Anders Jorud Meyer

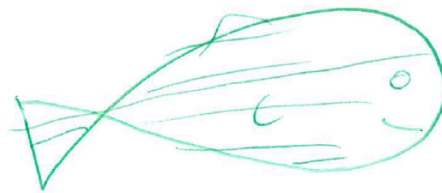# The Effects of Temperature and Oxygen Acclimation on Somatic Growth and Appetite in Zebrafish (Danio rerio)

Master's thesis in Biology
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Master's thesis

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The Effects of Temperature and Oxygen Acclimation on Somatic Growth and Appetite in Zebrafish (Danio rerio)

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Abstract

Stochastic heating events and abrupt changes in oxygen saturation are becoming more and more common in coastal areas around the globe. How these changes affect the physiology and well-being of ectotherms, especially fish, is paramount to predict future effects of climate change on costal fish populations and ectotherms in general.

In this study, fish was acclimated to extreme situations, mimicking trends being observed in the wild today, testing parts of the aerobic scope protection hypothesis presented by Jutfelt et. al. We tested if an increase in oxygen available in the surrounding water could increase the aerobic scope (AS) of zebrafish (*Danio rerio*). This increase in AS may allow for larger specific dynamic action, larger meals, and therefore an increase in growth rate compared to fish acclimated to normoxic oxygen saturation. A decrease in AS in fish acclimated to hypoxic oxygen saturation compared to normoxic treatments is also predicted.

A population of zebrafish was acclimated to what is considered outside of optimal living environments in regard to temperature and oxygen saturation. These fish were divided into two groups subjected to two different temperature treatments, lower than optimal and higher than optimal temperatures. 20°C and 34°C, respectively. These two temperature groups were further divided into three oxygen saturation groups – Hypoxia, normoxia, and hyperoxia. 50% DO, 100% DO, and 200% DO dissolved oxygen, respectively.

Variations in growth rate for both length and mass between the two temperature treatments, but also within the temperatures between the various oxygen treatments was observed. The AS of fish acclimated to hyperoxia was found to decrease compared to normoxia, this indicates other underlying mechanisms than oxygen saturation suppressing the AS at higher than optimal temperatures. And even illustrating a potential toxic/negative effect of hyperoxia at higher than optimal temperatures.

Sammendrag

Uforutsigbare hetebølger og brå endringer i oksygenmetning blir mer og mer alminnelig i kystområder rundt omkring på Jorda. En av forutsetningen for å kunne forstå hvordan ektoterme dyr vil påvirkes av fremtidig klimaendring, er å forstå effekten av nettopp disse faktorenes påvirkning på livet i havet. En bred forståelse av disse variablene vil være til hjelp for senere å forstå endringene vi har i vente.

Under dette eksperimentet ble fisk akklimmert til ekstreme situasjoner i et forsøk på å etterligne trendene observert i det ville i dag. Eksperimentet er en mindre bit av puslespillet for å teste "Aerobic Scope Protection Hypothesis" presentert av Jutfelt et. al. i 2019. I dette studiet ble det testet om en økt mengