was used) from either the CO_2 treatment tank or the control tank and transferred to the novel arena (Figure 2.2). The testing arena consisted of a 40 x 31.8 x 15.2 cm white basin that contained water from the treatment raceway of the fish being tested. Using the camera described above, the fish's activity was recorded for 10 -min. White screens were placed around the testing arena to minimize external factors that may impact the

fish's behaviours. Additionally, noises in the area of the assay were kept to a minimum to avoid startling the fish. Videos were processed using automated software (EthoVision XT 15, Wageningen, Netherlands) to calculate distance moved (body length) and velocity in body lengths per second (BL s^{-1}) over the course of the trial.

Statistical analyses

To test the effects of CO_2 on MMR, SMR, AS, and body mass, a linear mixed effects model was used (Bates et al. 2015). For these models treatment and species were used as an interactive linear predictor and day was included as an additional fixed effect. The raceway the fish was housed in was included as a random effect. To evaluate the effects of the CO_2 treatment on the ratio of stomach weight to body weight, a generalized linear model (beta distribution) was used (Brooks, et al. 2017). Treatment and species were used as an interactive linear predictor. For the behaviour and novel tank tests, a linear model was used (Bates et al. 2015). Treatment, species, and day were used as interactive linear predictors. Statistical analyses were performed using R, version 4.1.1 (R Foundation for Statistical Computing, 2022). For all analyses significance was accepted at $p < 0.05$ and all values in the text are presented as mean ± standard error. Model residuals were analyzed after every model to ensure that model assumption for normality and homogeneity were met.

Results

Metabolism

Species differences in metabolic phenotypes were observed but were only found to be attributed to exposure to CO_2 in one species Arctic charr exposed to CO_2 halved their SMR when compared to Arctic charr held in ambient conditions (Figure 2.3A; Linear mixed effects model, $p < 0.05$). Significant effects of CO_2 were not found for MMR (Figure 2.3B; Linear mixed effects model, $p > 0.05$) nor for AS (Figure 2.3C; Linear mixed effects model, $p > 0.05$). The species differences observed indicate that brook charr and rainbow trout had the highest SMR compared to Arctic charr (Figure 2.3A; 23.33 ± 1.01 mgO_2/kg/hr, 23.66 ± 1.35 mgO_2/kg/hr, and 13.12 ± 1.37 mgO_2/kg/hr respectively) and MMR (Figure 2.3B; 170.66 ± 6.91 mgO_2/kg/hr, 127.02 ± 6.83 mgO_2/kg/hr, and 106.92 ± 13.62 mgO_2/kg/hr respectively). Arctic charr had the lowest and broadest AS (Figure 2.3C; 93.77 ± 13.56 mgO_2/kg/hr). No effect of day within the exposure period was found among any of the species (Linear mixed effects model, $p > 0.05$).

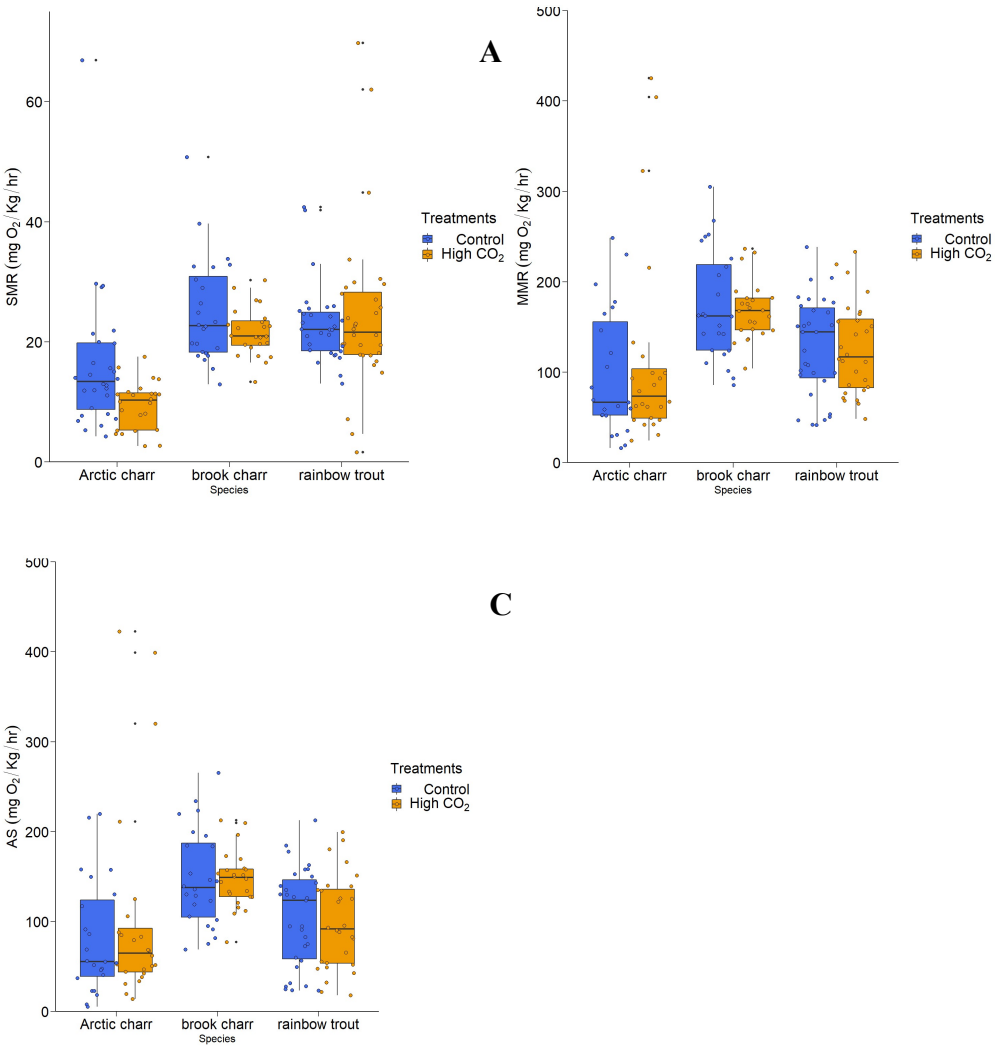

Figure 2.3. The standard metabolic rate (SMR)(A), maximum metabolic rate (MMR)(B), and aerobic scope (AS)(C) of Arctic charr (*Salvelinus alpinus*, Control n = 28, CO_2 n = 28), brook charr (*Salvelinus fontinalis*, Control n = 28, CO_2 n = 28), and rainbow trout (*Oncorhynchus mykiss*, Control n = 32, CO_2 n = 32) under the Control (blue) or the CO_2 treatment (orange). The horizontal bars depict the 25 % quartile, median, and the 75 % quartile. The lowest whisker indicates the minimum value determined by the 25th percentile minus 1.5 times the interquartile range and the highest whisker indicates the maximum value determined by the 75% percentile plus 1.5 times the interquartile range, excluding outliers. Outliers are values that are lower then the minimum and the maximum. The coloured points represent data points used to create the box

plots and the black dots represent outliers. The asterisks indicate statistical significance (Estimate 6.68, Std. Error 2.90, d.f. 9.34, t-value 2.31, $p = 0.046$).

Feeding

There was no evidence that exposure to CO_2 affected the dried stomach weight to dried body weight ratio for any of the species (Figure 2.4; Generalized linear model, $p > 0.05$). Ignoring the effects of CO_2 overall, there was strong evidence that Arctic charr and brook charr had the lower dried stomach to dried body weight ratios than rainbow trout (Generalized linear model, $p < 0.001$). On average the stomachs of the Arctic charr accounted for 2.1 ± 0.11 % of total dry mass (Figure 2.4). Similarly, the brook charr stomachs accounted for 2.1 ± 0.13 % of total dry mass (Figure 2.4). Rainbow trout stomachs accounted for 3.2 ± 0.10 % of total dry mass (Figure 2.4).

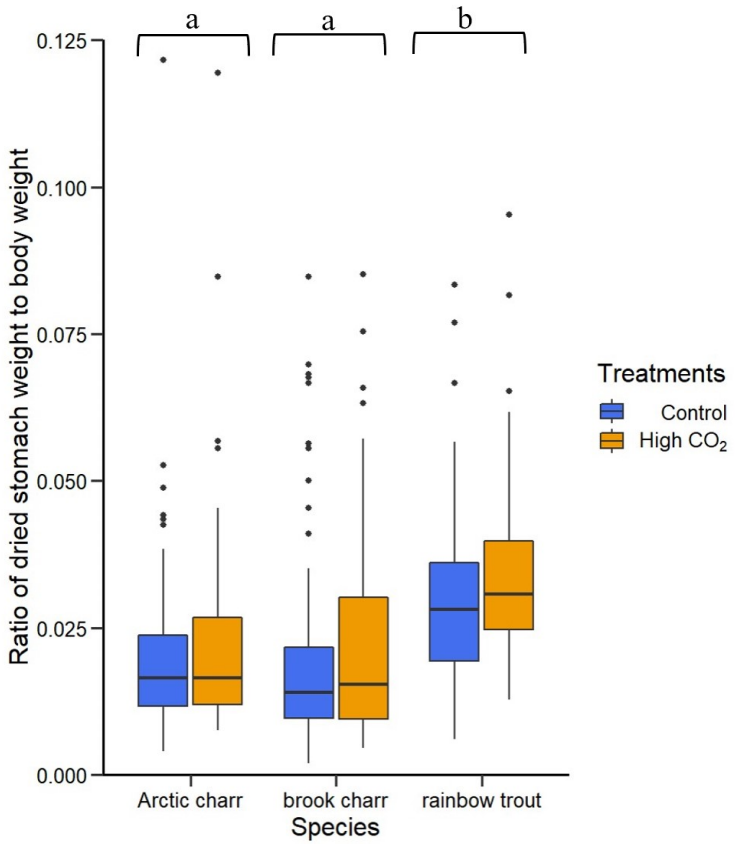

Figure 2.4. Ration of dried stomach weights to dried body weights of Arctic charr (*Salvelinus alpinus*, Control n = 92, CO_2 n = 97), brook charr (*Salvelinus fontinalis*, Control n = 96, CO_2 n = 96), and rainbow trout (*Oncorhynchus mykiss*, Control n = 100, CO_2 n = 95) under the control (blue) or the CO_2 (orange). The horizontal bars depict the 25 % quartile, median, and the 75 % quartile. The lowest whisker indicates the minimum value determined by the 25th percentile minus 1.5 times the interquartile range and the highest whisker indicates the maximum value determined by the 75% percentile plus 1.5 times the interquartile range. The black dots represent outliers. Outliers are values that are lower then the minimum and the maximum. Different letters indicate statistical significance. (Estimate 0.40, Std. Error 0.07, z-value 5.33, $p = 6.6 \times 10^{-8}$).

Body mass

No evidence was found that exposure to CO_2 affects body mass (Figure 2.5, Linear mixed effects model, $p > 0.05$). Arctic charr gained an average of 0.9 ± 0.066 g (Figure 2.5A). Brook charr gained an average of 0.56 ± 0.103 g (Figure 2.5B). Rainbow trout gained 0.86 ± 0.037 g (Figure 2.5C). Among the species, there was no indication that they had different body masses (Linear mixed effects model, $p > 0.05$). The day in which the fish were weighted did show strong evidence of increasing body mass in all species, with Arctic charr overall gaining 4.2% of their total body weight, rainbow trout gaining 3.6 %, and brook charr gaining 9.5 % over the 15-day time period (Figure 2.5; Linear mixed effects model, $p < 0.05$).

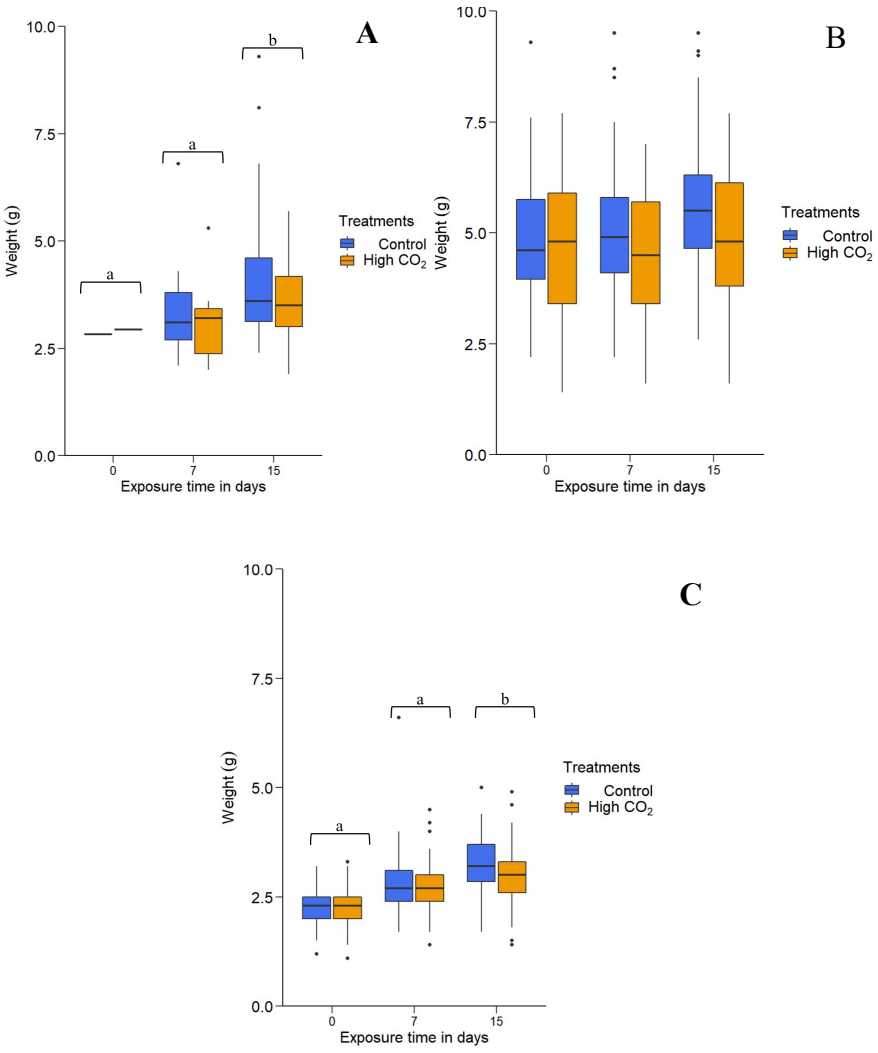

Figure 2.5. Body mass of Arctic charr (*Salvelinus alpinus*, initially n = average of 100 per treatment, final day Control n = 92 and CO_2 n = 97) (A), brook charr (*Salvelinus fontinalis*, initially n = 100 per treatment, final day Control n = 96 and CO_2 n = 96) (B), and rainbow trout (*Oncorhynchus mykiss*, initially n = 100 per treatment, final day control n = 100 and CO_2 n = 95) (C) under the control (blue) or the CO_2 treatment (orange) at 0, 7, and 15-day exposure times. The horizontal bars depict the 25 % quartile, median, and the 75 % quartile. The lowest whisker indicates the minimum value determined by the 25th percentile minus 1.5 times the interquartile range and the highest whisker indicates the maximum value determined by the 75% percentile plus 1.5 times the interquartile range. The black dots represent outliers. Outliers are values that are lower then the minimum and the maximum. Note, on day 0 of the Arctic charr a group of 200 Arctic charr were weighted on a balance scale in a large container with the average weight. Thus, the black bar for this species on day 0 represents the mean weight of the 200 fish. The double astricts indicates statistical significance from the single astricts (Panel A: Estimate 7.68 x 10^{-2}, Std. Error 2.92 x 10^{-2}, d.f. 1.35 x 10^{3}, t-value 2.63, p = 0.0086; Panel C: Estimate 4.78 x 10^{-2}, Std. Error 1.00 x 10^{-2}, d.f. 1.35 x 10^{3}, t-value 4.78, p = 1.94 x 10^{-6}).

Escape response

No evidence was found to suggest that the C-start escape response was influenced by exposure to CO_2 (Figure 2.6; Liner model, $p > 0.05$). Brook charr showed the fastest response to the stimuli compared to Arctic charr and rainbow trout which responded much the same after the eighth day of exposure to the CO_2 treatment (Figure 2.6). However, there is strong evidence that as the time within the exposure continued the time it took Arctic charr to complete the C-start increased (Figure 2.6; Liner model, $p < 0.05$). There is also evidence that brook charr escape time responses were quicker with an average of 0.014 seconds quicker than the Arctic charr (Linear model, $p < 0.05$).

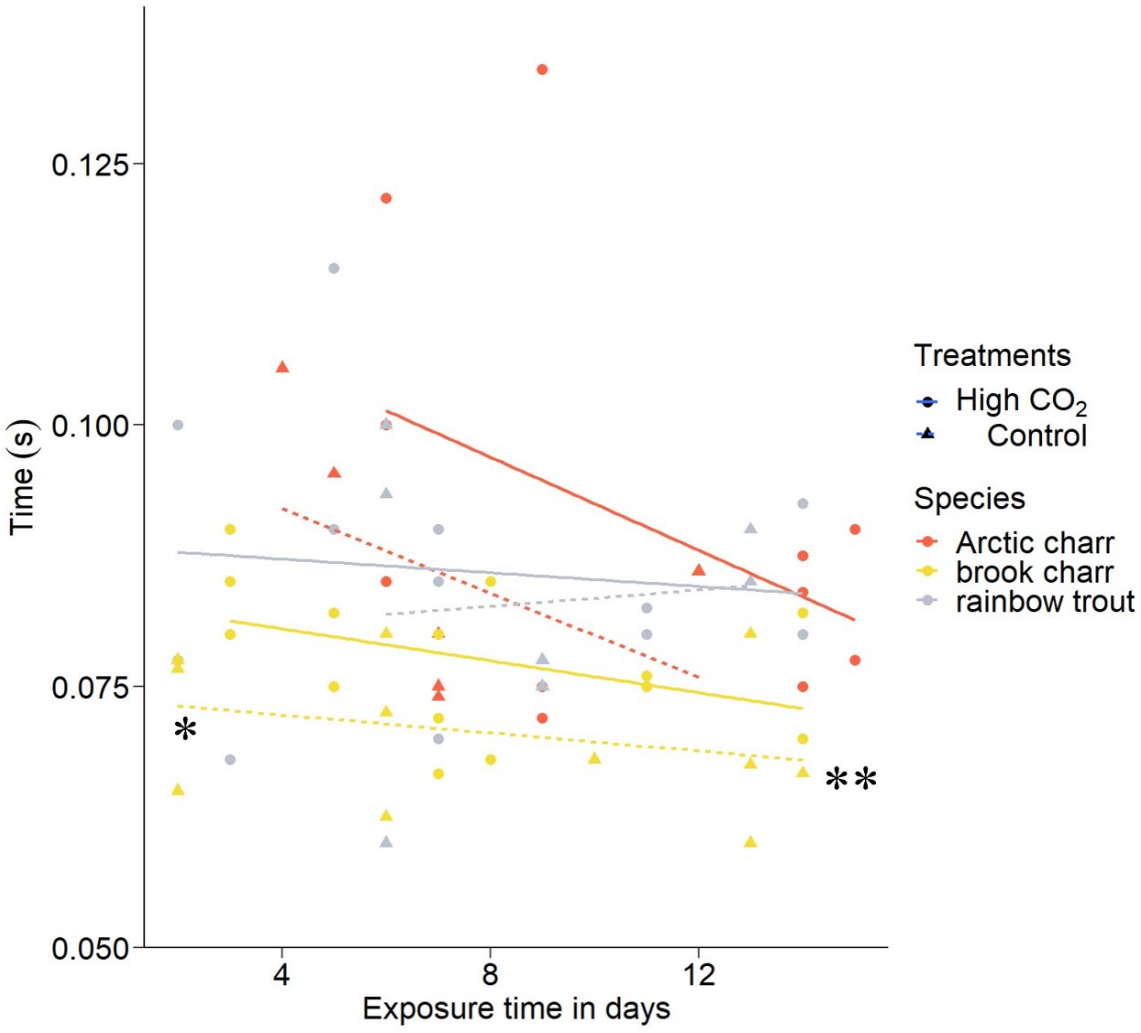

Figure 2.6. The time in seconds which it took Arctic charr in red (*Salvelinus alpinus*, Control n = 9, CO_2 n = 11), brook charr in yellow (*Salvelinus fontinalis*, Control n = 11, CO_2 n = 14), and rainbow trout in gray (*Oncorhynchus mykiss*, Control n = 9, CO_2 n = 12) to go through both stages of a C-start escape response under the two different treatments, control (triangle shape with dotted line) or CO_2 (circle shape with solid line) across a 15-day exposure period. The coloured lines represent smoothers using a linear model method. The double asterisks indicate statistical significance from the single asterisks (Estimate -0.002, Std. Error 0.001, t-value - 2.16, p = 0.036).

Novel tank

There was no evidence that CO_2 exposure altered the distances traveled for any of the species (Figure 2.7; Linear model, $p > 0.05$). On average rainbow trout swam an average of 51.15 ± 6.14 body lengths (BL), Arctic charr swam slightly less at an average of 38.11 ± 1.23 body lengths (BL), and finally, the brook charr swam an average of 6.54 ± 1.23 body lengths (BL) (Figure 2.7). There was evidence that rainbow trout and Arctic charr traveled 87 % and 82 % further than brook charr, respectively (Linear model, $p < 0.05$).

Volitional swimming speed did not differ due to CO_2 treatment (Figure 2.8; Liner model, $p > 0.05$). The results follow a similar pattern to the distance moved with Arctic charr and rainbow trout moving the greatest velocity on average at 0.064 ± 0.005 and 0.078 ± 0.010 body lengths per second (BL/s) respectively (Figure 2.8). Brook charr had the slowest velocity at an average of 0.011 ± 0.002 body BL/s (Figure 2.8). Consequently, there was also evidence that Arctic charr and rainbow trout were 0.053 and 0.067 body lengths per second faster than the brook charr (Linear model, $p < 0.05$).

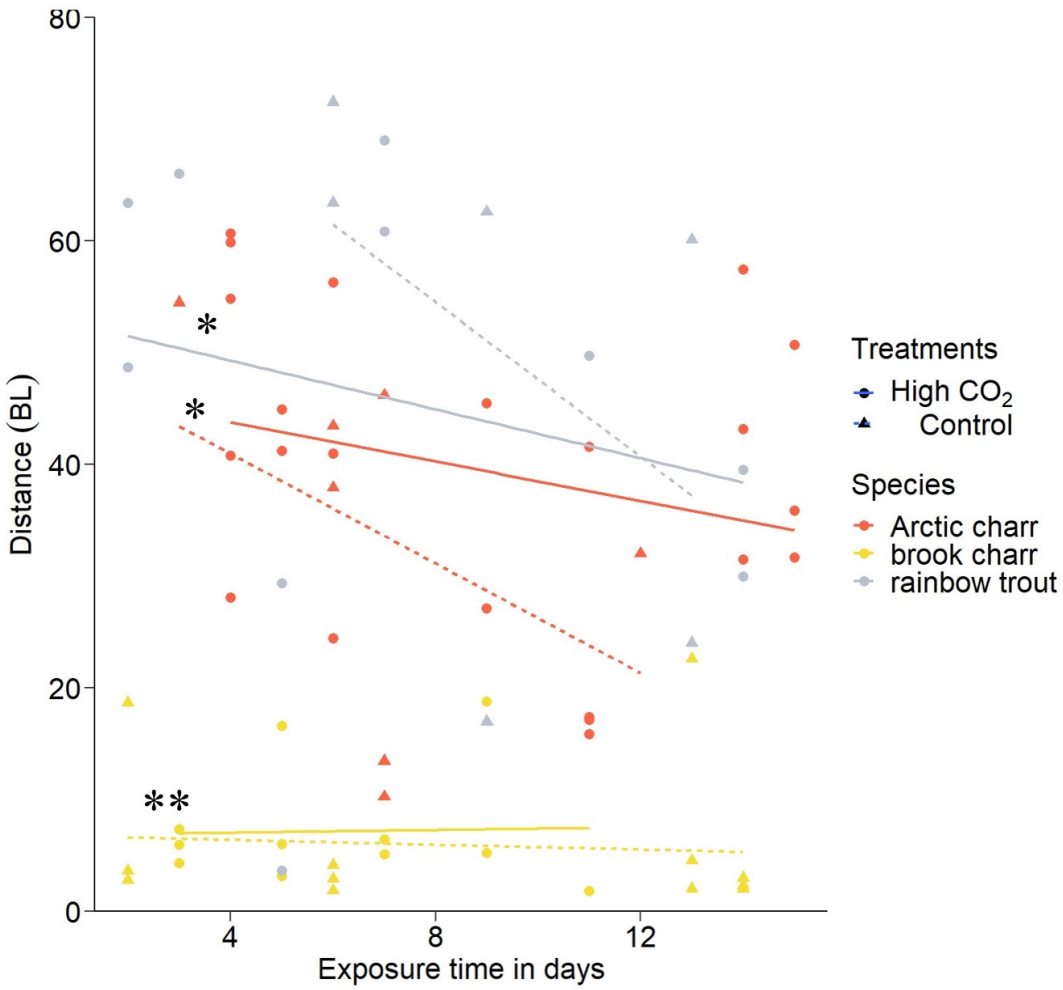

Figure 2.7. The distance Arctic charr in red (*Salvelinus alpinus*, Control n = 12, CO_2 n = 22), brook charr in yellow (*Salvelinus fontinalis*, Control n = 12, CO_2 n = 12), and rainbow trout in grey (*Oncorhynchus mykiss*, Control n = 9, CO_2 n = 11) traveled in body lengths (BL) during a novel tank behavioural experiment under either control (triangle shape with dotted line) or CO_2 (circle shape with solid line) treatment over a 15-day exposure period. The coloured lines represent smoother using a linear model method. The double asterisks indicate statistical significance from the single astricts (brook charr and Arctic charr, Estimate 40.54, Std. Error 15.00, t-value – 2.70, p = 0.009; brook charr and rainbow trout, Estimate 52.67, Std. Error 15.84, t-value 3.33, p = 0.002).

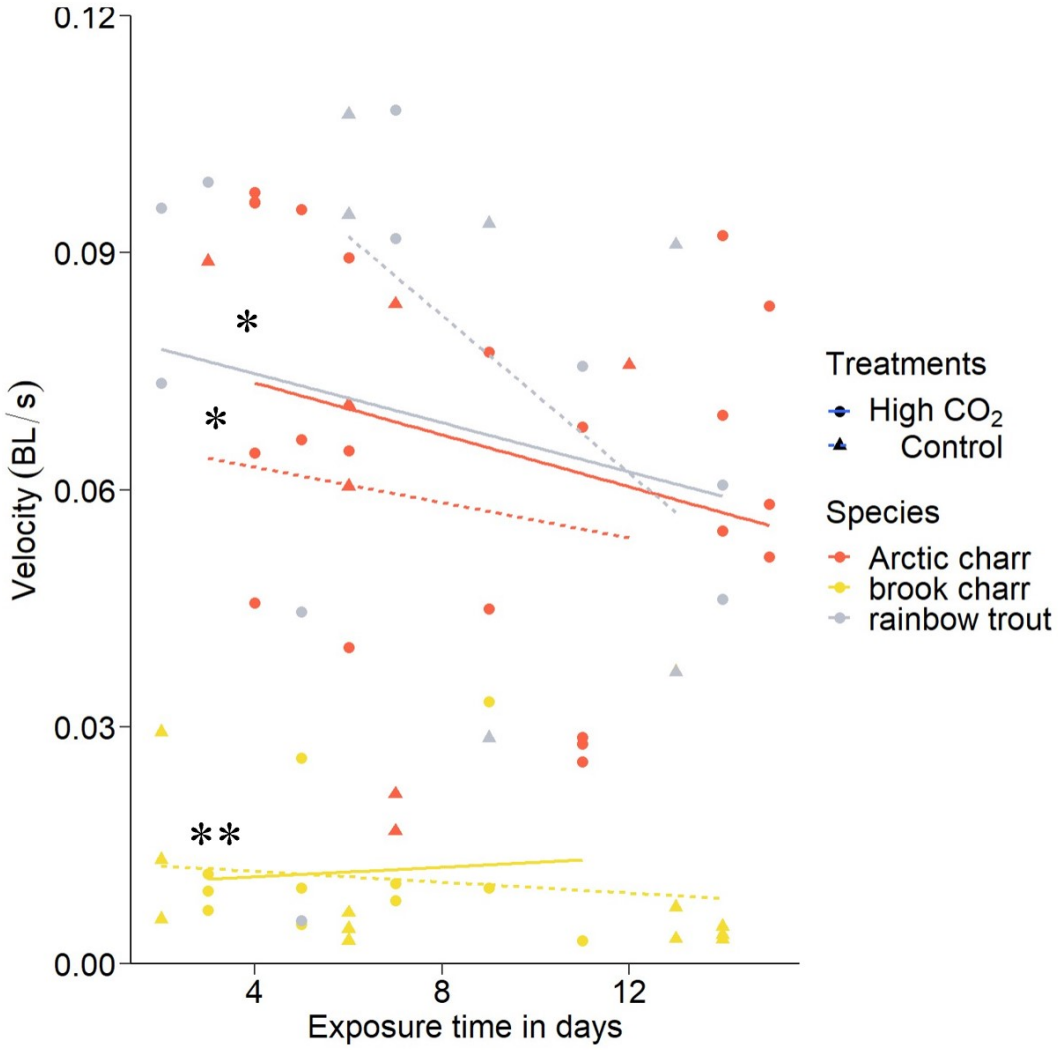

Figure 2.8. The velocity Arctic charr in red (*Salvelinus alpinus*, Control n = 12, CO_2 n = 22), brook charr in yellow (*Salvelinus fontinalis*, Control n = 12, CO_2 n = 12), and rainbow trout in grey (*Oncorhynchus mykiss*, Control n = 9, CO_2 n = 11) traveled in body lengths per second during a novel tank behavioural experiment under either control (triangle shape with dotted line) or CO_2 (circle shape with solid line) treatment over a 15-day exposure period. The coloured lines represent smoothers using a linear model method. The double asterisks indicate statistical significance from the single asterisks (brook charr and Arctic charr, Estimate 0.07, Std. Error 0.02, t-value – 2.89, p = 0.005; brook charr and rainbow trout, Estimate 0.08, Std. Error 0.03, t-value 3.14, p = 0.003).

Discussion

Exposure to elevated CO_2 had limited impacts on juvenile salmonids. Of all the metrics monitored, only SMR of Arctic charr showed evidence that the CO_2 treatment had an effect, specifically, a 1.5-fold increase in SMR of Arctic charr exposed to elevated CO_2. This increase in SMR has been observed in other studies investigating salmonids (e.g., Khan et al., 2018) but not all (e.g.,Sadoul et al., 2017). For example, a freshwater study using rainbow trout exposed to high levels of CO_2 (40 mg/L) demonstrated approximately 2 μmol/kg/h decrease in routine oxygen consumption rate (Sadoul et al., 2017); while, another study on rainbow trout observed an increase of approximately 8μmol/kg/h in oxygen consumption within the first hour of exposure to 400-450 Torr of CO_2 (Thomas et al., 1983). Another freshwater study looking at Atlantic salmon (*Salmo salar*) exposed to elevated CO_2 and a decrease in pH, displayed an increase in SMR by approximately 20 mgO_2/kg/h (6.25×10^{-4} μmol/kg/h converted; Khan et al., 2018). Thus, even among the limited literature, there is no consensus on how the metabolic rate is affected by elevated CO_2.

A likely explanation for the increase in SMR found in Arctic charr in my study relates to the increased energetic costs on an individual as a result of exposure to elevated CO_2 (Heuer and Grosell, 2014). Fish gills are more permeable to CO_2 than hydrogen protons, which promotes acidosis in the internal environment of the fish (Lefevre, 2019). Briefly, acidosis occurs as metabolic CO_2 is produced in the mitochondria and diffuses down its concentration gradient eventually reaching the blood (Brauner et al., 2019). From the blood, the CO_2 is transported to the gills where it passes through the gill plasma membrane aided by carbonic anhydrase (CA) and water (Esbaugh and Tufts, 2006; Henry and Hemin, 1998). At the gills, CO_2 and O_2 gases are exchanged across the epithelium where the offloading of CO_2 occurs from the red blood cells

and causes a decline in HCO_3^- (the compound used to help with offloading; Henry and Heming, 1998). This reaction is bi-directional, an increase in CO_2 can cause the reaction to be pushed into acidosis as more of the basic compound is released to help reduce the amount of CO_2 within the body. Elevated CO_2 activates these ion channels to maintain normal blood pH and homeostasis, but at the cost of losing energy (Brauner et al., 2019). This increase in energy use may be why we are seeing an increase in SMR within the Arctic charr; however, it does not explain why an increase in SMR was only observed in Arctic charr. It is possible that because we used hatchery-reared fish, past exposure (both within their lifetime and generational) has improved their capacity for intracellular pH regulation (Brauner et al., 2019).

The lack of evidence in my experiment that CO_2 influences growth, feeding rate, and behavioural was not overly surprising considering the wide array of effects seen within the literature. For example, in my study, no evidence of reduced body mass was seen between the treatments of any species. This is in stark contrast to countless other studies in which a negative growth rate was measured between CO_2 treatments in other salmonids (Fivelstad et al., 1999; Gil Martens et al., 2006; Good et al., 2010; Last et al., 2011). However, studies have seen no effect on growth or an increase in growth as a response to elevated CO_2 (Fivelstad et al., 2003). Additionally, only the SMR of Arctic charr were seen to show evidence of a treatment effect, while MMR and AS did not. The AS is calculated from the subtraction of SMR from the MMR thus it is possible that the difference seen in the SMR of Arctic charr was not large enough to also alter the AS in this species. In Khan et al. (2018), during an acute exposure, MMR was significantly affected along with time to reach SMR and EPOC (Excess Post-Exercise Oxygen Consumption) in Atlantic salmon under CO_2 exposure of 0 mg/L and 10mg/L (converted to ~ 0 µatm and 5000 µatm). Furthermore, no behavioural changes were observed in this study for any

of the species. Again, this contrasts with multiple other studies which report a negative association between CO_2 and behavioural responses (Leduc et al., 2013; Nilsson et al., 2012; Ou et al., 2015). However, other studies have also observed no difference in the behaviour of freshwater fish exposed to elevated CO_2 (Midway et al., 2017; Tix et al., 2017b).

There are a number of explanations as to why few significant results were observed in the study. Fish are ectotherms, their physiology can be easily influenced based on the temperature of their environment. Salmonids are cold water fishes and there are species-specific preferred temperatures within this family. For example, brook charr has a temperature range of 10–20˚C and rainbow trout have a preferred temperature of 8–22 ˚C (Bear et al., 2007; Smith and Ridgway, 2019). Arctic charr, on the other hand, prefers cooler water and has a temperature range of 5–9 ˚C (Harris et al., 2020). Thus, the temperature used in this study (~10 ˚C) is not optimal for any of the species and is, in fact, too warm for Arctic charr. The combination of this higher temperature and the CO_2 treatment may explain why the SMR in the CO_2 treated group was higher than the control group as well as why no other species experienced a difference. For example, studies have already shown that temperature influences the metabolic rate (Durhack et al., 2021; Smith and Ridgway, 2019). This is normally due to the higher energetic cost of the biological mechanism as different proteins and enzymes do not function the same in extreme thermal conditions (Schulte, 2015), which leads to declines in fitness (Clarke and Johnston, 1999; Durhack et al., 2021; Neubauer and Andersen, 2019), growth rate (Bear et al., 2007; Jobling, 1997; Larsson, 2005), and feeding rate (Jobling, 1997; Larsson, 2005; Volkoff and Rønnestad, 2020).

An alternative explanation for not finding many effects of exposure on juvenile salmonids may be attributed to the short exposure time used in the experiments. Though some

studies on the effects of CO_2 exposure on freshwater fishes have used two-week long exposure periods (Hasler et al., 2016b) other studies have exposed fish to elevated CO_2 for over a month to just under a year (Skov, 2019). Due to the logistics of working with multiple species and a limited amount of time available on site at the hatchery 15 days was the longest exposure period we could have. Studies that have tested mass-specific metabolic rate and growth rate effects of CO_2 exposure over time have seen that it is only after two weeks when a significant difference in growth rate and MMR were observed (McCormick and Regish, 2018; Ou et al., 2015). The delay in response to elevated CO_2 may have to do with the exposure timeline. Extending the exposure time period in future experiments may clarify if the lack of significant findings was due to the use of a short exposure period.

The experimental level of CO_2 used may have also been too low for effects to be observed. Generally, there are three major categories CO_2 concentrations can be placed in. The first is concentrations used to explain what may happen due to predicted atmospheric CO_2 levels of approximately 1000 μatm (i.e., a value predicted in climate change models; McNeil and Matsumoto, 2019). Many of the studies that use a climate change target are done in marine ecosystems where it is easier to predict future levels. This is not the case for freshwater ecosystems where several factors must be considered to predict future CO_2 levels, including some lakes already surpassing these levels (Hasler et al., 2016a). A second target concentration used is associated with aquaculture. Overall higher levels of CO_2 are observed in aquaculture due to high stocking densities and bacterial respiration (Fivelstad, 2013; Fivelstad et al., 1999; Fivelstad et al., 2003; Gil Martens et al., 2006; Hosfeld et al., 2008; Khan et al., 2018). This is so well known that dedicated CO_2 strippers or degassing units are often found in these facilities. The third category of CO_2 concentration used are those related to the use of CO_2 as an invasive

species barrier (Suski, 2020). These concentrations of CO_2 can range from 29.0 mg/L to 798 mg/L (converted to 20000 µatm ~ >200000 µatm) depending on the species and the freshwater properties (Cupp et al., 2020; Schneider et al., 2018; Schneider et al., 2019). In my study, the level of CO_2 used in the elevated exposure treatment was based on a tripling of current source lake levels. As noted above, it is difficult to predict what might happen in a specific freshwater system as a result of climate change, and therefore I targeted a number that was clearly elevated, but within reason as to not induce mortality before the two-week experimental period was over. A future study should aim to seek thresholds where metabolic, growth, and behavioural responses do occur.

The use of hatchery reared fish in my experiments may have also affected my ability to detect responses to elevated CO_2. Many aquaculture facilities require constant monitoring and equipment to maintain water quality due to the concentration of metabolic CO_2 and bacteria-produced CO_2 in high-density aquaculture (Skov, 2019). Water quality data linked to CO_2 (e.g., pH and alkalinity) is not collected at the hatchery where my experiment took place, thus it is not known what levels of CO_2 the fish used in my experiments were exposed to prior to the experiments. It is possible that fish had been exposed to elevated CO_2 and this may have prompted two different types of adaptation. The first type of adaptation is within generational phenotypic plasticity. Very little has been done on this topic, but in marine spiny damselfish (*Acanthochromis polyacanthus*) they have shown that within the first 4-days, 184 genes indicated a degree of response within a cohort of fish in elevated CO_2 (Schunter et al., 2018). Specifically, the genes that were enriched are ATPase-related processes indicating a high energetic cost at the start of the exposure (Schunter et al., 2018). It may be possible that due to the condition and age of the fish used in this study they may have already undergone phenotypic

changes to balance the effects of elevated CO_2. The second type of adaptation is across generational changes. The hatchery uses brood stocks to produce the current generation of fish, it is possible generational exposures to elevated CO_2 in hatcheries has led to fish that are better adapted to high CO_2 conditions. Though it is unclear how fish are selected to become brood stock at the Whiteshell Hatchery, it is possible that fish that perform best in the high CO_2 conditions of a hatchery setting are also the fish that would be more tolerable to high CO_2 and used for breeding (Ellis et al., 2017; Munday et al., 2019). Furthermore, multiple studies looking at marine reef fishes have found that if the parental generations are exposed to high CO_2 conditions certain adaptive traits are shared with offspring (Miller et al., 2012; Monroe et al., 2021). It is also possible that a combination of both intergenerational and generational effects are at play when it comes to tolerance of CO_2 in fish (Schunter et al., 2018), and future studies should investigate these effects.

One of the main reasons we studied three salmonid species was that we wanted to understand how responses to CO_2 might be influenced by taxonomic relationships. Unfortunately, with limited significant responses, an examination of the influence of phylogeny is not possible. Additionally, the taxonomy of fishes is complex and constantly under review, with salmonids in North America being no different. Currently, it is thought that rainbow trout phylogeny is related to that of pacific salmon (*Oncorhynchus spp.*; Jonsson et al., 2019). This genus has a southern range that can stretch all the way to Mexico (Penaluna et al., 2016). Unlike the *Oncorhynchus*', the charrs (*Salvelinus*) have a northern circumpolar distribution (Power, 2002). Within this genus, I used two species, Arctic charr and brook charr, in which brook charr has a larger, more southern range. Consequently, each species will experience different water routes with varied temperatures which can influence the rate in with CO_2 can dissolve into the

water due to the principles of gas exchange. These species also have different life histories. For example, size wise, Arctic charr only has a maximum size of 110 cm and 20 kg (Kottelat and Freyhof, 2007), brook charr have a max size of 86 cm and 8 kg (Machacek, 2015; Skelton, 1993), and rainbow trout have a max size of 122 cm and 25 kg (Hutchings and Morris, 1985; Jonsson, 2013). This is significant as some larval fish are more sensitive to elevated levels of CO_2 compared to adults (Ishimatsu et al., 2004). Understanding how CO_2 may affect different species is important as levels of CO_2 increase, information such as this can help make informed management discissions on vulnerable populations.

Within the context of elevated CO_2 for freshwater fish, the results of my study have implications for fisheries management and aquaculture. It is only a matter of time before salmonids within the natural population and aquaculture will be exposed to elevated CO_2 due to natural sources such as environmental variation (Hasler et al., 2016a), climate change (McNeil and Matsumoto, 2019), hatchery rearing for stocking programs (Ou et al., 2015), and more intensive aquaculture (Gil Martens et al., 2006; Khan et al., 2018). If they are exposed to elevated CO_2 for an acute period no strong effects will likely be noted in growth rate, metabolism, feeding rate and behavioural responses, as displayed in this study. The source water used already had a high amount of dissolved CO_2 in it (approximately 1100 µatm) thus my elevated CO_2 group aimed to be triple the control values at approximately 5236 µatm. This level of µatm may seem high in relation to other freshwater studies but many of these studies have a lower base line and thus the elevated treatment can be 4 to 30 times that of the baseline (Fivelstad et al., 2003; Khan et al., 2018; Ou et al., 2015). Additionally, the time frame of my study was two weeks which I am considering an acute exposure as many other CO_2 studies have much longer time frames of weeks to years (Skov, 2019). No observable negative effects of CO_2

were found within the time frame but by extending the time frame negative effects may appear in the growth rate, metabolic rate, and behaviour (Heuer and Grosell, 2014; Leduc et al., 2013; Lefevre, 2019; Skov, 2019). These negative effects have implications on the survival for young juvenile salmonids both from a physiological sense and from a behavioural sense. This study indicates that even under elevated levels of CO_2 juvenile fish from these populations and under these experimental conditions will not experience negative affects when exposed for an acute period, therefore they are able to function without repercussions in elevated levels of CO_2. However, work is still needed to understand how CO_2 may alter other freshwater fish species growth, metabolism, feeding rate, and behaviour. Specifically, work related to within generation and across generations adaptation to elevated CO_2 should be investigated as many stocking fish, as well as laboratory fish come from hatchery conditions. As research in this field continues, data sets such as this can help to better understand how our freshwater ecosystems will be changing as atmospheric CO_2 increases due to climate change.

Chapter 3: Using Histology to Explore Possible CO_2 Induced Tissue Damage in Salmonids

Abstract

The amount of carbon dioxide (CO_2) within our atmosphere is increasing and more of it is ending up in our freshwater systems. A limited number of studies have looked at how CO_2 effects fish at the tissue level. To study these effects Arctic charr (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*), and brook charr (*Salvelinus fontinalis*) were exposed to either control levels of CO_2 (1400 µatm) or elevated levels of CO_2 (5236 µatm) for 15 days. At the end of the 15 days, the fish were euthanized and the 2nd gill arch, hearts, and livers were collected from the fish. Through histological analysis, changes within the tissues were noted. Other factors included secondary lamellae length, and the thickness of the compact myocardium. When looking at the combination of changes occurring within the gills and liver there was an effect of CO_2 on Arctic charr. There were no other effects seen within the other tissue samples taken. Ongoing research dedicated to examining how elevated CO_2 may affect internal tissues of fish will allow for a more comprehensive understanding of how these species may fair with ongoing climate change and within aquaculture facilities.

Introduction

Climate change is a large and global issue that encompasses many aspects of our world. One of the greenhouse gases that contributes to climate change is carbon dioxide (CO_2). Atmospheric levels of CO_2 have increased by 68 % since the industrial era (IPCC, 2014). These CO_2 levels have resulted in increased air and water temperatures, extreme weather events, melting poles, and ocean acidification (Rhein et al., 2013). CO_2 levels are not likely to decrease in the foreseeable future but are more likely to increase in the coming years due to anthropogenic sources. The continued accumulation of CO_2 levels within our atmosphere will only cause further environmental damage.

Atmospheric CO_2 ultimately gets deposited into carbon sinks such as freshwater rivers and lakes (Bastviken et al., 2011; Grasset et al., 2020; St Pierre et al., 2019), or it can enter freshwater via repiration (both allocthonous and autocthonous; Sobek et al., 2003). Once in water, CO_2 gas dissolves and forms carbonic acid, which then becomes bicarbonate. Depending on pH, more or less CO_2 gas will remain and acidification is possible (Wetzel, 2001). There are also abiotic factors that can influence the extent of acidification that occurs including the source of the water, the speed at which the gas dissolves in, and the surrounding geology of the area (Wetzel, 2001). Additionally, biotic factors such as the amount of dissolved organic matter (DOM) and dissolved inorganic carbon (DIC) can also add to increased levels of CO_2 within freshwater. Knowing how all these factors play a role in determining the effects of partial pressure of CO_2 (pCO_2) in the fresh water environment helps determine the levels of CO_2 in which organisms living there can experience.

Organisms within freshwater ecosystems that experience acidification can be negatively impacted. Fish have a close connection with their environment via a constant stream of water

over their gills. There have been numerous studies looking at how acidification affects fish behaviour, growth rate, and metabolic rate. When looking at behaviour associated with elevated CO_2 it is important to be critical of some of the findings. Some studies looking at ocean acidification display large size effects and have been challenged due to unrepeatable outcomes using the same methods (Clark et al., 2020). When narrowing this field there is evidence of altered alarm cue responses (Tix et al., 2017b), and loss of olfaction (Elvidge and Brown, 2014; Munday et al., 2009). Interestingly, it has also been seen that some of these behavioural affects maybe reversable once fish are placed back in low CO_2 environments (Hasler et al., 2016b; Tix et al., 2017b). Some of the major findings within growth rate studies are decreases in growth (Fivelstad, 2013; Fivelstad et al., 2003; Khan et al., 2018; Ou et al., 2015), no effects on growth rate (Cattano et al., 2018; Hosfeld et al., 2008), or an increase in growth rate (Fivelstad et al., 1999). Similar results can be seen in metabolic rate, with increase (Khan et al., 2018) or a decreasing (Ou et al., 2015) rates as a result of high CO_2. However, among all these different aspects there is no clear consensus on the outcomes specifically related to CO_2 exposure. The reasons for organismal responses (or lack of) have not been investigated at the sub-organismal level where tissue damage or pathology may be occurring, regardless of whether CO_2 has been found to have an effect at the organismal level.

One way to examine how CO_2 may be affecting fish at the sub-organismal level is by observing tissues from multiple organs using histological techniques. These techniques allow for a microscopic assessment of tissues to observe possible effects of elevated CO_2 at the cellular level. With the change in pH happening externally (as CO_2 enters the water), internal mechanisms must be put in place to maintain homeostasis within the body (Brauner et al., 2019). A study examining how increasing acidification may affect Atlantic cod (*Gadus morhua*)

observed severe tissue damage in 12% of the medium pCO_2 group (1800 µatm) and 75% of the high pCO_2 group (4200 µatm; Frommel et al., 2012). Another study looking at rainbow trout under elevated CO_2 saw many lesions throughout different tissue types, such as the skin, gills, liver, kidney, and spleen 24 mg/L over 6 months (Good et al., 2010). They concluded that even with these lesions, the overall survival of the fish was not affected (Good et al., 2010). As animals are integrative systems, it is important to know how stressors, such as elevated CO_2, influence multiple organ systems.

Common tissues sampled in fish are gills, and liver. Gills are commonly sampled in ocean acidification studies as they are vital for ion regulation and gas exchange. For example, studies observing the gills of fish exposed to acidification saw hypertrophy under 2500 µatm for 56 days, 1010 µatm for 120 days, and 2300 µatm for 14 days (Hermann et al., 2019; Noor et al., 2019; Thomas and Achiraman, 2021), aneurysms (Tegomo et al., 2021), and even necrosis of the tissue (Thomas and Achiraman, 2021). Another major tissue that is commonly looked at when conducting histological examinations is the liver. The liver is a major organ responsible for supplying energy to the body, among other important functions (Bruslé et al. 1996). The common use of the liver could also be due to its ability to react to acute-phase responses (Gabay and Kushner, 1999) and that it is a major point of interaction for exotoxins (Bols et al., 2001). Acidification has been shown to cause severe liver damage (Frommel et al., 2012; Tegomo et al., 2021) and failure in protein synthesis within marine fishes (Dickinson et al., 2012), but has yet to be described in a freshwater fish.

An uncommon tissue that is occasionally sampled is the heart. Many studies that look at hearts in fish record the size of the heart in terms of ventricle mass (Eliason and Farrell, 2016; Farrell et al., 2007). However, the goal of many of these studies is not to determine tissue

damage, such as necrosis, but rather look at the heart sizes and compare them to the cardiac load a fish may experience. More recently with the on-going conversation of climate change there has been a few studies looking at how temperature may affect fishes' hearts. From these studies a hypothesis of heart remodeling has evolved, in which a fish's heart will increase its amount of compact myocardium when faced with a temperature stressor (Klaiman et al., 2011). It is thought that the increasing of this tissue is done to help maintain function even under these stressful conditions. Acidification can also be a stressful event but it is unknown if it is a strong enough stressor to initiate a compensation type mechanism.

The goal of this study was to determine if freshwater salmonids are affected by internal elevated CO_2 during the rearing stage (free-swimming stage, age 0). My first objective was to quantify the severity of tissue damage due to CO_2 exposure in Arctic charr (*Salvelinus alpinus*), brook charr (*Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*) using histological techniques. I hypothesize that fish exposed to elevated CO_2 will experience an array of negative damaging affects on the tissues being tested. I predicted that damage such as aneurysms may be found in the gills (Noor et al., 2019), while the liver may experience necrosis (Frommel et al., 2012), and the heart will see an increase in compact myocardium in order to maintain function in fish under the stressful external environment of the CO_2 treatment (Klaiman et al., 2011). Overall, I expect that there will be variation among the responses to the CO_2 treatment among the three species used, which may relate to the CO_2 levels they experienced during their evolutionary histories.

Methods

Study animals and animal care

Experiments were completed between June 4 and July 28, 2021 and divided into three 15-day CO_2 exposure periods. Exposure periods were completed one species at a time, starting with juvenile Arctic charr *(Salvineus alpinus*; mass = 2.9 ± 0.7 g, total length = 6.1 ± 0.2 cm; Nauvuk Lake strain); then juvenile rainbow trout (*Oncorhynchus mykiss*; mass = 2.3 ± 0.4 g, total length = 5.9 ± 0.4 cm; Nipigon River strain); and, finally juvenile brook charr (*Salvineus fontinalis*; mass = 4.7 ± 1.3 g, total length = 7.8 ± 0.8 cm; Nipigon River strain). These fish were from the 2020 fall spawning season. All the fish used in the study were reared and experimented on at the provincial Whiteshell Hatchery, Whiteshell, Manitoba, Canada. Over the course of the study, fish were fed Skretting number 1 crumble (Skretting Canada, Vancouver, British Columbia) at two percent of their body weight per day. A day before a CO_2 exposure period began, fish were netted from outdoor holding raceways and assigned to one of four indoor raceways (4.3 x 0.30 x 0.30 m) with flow through water from West Hawk Lake, Manitoba. Lake water first passed through UV light and bio-filters to remove microbes and reduce nutrient loads before entering the raceways.

Half of the fish (n = 100) were treated with ambient lake CO_2 (n = 2 raceways), and half with elevated CO_2 conditions (n = 2 raceways). Elevated CO_2 was maintained at 5237 ± 1532 µatm using compressed CO_2 gas bubbled into the water using air stones (38 x 8 cm, a max flow rate of 9 L/min, Point Four Micro bubble diffusers, Cary, North Carolina, USA) controlled by pH pinpoint meters set to a low point of 6.2 and the high of 6.4 with a solenoid valve (American Marine Inc., Ridgefield, Connecticut, USA; Table 1; Figure 1). The level of CO_2 used was 5 times higher than the current lake levels and was intended to represent future lake levels if

dissolved CO_2 rises in the watershed (Figure 1). The partial pressure of CO_2 was monitored in the raceways using a modified infrared CO_2 probe (GM70, Vaisala, Helsinki, Finland; Table 1; Johnson et al., 2010). Water samples were titrated twice daily using a digital titration kit to determine total dissolved CO_2 (mg/L)(Hach model CA-DT; 10-1000 mg/L, Hach Company, Loveland, Colorado, USA). Additionally, pH, ammonia, and alkalinity were also monitored. Temperature and dissolved oxygen (DO % saturation) were also measured twice daily using YSI Pro 1020 dissolved oxygen and pH meter (Xylem Inc, Yellow Springs, Ohio, USA).

Histological tissue preparation and staining

After the 15-d exposure period, 14 fish from each species and treatment were euthanized by abrupt cerebral percussion and severing of the spinal cord behind the opercula. The 2nd gill arch of the left side of the fish was taken as the second gill arch is commonly selected for histological analysis and, the liver, and the heart were removed and placed in 10 % neutral buffered formalin and stored in an onsite fridge at 4 ˚ C. After approximately, 6 weeks tissues underwent a series of dehydration starting with 70 % ethanol and ending with 100 % ethanol before being placed in clearing solvent. Tissues were then placed into molds in which melted paraffin wax was added. When the molds cooled, the wax chucks were removed from the molds and sliced using a microtome (Leica biosystems, Buffalo Grove, Illinois, USA) into 5 µm sections. Sections were then mounted on to precleaned frosted 25 mm x 75 mm x 1 mm glass microscope slides (VWR International, Radnor, Pennsylvania, USA).

Slides were then placed into clearing solvents to remove the wax and then went through four ethanol washes starting with 100 % to 80 % to rehydrate before placing in deionized water prior to staining. Alcian blue which stains the cartilage in the gills, Haematoxylin staining the nuclear components, and Eosin which stains cytoplasmic were the stains used and applied following standard protocols (Luna, 1968). Slides were then placed on a light microscope (CX41 Upright Microscope, Olympus Corporation, Tokyo, Japan) with images taken using an Infinity Analyze 2-1 digital camera (Lumenera Corporation, Ottawa, Canada). Gill tissue was examined for histological lesions within the lamellae as well as changes in lamellar length (Noor et al., 2019), heart tissue was examined for any signs of heart remodeling (increases in the thickness of the compact myocardia; Klaiman et al., 2011), and livers were assessed for signs of damage such as necrosis due to the CO_2 exposure (Frommel et al., 2012).

Organ index

The histological changes were evaluated qualitatively and quantitatively using a protocol described by Bernet et al. (1999). For the gill and liver, 5 slices of each tissue were examined per individual. Scores from 1–6 were assigned based on the percentage of the tissues experiencing changes. In liver, most of these changes come from glycogen-type vacuolation occurring. In my study < 15 % of the field of view were considered unchanged and > 85 % of the field of view were used to help determine a severe occurrence of possible vacuolation in the liver. In the gills, if < 5 % of the secondary lamellae are affected the tissue was considered unchanged and if > 85 % secondary lamellae are affected the tissue was considered to have a severe occurrence. The importance factors range from 1–3 with higher importance factors being placed on the changes that represent the greatest potential for impact on fish health (Bernet et al., 1999). The individual organ index for the liver and gill were assessed for changes, then the scores in addition to the importance factors were entered into the equations (Equation 1). These individual organ indexes can be summed to create a total organ pathological index (Equation 2).

$$I_{org} = \Sigma_{rp} \Sigma_{alt} (a_{org\ alt} \times w_{org\ rp\ alt})$$

Equation 1. Organ index (I_{org}) for the change within the gill and liver. In which $_{org}$ = organ, $_{rp}$ = reaction pattern, $_{alt}$ = change, a = score value, and w = importance factor. This is an equation proposed in Bernet et al 1999.

$$Tot - I = \Sigma_{org} \Sigma_{rp} \Sigma_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})$$

Equation 2. Total index (I_{org}) for the change within both the gill and liver. In which $_{org}$ = organ, $_{rp}$ = reaction pattern, $_{alt}$ = change, a = score value, and w = importance factor. This is an equation proposed in Bernet et al 1999.

Gill lamellar length

To determine the secondary lamellae length ten random points were generated in ImageJ (Blair et al., 2016; Ong et al., 2007). The secondary lamella closest to the points were measured using the measuring tool in ImageJ. These lengths were recorded per slide and an average of the 10 lengths was taken.

Compact myocardium

Heart samples were uploaded to ImageJ v 1.8.0 (image processing and analysis in java) in which three points were generated in ImageJ along the posterior outer edge of the ventricle (Johansen et al., 2017). Using a measurement tool within ImageJ, the thickness within this section was recorded. From that, an average for each heart sample was compared. A reference on how to identify the compact myocardium apart from the sponge myocardium is provided (Figure S4).

Statistical analyses

To test the effects of CO_2 on the fish gill index, liver index, and total index, a generalized linear model was used (Brooks et al., 2017). For these models, the treatment and species were used as an interactive linear predictor. To evaluate the effects of the CO_2 treatment on the compact myocardium and the secondary lamellar lengths, a linear model was used (Bates et al., 2015) Again, treatment and species were used as the linear predictors. Statistical analyses were performed using R, version 4.1.1 (R Foundation for Statistical Computing, 2022). For all analyses significance was accepted at $P < 0.05$ and all values in the text are presented as mean ± standard error. Model residuals were analyzed to ensure that the model assumptions of normality and homogeneity are met.

Results

Organ index

There was no evidence that the gill pathological index was affected by CO_2 in any of the species (Figure 3.1; Generalized linear model, family = Poisson, $p > 0.05$). Though there was no evidence, Arctic charr from the CO_2 treatment showed the greatest number of changes to the gills (Figure 3.1), which primarily consisted of aneurysms within the secondary lamellae (Figure 3.2a).

The liver pathological index displayed no evidence that CO_2 had an effect within any of the species (Figure 3.3; Generalized linear model, family = Poisson, $p > 0.05$). There was no evidence to support that there was a species difference across either treatment (Figure 3.3, Generalized linear model, family = Poisson, $p > 0.05$).

The total pathological index displayed weak evidence that only Arctic charr had differed between the treatments (Figure 3.4, Generalized linear model, family = Poisson, $p < 0.05$). The mean total index for the CO_2 treatment in Arctic charr was double that of the control treatment (Figure 3.4). Brook charr and rainbow trout both showed no evidence that treatment affected the total pathological index for either species (Figure 3.4; Generalized linear model, family = Poisson, $p > 0.05$). There is also no evidence that species differed in their total pathological index (Figure 3.4, Generalized linear model, family = Poisson, $p > 0.05$).

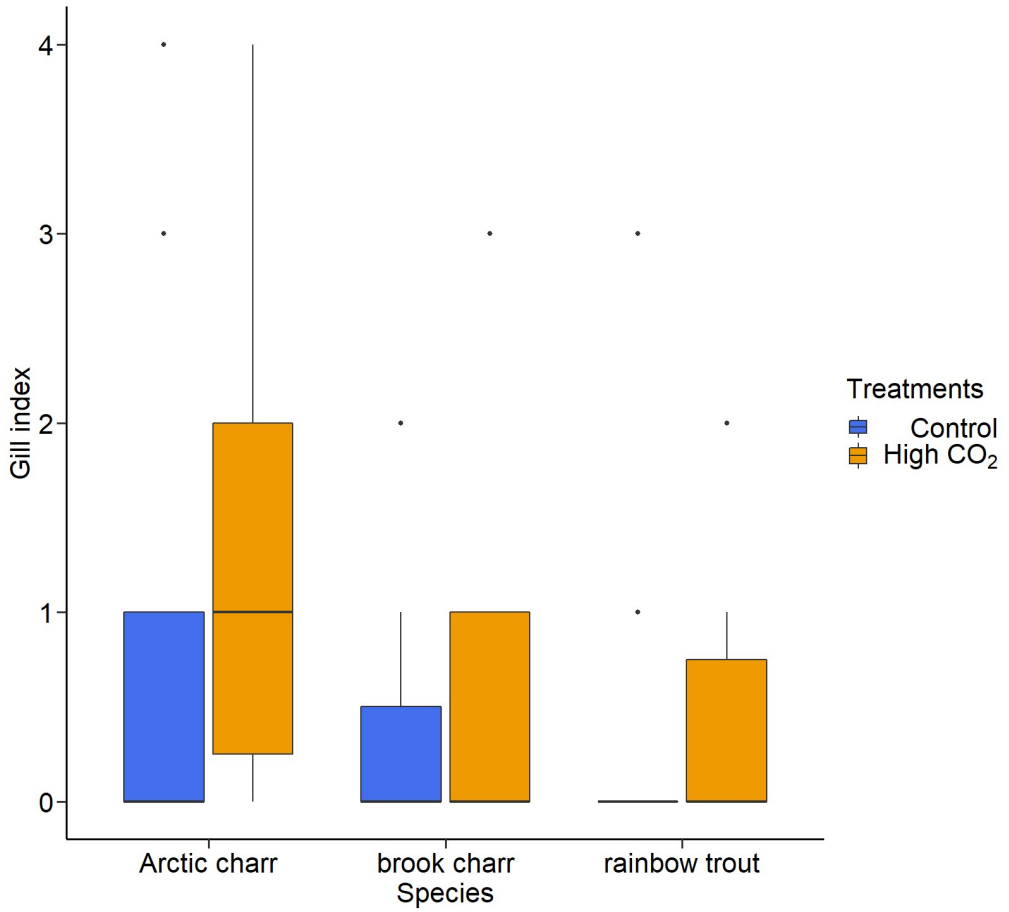

Figure 3.1. Gill index score based on circulatory disturbances and structural changes in Arctic charr (*Salvelinus alpinus*, Control n = 14, CO_2 n = 14), brook charr (*Salvelinus fontinalis*, Control n = 15, CO_2 n = 13), and rainbow trout (*Oncorhynchus mykiss*, Control n = 15, CO_2 n = 14) gills. The horizontal bars depict the 25 % quartile, median, and 75 % quartile. The lowest whisker indicates the minimum value determined by the 25th percentile minus 1.5 times the interquartile range and the highest whisker indicates the maximum value determined by the 75% percentile plus 1.5 times the interquartile range The black dots indicate outliers.

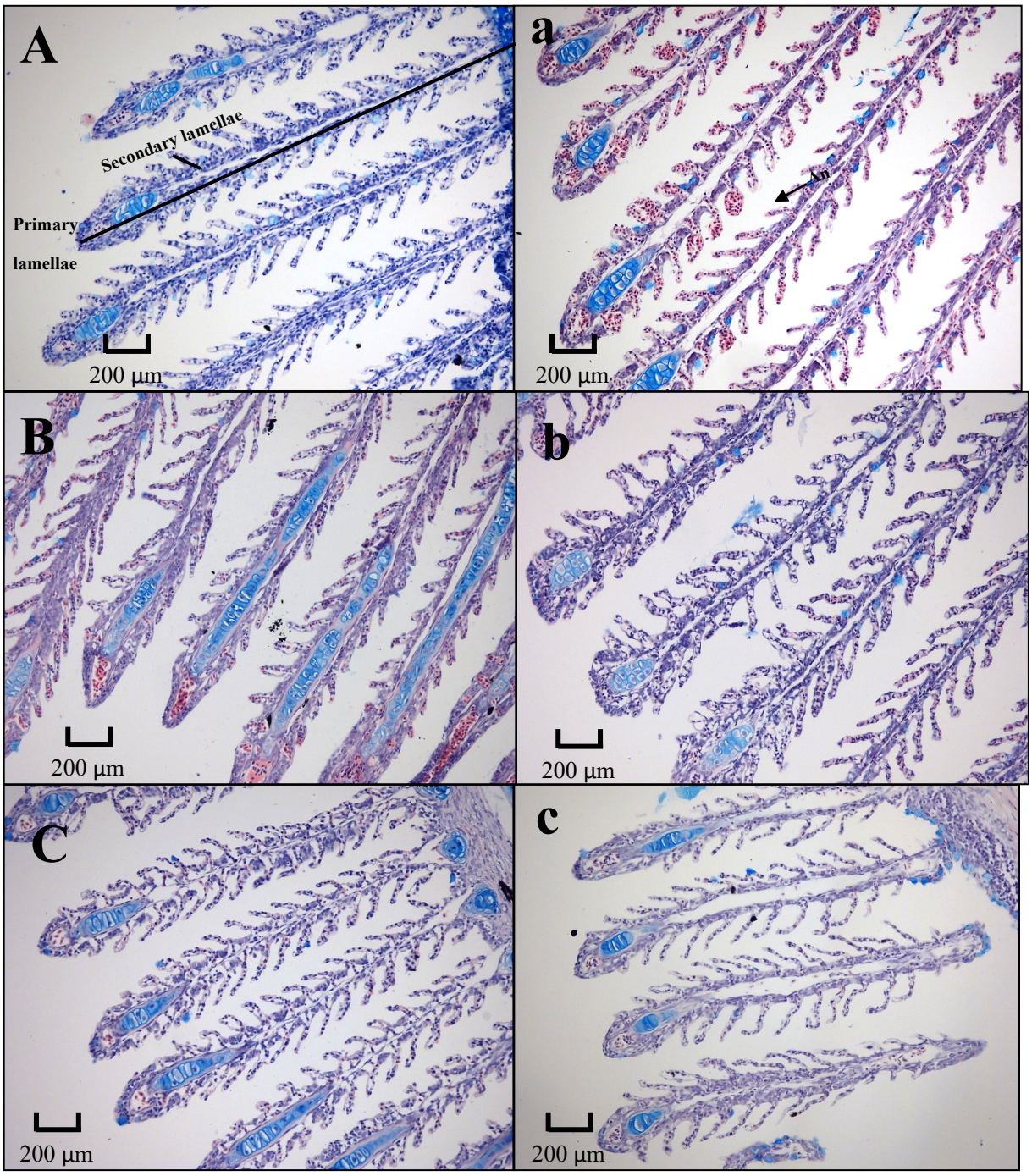

Figure 3.2. Reference images of gill sections from Arctic charr (*Salvelinus alpinus*) (A, a), brook charr (*Salvelinus fontinalis*) (B, b), and rainbow trout (*Oncorhynchus mykiss*) (C, c) from either the control treatment fish (uppercase letter) or CO_2 treatment fish (lowercase). The gills are all from the 2nd gill arch on the left side of the fish. The lines represent primary and secondary lamellar. The black arrow points towards an aneurysm (An). The tissue was sliced to 5 µm thickness and placed under 40 X magnification. Scale bars represent 200 µm.

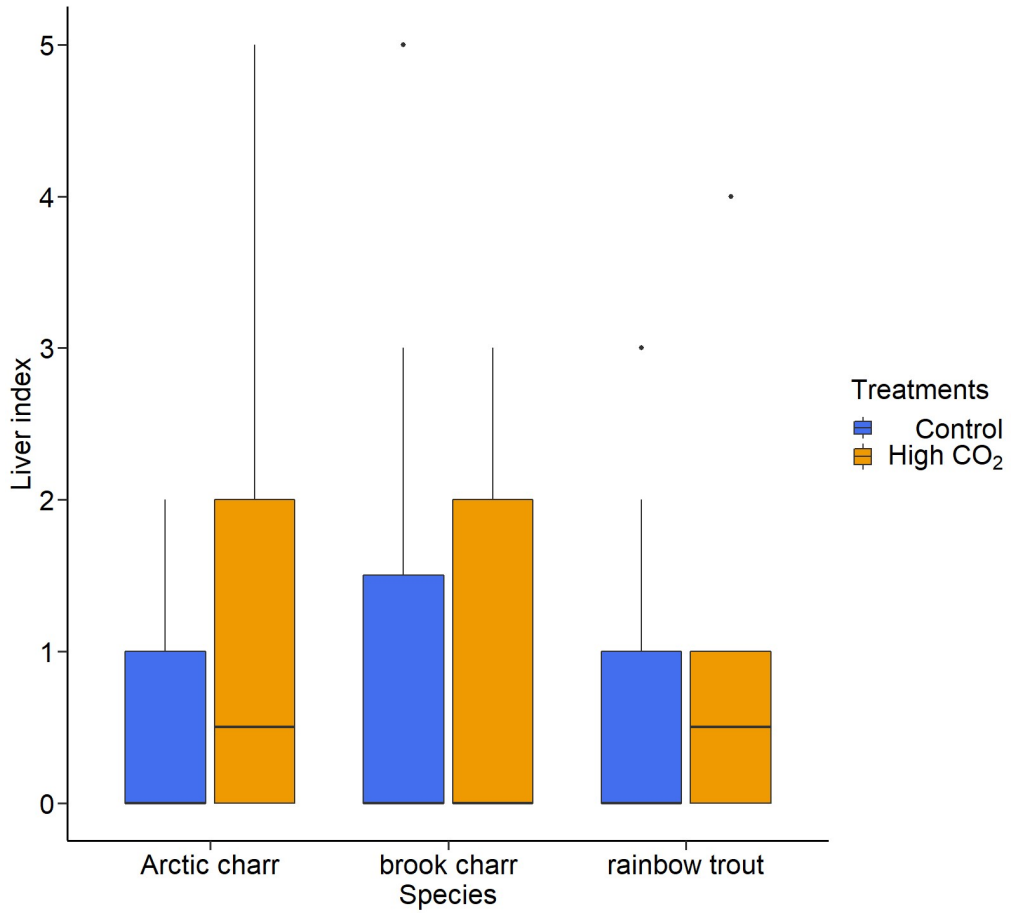

Figure 3.3. Liver index score based on architectural and structural changes in Arctic charr (*Salvelinus alpinus*, Control n= 14, CO_2 n= 14), brook charr (*Salvelinus fontinalis*, Control n = 15, CO_2 n = 13), and rainbow trout (*Oncorhynchus mykiss*, Control n = 15, CO_2 n = 14) livers. The horizontal bars depict the 25 % quartile, median, and 75 % quartile. The lowest whisker indicates the minimum value determined by the 25th percentile minus 1.5 times the interquartile range and the highest whisker indicates the maximum value determined by the 75% percentile plus 1.5 times the interquartile range The black dots indicate outliers.

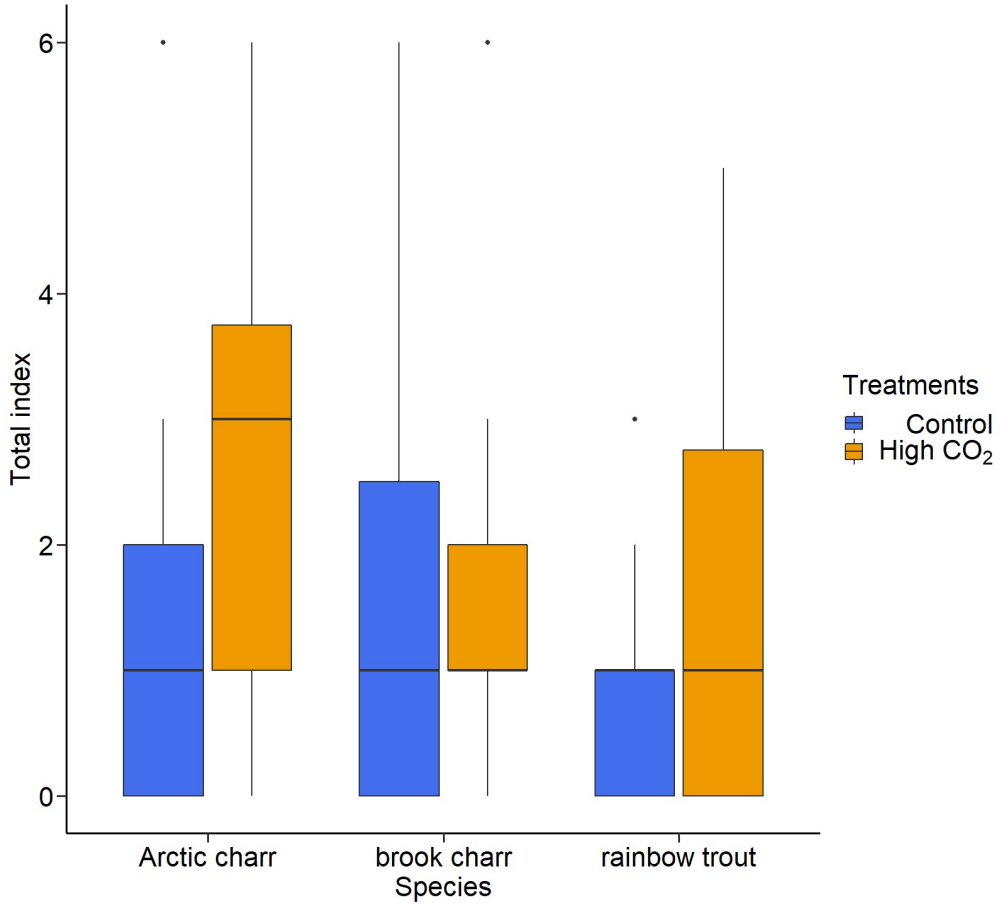

Figure 3.4. Total index which is the sum of the gill index and liver index scores for Arctic charr (*Salvelinus alpinus*, Control n = 14, CO_2 n = 14), brook charr (*Salvelinus fontinalis*, Control n = 15, CO_2 n = 13), and rainbow trout (*Oncorhynchus mykiss*, Control n = 15, CO_2 n = 14) livers. The horizontal bars depict the 25 % quartile, median, and 75 % quartile. The lowest whisker indicates the minimum value determined by the 25th percentile minus 1.5 times the interquartile range and the highest whisker indicates the maximum value determined by the 75% percentile plus 1.5 times the interquartile range. The black dots indicate outliers.

Secondary lamellae length

Only CO_2 exposed Arctic charr displayed evidence of a differences in secondary lamellae lengths when compared to controls (Figure 3.5; Linear model, $p < 0.05$). Secondary lamellae lengths were longer by 15 % in the CO_2 treatment (Figure 3.5). There was evidence of a species difference with brook charr having the longest secondary lamellae (Figure 3.5; Linear model, $p < 0.05$).

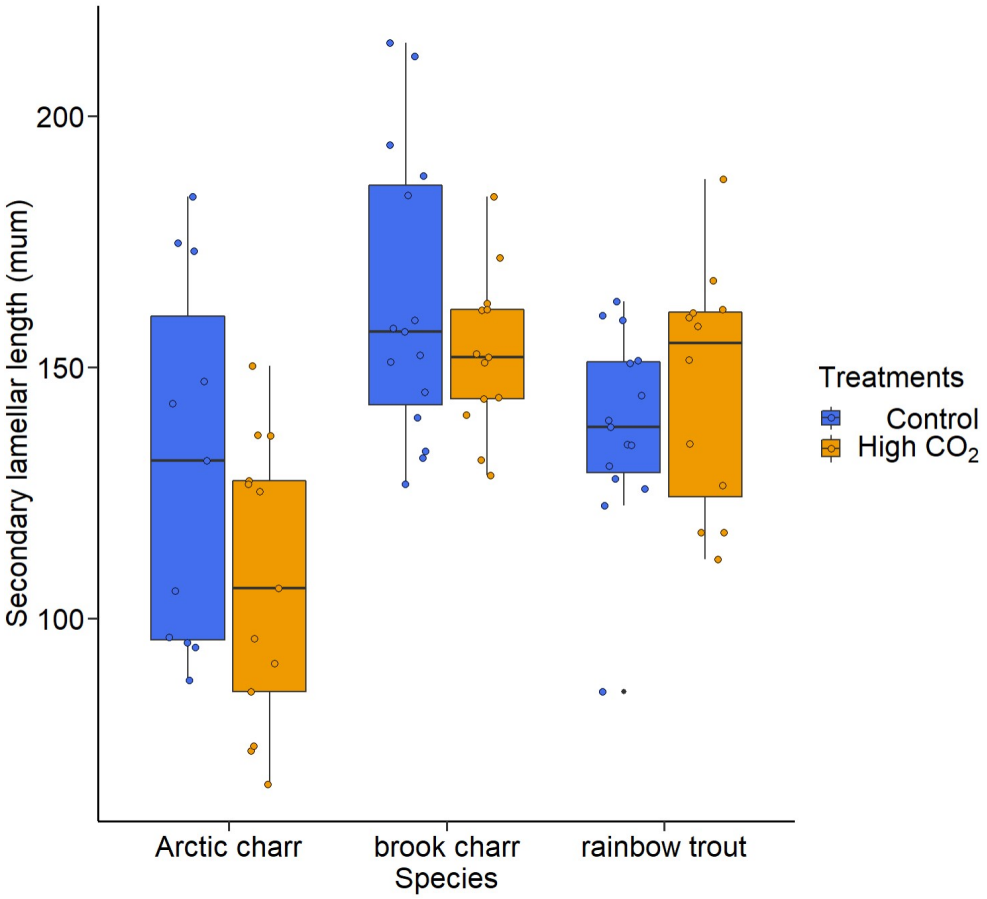

Figure 3.5. Secondary lamellar length in micrometer (µm) for Arctic charr (*Salvelinus alpinus*, Control n = 14, CO_2 n = 14), brook charr (*Salvelinus fontinalis*, Control n = 15, CO_2 n = 13), and rainbow trout (*Oncorhynchus mykiss*, Control n = 15, CO_2 n = 14) livers. The horizontal bars depict the 25 % quartile, median, and 75 % quartile. The lowest whisker indicates the minimum value determined by the 25th percentile minus 1.5 times the interquartile range and the highest whisker indicates the maximum value determined by the 75% percentile plus 1.5 times the interquartile range The black dots indicate outliers. The coloured dots represent individual data point per each treatment and species.

Compact myocardium thickness

There was no evidence that CO_2 treatment affected the thickness of compact myocardium in any of the species used (Figure 3.6; Linear model, $p > 0.05$). On average Arctic charr had a compact myocardium thickness of 66.71 ± 4.12 µm, while rainbow trout had an average thickness of 68.18 ± 3.65 µm, and brook charr had an average of 77.98 ± 4.30 µm under the control treatment (Figure 3.6). There was evidence of a species difference between rainbow trout and brook charr (Linear model, $p < 0.05$).

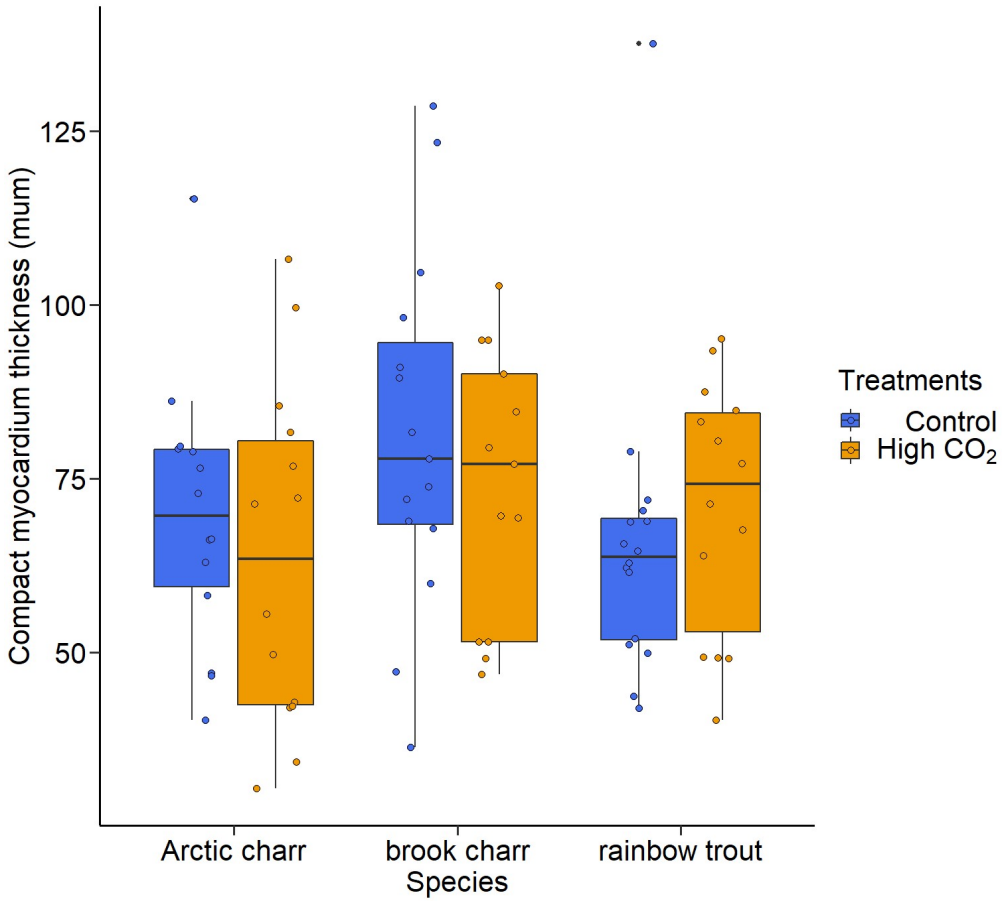

Figure 3.6. Amount of compact myocardium thickness in micrometer (µm) of Arctic charr (*Salvelinus alpinus*, Control n = 14, CO_2 n = 14), brook charr (*Salvelinus fontinalis*, Control n = 15, CO_2 n = 13), and rainbow trout (*Oncorhynchus mykiss*, Control n = 15, CO_2 n = 14). The horizontal bars depict the 25 % quartile, median, and 75 % quartile. The lowest whisker indicates the minimum value determined by the 25th percentile minus 1.5 times the interquartile range and the highest whisker indicates the maximum value determined by the 75% percentile plus 1.5 times the interquartile range. The black dots indicate outliers. The coloured dots represent individual data point per each treatment and species.

Discussion

The two factors in which evidence was found that the CO_2 treatment effect was within the total organ index and the length of the secondary lamellae in Arctic charr. These results indicate that a tissue level response is occurring within this species due to the elevated CO_2. However, the fact that evidence of a CO_2 effect only appears in the total index shows how both tissues that make up this total index (gills and liver), individually display a change but not a large enough change to be significant on its own. These changes may indicate the early signs of a system failure. For example, increasing the length of the secondary lamellae requires energy to occur. The liver within the fish is one of the major sources of metabolic energy (Wolf and Wolfe, 2005), but under CO_2 conditions, changes were made to this source. Over time this change may get worse and the fish my have to compensate for this loss in organ function due to the elevated CO_2. This additive effect may be what is occurring within the Arctic charr as individually the gills and liver do not display evidence of a treatment effect but when summed together the effect of CO_2 is present. Thus, these small changes in the organs may start a chain reaction that can cause a larger whole body compensation mechanism to occur.

Liver changes can be serious at times, but can also display non-life-threating changes (Wolf and Wolfe, 2005). In trout that are exposed to allyl formate there is a severe necrosis in the liver (Droy et al., 1989), whereas other trout exposed to tri-*n*-butyltin chloride and bisoxide just have a reduction in the hepatic glycogen, or glucose storage cells, within the liver (Seinen et al., 1981). Thus, both studies displayed an affect of treatment but to varying degrees of severity on the fish. The increase in glycogen seen could be occurring due to a decrease in glycogen being broken down, as was seen in medaka (*Oryzias laptipes*) and guppies (*Poecilia reticulata*; Wester and Canton, 1987; Wester et al., 1990). The increase in CO_2 may have caused this to happen,

however, all the species used in this study experienced similar amounts of glycogen in the liver. These vacuolations were seen in both treatments of this study (Figure S4). Glycogen vacuolations are a common response that occurs in fish livers when exposed to toxins (Ferguson, 1989). Another theory as to why they are occurring within both treatments could be related to how fishes living within a captive environment experience vacuolation as a result of overfeeding (Penrith et al., 1994). This has implications for this study as all the fish used were raised within a captive hatchery environment. Thus, it is difficult to distinguish if the glycogen vacuolation was due to the treatment or a factor attributed to captive feeding practices.

The fish gills also experienced changes commonly observed in ecotoxicology. Anything harmful found within the water can encounters the gills without crossing countless barriers. In this study the major changes observed were aneurysms forming at the end of the secondary lamellae (Figure 3.2, a). These aneurysms have been seen in other species when exposed to toxins (Barker et al., 1994; Spies et al., 1996; Teh et al., 1997). It is thought that the aneurysms are probably the result of the death of pillar cells in the secondary lamellae where the loss of these structural cells allow for blood to fill and pool at the end of the lamellae (Hassaninezhad et al., 2014; Strzyżewska-Worotyńska et al., 2017; Van Den Heuvel et al., 2000). Damage to the gills, such as aneurysms, can leave the gills "leaky" to ions as the gills are one of the main areas of ion transfer (Van Den Heuvel et al., 2000). Damage such as this can provoke an adaptive tissue response in order to prevent the loss or addition of ions (Van Den Heuvel et al., 2000). Freshwater fish have to constantly prevent ion loss through the gills as ions wish to diffuse out of the body down their concentration gradient to an area of low concentration, their environment. This may be a double edge sword for the species used in this study as the CO_2 within the environment is causing the damage to the gills, at the same, putting more pressure on ion

regulation as fish try to compensate for the decrease in pH (Brauner et al., 2019). The offloading of pCO_2 from the red blood cells causes a reduction in the intracellular HCO_3^- due to the processes related to removing this pCO_2 (Henry, R. P. and Heming, T. A. 1998). The acidosis elevates the amount of H^+ ion and HCO_3^- in the plasma to compensate for the increase in CO_2, this is primarily done through the gills (Esbaugh and Tufts, 2006). These processes create a constant strain on the fish contributing to increased energy put towards ion regulation. Given that both the control fish and the CO_2 treatment fish were in water originating from a source that had elevated CO_2 before the study was conducted might explain why aneurysms were observed in both treatments.

High levels of CO_2 can be considered a stressful event in which fish may need to cope with. Fish exposed to temperature stressors have been observed to increase their amount of compact myocardium to maintain cardiovascular function (Klaiman et al., 2011). However, in this study the compact myocardium did not change when placed under this stressor. A theory as to why no change in the compact myocardium was observed could be due to no compensation mechanism being required. The fishes used in this study also underwent metabolic testing and only Arctic charr under CO_2 conditions showed little evidence that SMR increased compared to the control group (Traynor, 2022, thesis chapter 2). This may indicate that the heart does not require an increase in its amount of compact myocardium to maintain heart function under this stressor. Another reason as to why no changes occurred may have to do with the nature of the CO_2 stressor. The theory of heart remodeling is based on temperature as a stressor in which also has implications on growth rates (Jobling, 1997), metabolic response (Clarke and Johnston, 1999), and performance (Pörtner, 2010) of ectotherms such as fish. Similar to temperature stressors some studies looking at CO_2 as the major stressor in fish have found decreases in

growth (Fivelstad et al., 1999; Gil Martens et al., 2006; Good et al., 2010; Last et al., 2011), changes in metabolism (Lefevre, 2019), and performance (Heuer et al., 2019; Lefevre, 2019). However, using CO_2 as a stressor can produce highly variable results unlike temperature which shows a consistent pattern. A final thing to consider is that prior to the experiment the amount of CO_2 these fish were exposed to is unknown. It may be possible that due to the condition and age of the fish used in this study they may have already developed ways to mitigate the possible effect of CO_2, such as increasing compact myocardium thickness.

Even though the study showed little evidence of an CO_2 effect on compact myocardium there was a species difference. As proposed by Farrell et al. 1988, there are a few factors that effect cardiac growth, the first factor being water temperature. There have already been countless studies that have looked at how an increase or decrease in water temperature can cause an increase in compact myocardium (Farrell, 1987; Graham and Farrell, 1989; Klaiman et al., 2011). The species used in this experiment are all described as colder water fishes, but there are still temperature differences preferences between these species. For example, Arctic charr has a much more limited range spanning the Arctic, sub-Arctic, and temperate climates (Klemetsen et al., 2003). Whereas brook charr has a more southern range, living in every province in Canada except the territories (MacCrimmon and Campbell, 1969). Rainbow trout have the largest distribution, as they are found in every Canadian province both through historical distribution and introduction for sports fisheries (MacCrimmon, 1972). The water temperature used in this study was ~ 10 ˚ C which is in range for all these fish, but Arctic charr may have had a slight thermal challenge due to their preferred temperatures being 5 – 9 ˚ C (Harris et al., 2020). Therefore, not only was there a CO_2 challenge, but Arctic charr may have experienced a slightly

elevated thermal environment which might explain be why there is a species difference in the amount of compact myocardium.

Another factor that can affect cardiac growth is whole-body growth rate (Farrell et al., 1988). All the fish used within the experiment are juvenile fish (< 1 year old) and were raised on site at the hatchery. A study observing 1-year-old fish found that 30 % of the heart is compact myocardium, but 4-year-old fish have 45 % compact myocardium indicating that while the fish are young their hearts experience rapid growth (Farrell et al., 1988). Additionally, the base weight of the fish used was slightly different from one another. This is significant as the ventricle continues to grow isometrically with the body mass (Farrell et al., 2007; Farrell et al., 2012). Slight differences between the body masses may be attributed to the species difference being seen in the growth of the compact myocardium. The rate at which species grow and allocate their energy can be very different. For example, some Arctic charr show an extreme growth efficiency of almost 60 % food conversion (Larsson et al., 2005). Brook charr on the other hand has a conversion rate of 36 % for lipids and 41 % for proteins for fish held at similar temperatures (McMahon et al., 2007). Conversely, juvenile rainbow trout, of a similar size to this study, have the lowest gross efficiency of under 30 % (Wurtsbaugh and Davis, 1977). These different growth efficiency rates among the species may relate to their slight size difference and possible compact myocardium differences.

A final factor that can affect heart size is the amount of cardiac load that is placed on the fish (Farrell et al., 1988). For example, a study looking at the percentage of compact myocardium and the migration effort in sockeye salmon (*Oncorhynchus nerka*), found that generally with greater migration effort came a larger amount of compact myocardium (Eliason et al., 2011). Commonly, active fish have a large amount of compact myocardium but the exact

amount even between related species can be different (Eliason et al., 2011; Farrell et al., 2009; Pieperhoff et al., 2009). All three species used in this study are active salmonid species but have different life histories with varying cardiac loads. For example, Arctic charr migration can live within a river system but some populations are anadromous and undergo long migrations from the sea to freshwater streams (Klemetsen et al., 2003; Tallman and Saurette, 1996; Young et al., 2021). However, as with most things, there is a wide array of variation in the life histories of Arctic charr (Klemetsen et al., 2003; Rikardsen and Elliott, 2000; Rikardsen et al., 2004). Rainbow trout is another species that can be anadromous but only under very specific conditions, instead the majority spawn in freshwater (Kendall et al., 2015; Rosenberger et al., 2015). There are a few populations of sea-run brook charr that have been reported on the east coast of Canada but many Canadian brook trout populations are exclusively freshwater and have short migration distances (Curry et al., 2002; Doucett et al., 1999; Wilder, 1952). The rainbow trout and brook charr used in this study are related to land-locked populations from Ontario. These diverse life histories and cardiac loads across the species can result in species differences in the amount of compact myocardium observed.

Something that may need further investigation in the future is the amount of CO_2 needed to observe a consistent effect on all tissues. It may be possible that the amount of CO_2 used in this experiment was not elevated enough to enlist a response. Some other studies looking at the effects of CO_2 within aquaculture faculties use levels that can be 30 times that of the control group (Khan et al., 2018). Additionally, the time frame used in that study was longer, with the fish being exposed for 47 days (Khan et al., 2018). In my study, the CO_2 treatment was only triple that of the control and the exposure time period was only 15 days. Due to the hatchery facilities these fish were grown in, the levels of CO_2 were already elevated. It is possible that if

you compare the tissues of a healthy wild fish to fish within the high CO_2 treatment group differences between them may appear. However, if you compare the control group to the wild populations it is also possible differences will be seen and can be attributed to the hatchery setting. Studies looking at the effects of different levels of CO_2, in addition to exploring different exposure time points, would add valuable information to how these freshwater salmonids react to elevated CO_2 on a cellular level.

Apart from a few CO_2 effects, there were species differences seen. The species differences were seen between Arctic charr and the other two species in regards to secondary lamellae lengths as well as brook charr having the thickest compact myocardium. With longer secondary lamellae, the surface area in which gas exchange can occur increases. However, some fish can remodel their gills to increase or decrease the amount of exposed surface area available for gas exchange. For example, some studies have seen that under high temperatures and low oxygen the secondary lamellae length increases via apoptosis of interlamellar cell masses (Sollid and Nilsson, 2006). Oxygen was monitored twice daily and saturation remained high for all species (Table 1). The temperature was also kept constant for all the species at approximately 10˚ C (Table 1). The temperature used may have been high for Arctic charr as they prefer temperatures around 5–9 ˚C (Harris et al., 2020). Thus, the longer secondary lamellae length in Arctic charr compared to the other species may have to do with the temperature used in the study.

In conclusion, elevated levels of CO_2 have mixed effects at the tissue level of Arctic charr, brook charr, and rainbow trout. There was evidence that the total organ index in Arctic charr under the elevated CO_2 was higher than the control group. This may have to do with the compounding effects of changes in the gills and liver. When looking at the liver index and gill

index individually there was no evidence that CO_2 influenced these tissues. The major change seen within the gills were aneurysms at the tips of the secondary lamellae. This probably occurred due to pillar cells dying and blood still entering the secondary lamellae resulting in pooling at the tip due to the loss of structure (Hassaninezhad et al., 2014; Strzyżewska-Worotyńska et al., 2017; Van Den Heuvel et al., 2000). When looking at the amount of compact myocardium, there was no evidence that treatment had an effect. There are a few different ways that can cause the amount of compact myocardium to change including temperature, growth rate, and cardiac strain (Farrell et al., 1988). Even though there was limited evidence of a treatment effect there were species differences seen between the metrics being measured. This can be attributed to a variety of different aspects such as life history strategies. Additionally, the point during development in which these fish experience the elevated CO_2 is when the tissues are first developing, which may also provide further insight into the tissue level response of CO_2. As the levels of CO_2 within the earth's atmosphere are increasing, more CO_2 will end up in our freshwater ecosystems. Therefore, in the future, more work should be done on the effect CO_2 has on fish living within these environments, specifically effects of different concentrations of CO_2 on the tissue level response.

Chapter 4: General Discussion

When discussing climate change, many people assume this only relates to warming air temperatures; however, the mechanism at play is the rise of greenhouse gases such as CO_2. Many climate change studies on fish also mostly pertain to only increasing temperatures. Of the studies on the effects of CO_2 regarding fish, many of them are focused on marine fishes, even though freshwater systems are also experiencing this weak acidification caused by the elevated levels of CO_2. The aim of this thesis was to determine the effects of elevated CO_2 on multiple juvenile species of salmonids through multiple ecological and physiological studies.

My first goal of this thesis related to understanding how elevated levels of CO_2 may affect the behaviour and physiology of juvenile Arctic charr, rainbow trout, and brook charr (Chapter 2). I completed a control vs elevated CO_2 study where I monitored the behaviour and several energy-linked metrics. Through these experiments I found that overall elevated CO_2 had no effect on growth rate, feeding rate, and behaviour for all these species. There was evidence that the SMR was affected by the CO_2 treatment, but this was only the case for Arctic charr. The lack of an effect of CO_2 on these factors is not surprising as there is a high degree of variation among the results of CO_2 research with no clear pattern being observed. There are a few limitations in this first study regarding the use of the same temperature for all three, the short time frame used, as well as the relatively low difference between the control group and elevated CO_2 group.

My second goal was to understand if elevated CO_2 caused damage to internal organs such as the gills, liver, and heart within the three juvenile salmonids (Chapter 3). I did find the gills experienced aneurysms but there was no evidence to conclude it was solely due to the CO_2 treatment as they were found within both treatments. The liver also experienced vacuolation but

again there was no evidence that this was related to the CO_2 treatment. When looking at the total organ index, in which all organ damage is summed together, there is evidence that the CO_2 treatment had an effect. Thus, the vacuolation and aneurysms seen within liver and gills in the CO_2 was higher than that of the control treatment when summed together in Arctic charr. This finding may be an indicator that elevated CO_2 may not seriously affect individual tissues but may cause enough harm collectively to hinder whole-organism performance. The hearts were also assessed for an increase in compact myocardium, however, no evidence was found that CO_2 treatment influenced the thickness. Even though no large effects of CO_2 were seen within the tissues, my study does shed light on an area of research that is lacking, especially in freshwater species.

A common theme seen among both data chapters of my thesis is that there is a species difference. This species difference can likely be attributed to the different adaptation these salmonids have to perform in their preferred habitats. When looking at the phylogeny, all the fish within this study originated from a relatively recent common ancestor. From this ancestor speciation occurred as individuals started to exploit different habitats. These different habitats experience varying levels of CO_2. For example, water sheds within the Arctic have a mean CO_2 concentration of 21–28 μmol L^{-1} (converted to ~ 500 μatm and 720 μatm; Emmerton et al., 2016). Whereas, large lakes in low latitudes exhibit higher primary production due to the increased temperature, light, and high nutrient loading, leading to elevated levels of CO_2 (McNeil and Matsumoto, 2019). Thus, individuals may acclimatize to their changing environment by expressing a phenotype that has a high proficiency under these conditions (Munday et al., 2019). Genetic adaptation, involving genotypes that have higher fitness in these new conditions, can lead to a change within the population by shifting the baseline of the

population towards a higher overall fitness in these new environments (Hendry et al., 2011). Others have remarked that more studies should focus on whether phenotypic plasticity and/or evolution can buffer the direct effects of elevated CO_2 across the timescale of environmental change (Munday et al., 2013; Sunday et al., 2014). Therefore, determining the baseline of how CO_2 levels effects juvenile salmonids from different genera allows for insight as to what species may be more at risk due to increasing levels of CO_2. For example, in pink salmon a decrease in growth rate was only seen in fish that experienced levels of CO_2 of 2000 µatm (Ou et al., 2015). Additionally, in a study observing Atlantic salmon experiencing medium to high levels of CO_2 (19 mg/L and 32 mg/L, converted to ~ 11000 µatm and 18500 µatm) both experienced decreases in growth rate (Fivelstad et al., 1999). It is possible that the levels of CO_2 in my study were still within a range that each of the species had experienced in its evolutionary pass, thus they were able to cope.

This thesis also highlights the importance of using multiple species and multiple experiments as this allows for a more complete picture of what is occurring within the fish under elevated CO_2. The use of multiple species allows for easy comparison between species. It is difficult to find studies looking at the effects of elevated CO_2 within freshwater as well as ones that use comparable exposure levels. By using the same methods across all three species, easier comparisons can be made and conclusions drawn. The other highlight of this thesis is the wide array of tests used. By combining the use of external and internal assessments it allows for a broad view of what may be occurring in these fish under elevated CO_2. For example, the theory of heart remodelling in which a fishes' heart may increase its amount of compact myocardium in order to maintain its function under stressful conditions can be more closely examined through whole animal affects and lower levels of organisation. To do this, chapter 2 examined if

metabolic rate was affected by elevated CO_2 in all three species. Only SMR in Arctic charr was affected by the CO_2 treatment. This led me to wonder if there is some sort of compensation mechanism at work in order to maintain function under the stressor of elevated CO_2 such as the mechanism described in the theory of heart remodeling. Therefore chapter 3 focused on the tissue level responses. This allowed me to observe if the compact myocardium was increasing and if that explained why the fish were able to maintain their normal functions under the elevated CO_2. However, this was not the case. Another example is that instead of looking at the heart to explain the change in SMR occurring in Arctic charr, the secondary lamellae were shorter in the CO_2 treatment (Figure 3.5), thus there is less surface area for oxygen to enter the fish. These fish therefore need to increase ventilation to get the same amount of oxygen as the control group with the longer secondary lamellae, which in turn may increase the SMR. Doing approaches such as this allowed for a complete investigation of what maybe occurring.

There are a few aspects of this study that may warrant further investigation. The first being the effects of previous exposure. The fish used in this thesis were from a common provincial hatchery. These fish have lived within a hatchery system for their entire lives. Generally, hatchery facilities are found to have elevated levels of CO_2 due to the stocking density and metabolic CO_2 the fish produce. There have been some studies showing drastic effects of CO_2 on larval fish (Baumann et al., 2012; Frommel et al., 2012). Prior to arriving on site, the levels of CO_2 within the hatchery were unknown. Moreover, the hatchery is located within a section of the Canadian shield and uses source water from a local lake that has elevated CO_2 levels. The water used for the control group was approximately 1100 µatm of CO_2 which is high for a control group compared to other studies. Many other studies looking at the effects of elevated CO_2 use control groups under 1000µatm (Fivelstad et al., 1999; Fivelstad et al., 2003;

Fivelstad et al., 2004; Gil Martens et al., 2006; Khan et al., 2018; Ou et al., 2015). Thus, in the future it would be interesting to see how early life exposure to CO_2 may affect the fish later in life. Some studies have shown that species are able to recover after experiencing high levels of CO_2 (Hasler et al., 2016b; Tix et al., 2017b). Related to this a study looking at repeated exposure may also be able to shed light on how CO_2 may be affecting freshwater fish. Additionally, CO_2 within freshwater is not held at a stable level but instead oscillates hourly and the levels can change by almost 1000 μatm (Xu et al., 2019). A study looking at how fluctuating CO_2 may impact two species of coral reef observed that it can have beneficial impacts on oxygen uptake in one of the species but not the other (Hannan et al., 2020). CO_2 within freshwater can occasionally fluctuate more then that of marine water due to less ions available to buffer the weak acidification occurring (McNeil and Matsumoto 2019). Thus, fish within freshwater may experience these swings and may undergo more drastic changes in behaviour associated with these swings, but more studies mimicking the natural variation are needed.

Something else to note is that because the hatchery produced these juveniles from brood stock, it is possible that the hatchery has subliminally selected for fish that have high tolerances to CO_2 as these fish may perform better in these hatchery environments. This brings into question the possibility of a paternal effect occurring within the juveniles used. For example, studies have seen that the parents can alter the phenotype of the offspring based on the conditions the parents are exposed to (Burgess and Marshall, 2014). Thus, the possibility of parental effects occurring at the hatchery level can not be ignored. In order to get a complete picture of what is occurring more studies should investigate the possibility of a parental effect being seen within freshwater hatchery settings.

The timeline and concentration used within studies can be looked at further. My thesis experiments were all conducted within a 15-day exposure period. This is a short time frame when you compare it to other CO_2 studies that use time frames of weeks to months (Skov, 2019). It is possible that with prolonged exposure the effects of CO_2 maybe observed as other studies found that there was no change in metabolic rate within the first few weeks but overtime differences where observed (Ou et al., 2015). The concentration of CO_2 used in my thesis was triple that of the control group. CO_2 concentrations used within research generally falls into one of three categories. The first one being levels associated with climate change. These studies use concentrations associated with future CO_2 predictions which can generally be around 1000 µatm (IPCC, 2014). However, there can be a 4-fold difference between the control groups and the CO_2 treatment groups (Ou et al., 2015). Another common category of CO_2 concentration used is levels associated with aquaculture which can reach CO_2 concentration of 30 mg/L (~17,000 µatm converted; Skov, 2019). The final general category CO_2 research can be related to is levels associated with invasive species barriers. These barriers can exhibit concentrations of 42000 µatm in order for some species to exhibit stress signs and avoid the barrier (Dennis et al., 2015; Suski, 2020). Therefore, future studies should investigate the effects of different CO_2 concentrations along with the effects of the exposure time on freshwater fish.

As climate change continues the amount of CO_2 within freshwater environments will also continue to increase and studies pertaining to how these levels effect freshwater fish also need to increase. With such limited information available, my chapter 2 provides insight into how elevated CO_2 may affect the growth rate, metabolic rate, feeding rate, and volitional behaviour of three juvenile salmonids. The findings from this chapter indicated that there are no strong effects of CO_2 on these species and this population of fishes at the levels I exposed them to. With so

much variation being found across the literature these results agree with some studies and are in opposition to others. My chapter 3 focused on the internal effects CO_2 may have on the organs of these fish using histology. The results from this chapter indicate that extreme harm is not done to one individual organ but instead is evenly spread out among the tissues examined in this study. Apart from a few studies looking at the effects of ocean acidification on the livers of Atlantic cod, in which they observed serious damage, hardly anything else is known (Frommel et al., 2012). The combination of using multiple species as well as the use of external tests with the accompanying internal examination allows for a more complete understanding as to what elevated levels of CO_2 maybe doing to salmonids.

Possible future studies

Based on the findings in this study, there are two main paths for future explorations. The first pathway is that associated with climate change. There have been studies that look at how future levels of CO_2 may affect fish both in marine and freshwater environments; however, many of these studies apply a constant level of CO_2 throughout the exposure time frame. Some studies examining how CO_2 enters the freshwater environment have seen hourly changes occurring within the levels of CO_2 in their study sites (Xu et al., 2019). Thus, maintaining a constant level of CO_2 in these studies is required to get a good understanding of basic response, but oscillating the levels of CO_2 may better mimic what is natural occurring in freshwater environments. To better understand how future freshwater fish may experience climate change, a more integrative approach may be required. Studies that combine the effects of elevated CO_2 and elevated temperature may provide a more complete understanding of how our freshwater fish may fair under future predictions.

A second path in which future studies may want to explore is related to aquaculture. What sets apart CO_2 research in the field of aquaculture versus climate change is the species and CO_2 levels used. Species used in these studies are species commonly found in aquaculture such as Atlantic salmon. Additionally, the concentration of CO_2 generally reflects levels higher than that seen in climate change research due to the higher stocking density and fish waste. Thus, studies looking at elevated CO_2 within freshwater aquaculture may have to take a slightly different approach than that of the climate change studies. Examining how repeated exposure to elevated CO_2 within the unique setting of aquaculture studies, would add some insight into how juvenile fish are doing within these settings. Along with this, studies looking at possible parental effects the brook stock may pass to their young, may also provide an interesting look at how these commercially important species are adapting to elevated CO_2 found within these systems. Investigating how both juvenile fish and possible adaptations pass on to these fish from their parents may help aquaculture facilities improve or maintain the general health of these economically important fishes.

Therefore, there are many ways in which CO_2 research in freshwater fish can continue. Whether research within this field continues through the lenses of climate change, aquaculture, or invasive species barriers, the information from these studies will provide much needed data to an area of study that is lacking. Not only will this information fill in much needed knowledge gaps but also provide information about how an array of freshwater fish responded to elevated CO_2. The information gathered from these studies can be used by a variety of different groups to make informed management decisions. These decisions will vary in their implications but can stretch from environmental protections to industry standards.

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Sci. **7**, 436–453.

Supplementary

Table S1. Detailed list of the methodological information required to recreate the respirometry set-up, following the guidelines for reporting methods of aquatic respirometry (Killen et al., 2021).

Required information (Killen et al., 2021)	Answers
Equipment	
Provide body mass of animals at time of respirometry	Arctic charr = 0.0034 ± 0.001(mean ± SE) Brook charr = 0.0048 ± 0.001(mean ± SE) Rainbow trout = 0.0076 ± 0.001(mean ± SE)
Provide volume of empty respirometer	1.9 liters
Provide volume of tubing in any mixing circuit	0.105 liters
Describe how chamber mixing was achieved	This was an intermittently closed system but water was circulated using an aquarium pump (See methods).
Provide ratio of net respirometer volume to animal body mass	Arctic charr = 1:603 (0.0034 kg to 2.05 liters) Brook charr = 1:427 (0.0048 kg to 2.05 liters) Rainbow trout = 1:270 (0.0076 kg to 2.05 liters)
Provide material of respirometer	Plastic
Provide material of tubing	Silicon
Confirm volume to tubing in any mixing circuit is included in calculations of oxygen uptake rates	Yes
Describe placement of oxygen probe	It was placed in the exhaust tube before reaching the recirculation pump.
Provide type of oxygen probe and data recording	FireSting 3mm tip 10 cm long fiberoptic probes.
Provide sampling frequency of water-dissolved oxygen	3 seconds
Provide flow rate during flushing and recirculation	300 L/hr
Provide timing of flush/closed cycles	Closed period was 15 minutes and flush time was 3 minutes.
Provide wait time excluded from closed measurement cycles	2 minutes
Describe frequency and method of probe calibration	Probes were calibrated (0 and 100%) every two days during the exposure period.
State whether software temperature compensation was use during recording of water oxygen concentration	No

Measurement conditions	
Provide temperature during respirometry	10˚C ± 2 ˚C
State how temperature was controlled	A chiller set to 10˚C ± 2 ˚C was used in the water bath to maintain temperature.
Provide photoperiod during respirometry	A natural photo period for Manitoba from June to July was used.
Describe if the ambient water bath was cleaned and aerated during measurement of oxygen uptake	Yes, the water bath was drained and refilled at the start of every trial. An air stone along with recirculation pumps was included in the water bath.
Provide total volume of ambient water bath and any associated reservoirs	396 liters
Provide minimum water oxygen level reached during closed phase	83.7%
State whether chambers were visually shielded from external disturbance	Yes; the back side and front side of the chambers were blocked.
State how many animals were measured simultaneously, state whether they were able to see each other during measurements	3 animals were measured simultaneously, each in separate chambers. The chambers were placed 30 cm apart with no divider in-between them.
If multiple animals were measured simultaneously, state whether they were able to see each other	It would have been challenging for the individuals to see each other due to the distance and the aquarium pumps in the way.
Provide duration of animal fasting before placement in respirometer	24 hours
Provide duration of all trials combined	In total in took 48 days to complete all trials.
Provide acclimation time to the laboratory before respirometry measurements	Depending on the time point within the exposure period it was anywhere from 24 hour to 14 days.
Background respiration	
State whether background microbial respiration was measured and accounted for	Yes, background respirometry was measured and accounted for.
If background respiration was measured at beginning and/or end, state how many slopes and for what duration	The background respiration was measured during each trial.
State how changes in background respiration were modelled over time	Linearly, with bimodal decrease in O_2 after each day in which chambers were cleaned.
Provide level of background respiration (e.g. as a percentage of SMR)	20.8% of the mean SMR among all species.
State method and frequency of system cleaning	Each chamber was flushed and scrubbed clean at the end of each species trails.
Standard or routine metabolic rete	
Provide acclimation time after transfer to chamber	None, recording started immediately.
Provide time period within a trail over which oxygen uptake was measured	24 hours

State what value was taken as SMR	The lowest 10% of metabolic rate was used as SMR (See methods).
Provide total number of slopes measured and used to derive metabolic rate	~70 slopes were measured per individual.
State whether any time periods were removed form SMR data during acclimation periods of high activity	The first 4 hours was removed as this was the time after which a manual chase test occurred.
Provide r^2 threshold for slopes used for SMR	A r^2 threshold of 0.9 was used (Clark, 2022).
Provide proportion of data removed due to being outliers below r^2 threshold	No outliers within the data were removed.
Maximum metabolic rate	
State when MMR was measured in relation to SMR	MMR was generally measured before SMR.
State method used	Chase to exhaustion was used.
State what value was taken as MMR	The highest 99% of metabolic rate within the first four hours after transfer was used for MMR (See methods).
State length of activity challenge used for estimate MMR	5-minute manual chase test.
If MMR was measured post-exhaustion, state whether further air-exposure was added after exercise	No air exposure was added.
If MMR was measured post-exhaustion, provide time until transfer to chamber after exhaustion and time to start of oxygen uptake recording	<3 minutes elapsed after the manual chase test and before the recording started.
Provide duration of slope used to calculate MMR	~12-minutes of the 15-minute closed period was measured for MMR.
State slope estimation method for MMR	Rolling regression.
State how absolute aerobic scope and/or factorial aerobic scope is calculated	Allometrically mass-adjusted SMR and MMR.
Data handling and statistics	
Provide sample size	Arctic charr CO_2 n= 28, Control n = 28 Brook charr CO_2 n= 28, Control n = 28 Rainbow trout CO_2 n= 32, Control n = 32
State how oxygen uptake rates were calculated	They were calculated in R v. 4.1.1 using the respR package. Values were converted to O_2mg/kg/hr.
Confirm that volume of the animal was subtracted from respirometer volume when calculating oxygen uptake rates	Yes
Specify whether variation in body mass was accounted for in analyses and describe any allometric body-mass-correction or adjustment	Yes, body mass was included in the analysis as a way to standardize the metabolic rates of all individuals.

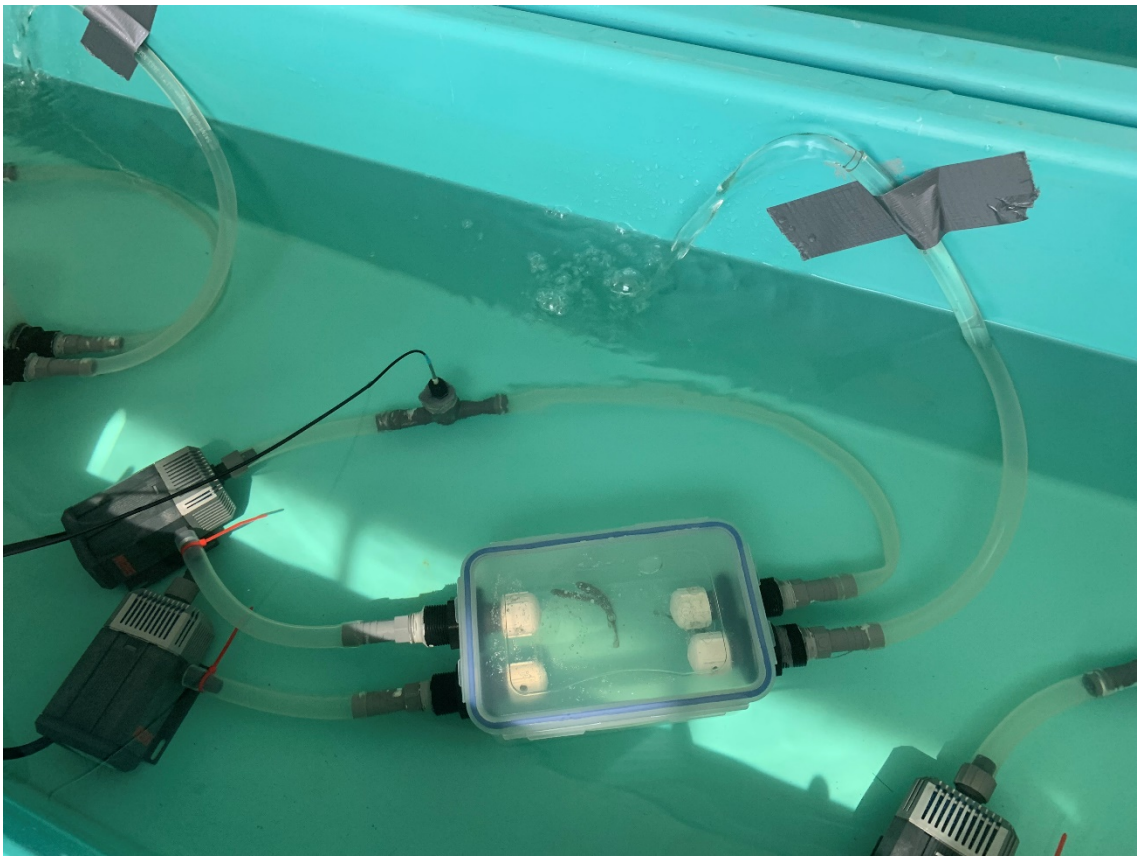

Figure S1. Image of the inhouse respirometry chambers used throughout the experiments.

Figure S2. Image of the respirometry chambers placed within the water bath at the WhiteShell hatchery.

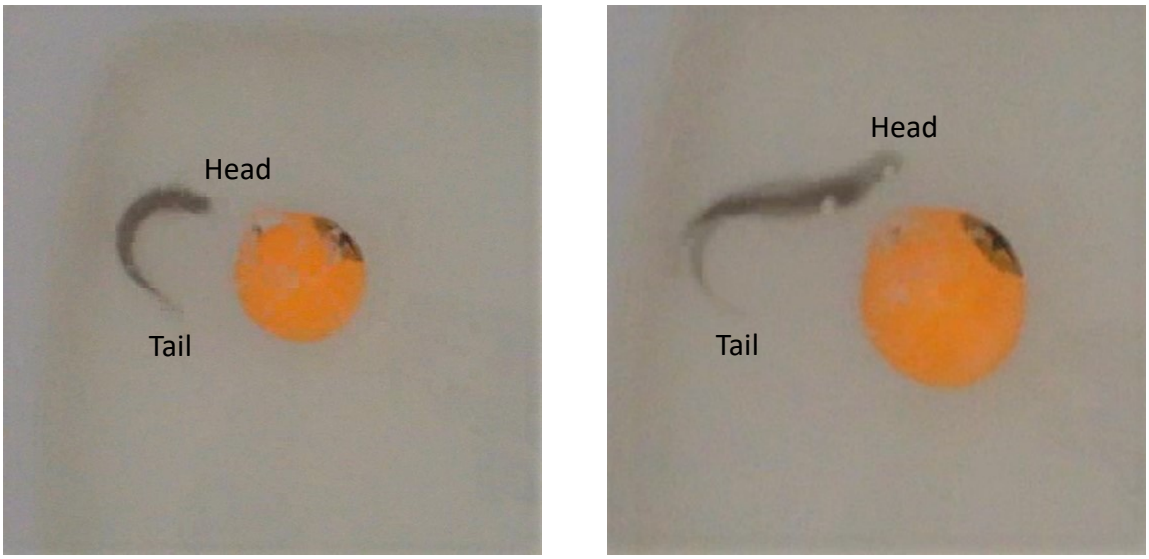

Figure S3. The C-start of an Arctic charr in freshwater. Note the stage 1 body bend in the fish that forms the characteristic “C” shape (A). In stage 2 the body forms an S-shape to form a propulsive stroke during which the center of the body is accelerated away from the initial path.

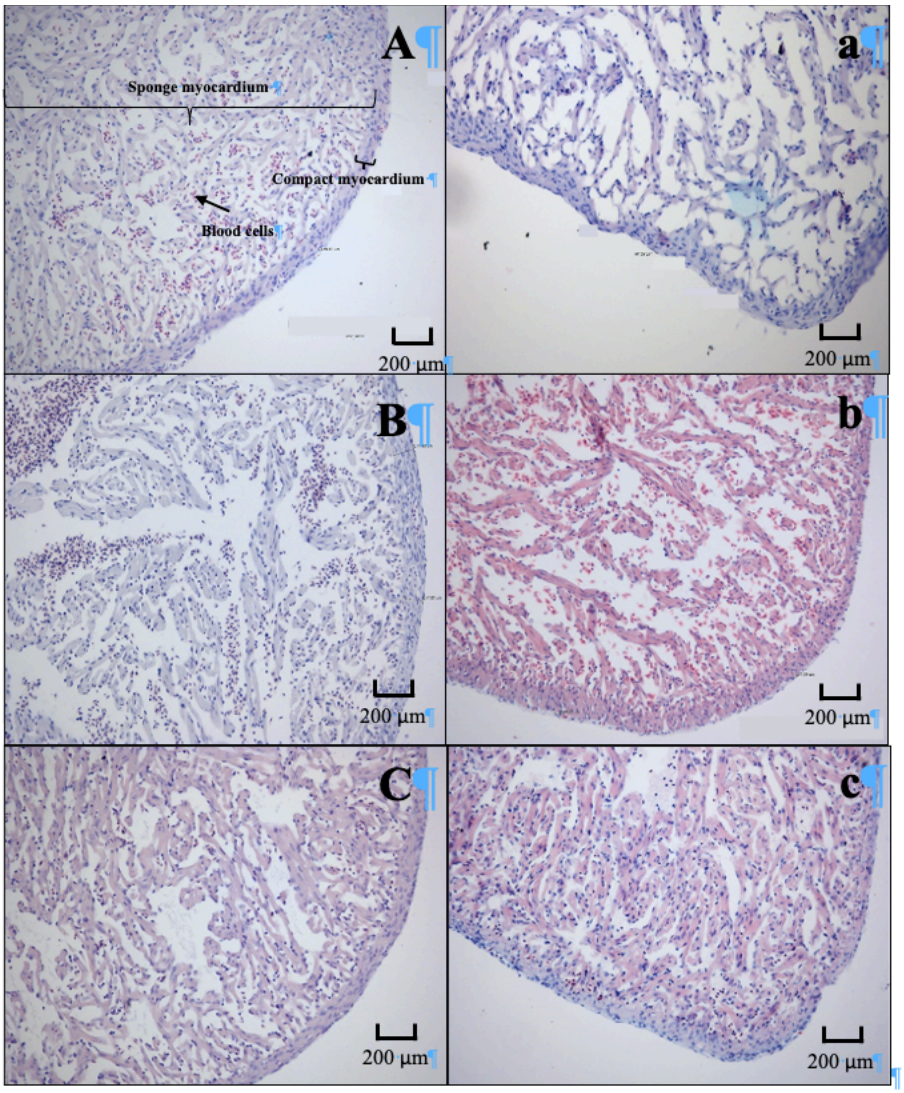

Figure S4. Reference images of heart sections from Arctic charr (A, a)(*Salvelinus alpinus*), brook charr (B, b)(*Salvelinus fontinalis*), and rainbow trout (C, c)(*Oncorhynchus mykiss*) from either the control treatment fish (uppercase letter) or CO_2 treatment fish (lowercase). The tissue was sliced to 5 µm thickness and placed under 40 X magnification. The back arrow points towards blood cells and the brackets represent the different myocardium types. Scale bars represents 200 µm.

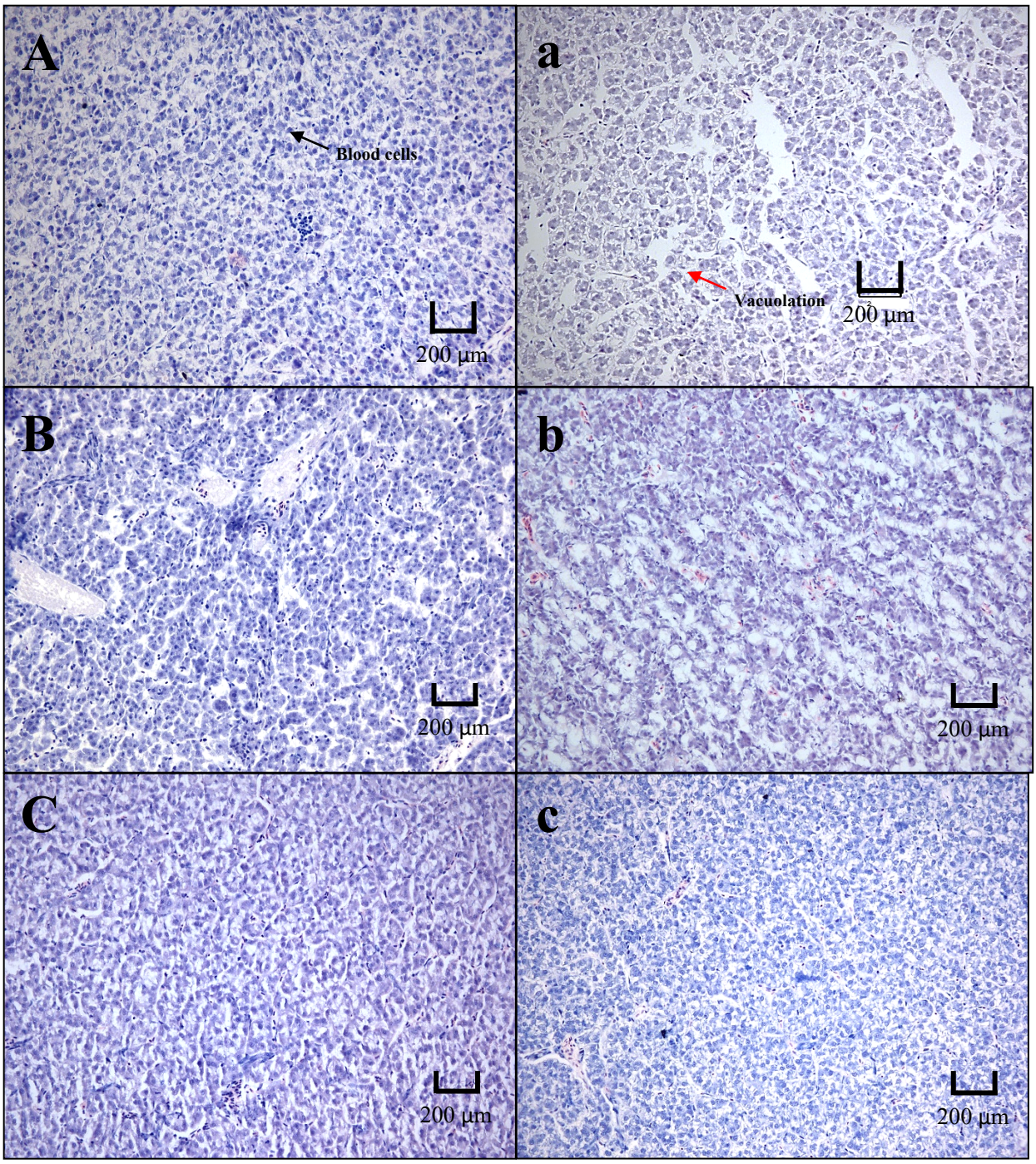

Figure S5. Reference images of liver sections from Arctic charr (A,a) (*Salvelinus alpinus*), brook charr (B,b) (*Salvelinus fontinalis*), and rainbow trout (C,c) (*Oncorhynchus mykiss*) from either the Control treatment fish (uppercase letter) or CO_2 treatment fish (lowercase). Tissues were sliced to 5 µm thickness and placed under 40 X magnification. The back arrow points towards blood cells and the red arrows indicate vacuolation. Scale bars represent 200 µm.

THE UNIVERSITY OF WINNIPEG

Vice-President,
Research and Innovation

PROTOCOL APPROVAL

TO: **Caleb Hasler**
Principal Investigator

FROM: **Desiree Vanderwel, Chair**
University Animal Care Committee (UACC)

Re: **Protocol #AE 15565**
Salmonid fishes and CO2 exposure

Effective: 01-May-2021 **Approval Type**: 1 year **Expiry:** 30-Apr-2022

University Animal Care Committee (UACC) has reviewed and approved the above research. UACC constitutes and operates in accordance with the current *Canadian Council on Animal Care (CCAC).* This approval is subject to the following conditions.

1. Approval is granted only for the research and purposes described in the application.
2. Any modification to the research must be submitted to UACC through WebGrants for approval before implementation.
3. Any deviations to the research or adverse events must be submitted to UACC as soon as possible.
4. This approval is valid for one year only and, if required, a Renewal Request ("Post Approval Activity") must be submitted through WebGrants and approved by the above expiry date.
5. Any unanticipated issues or events during this project that may increase the level of risk to animals, or has other ethical implications that may affect animals' welfare, must be reported to UACC without delay.
6. A Status Report must be submitted through WebGrants to UACC when the research is complete or terminated.
7. The University of Winnipeg may request to review research documentation from this project to demonstrate compliance with this approved protocol and the University of Winnipeg UACC *Policies and Procedures.*

Signed:

Desiree Vanderwel
Chair, UACC

April 20, 2021
Date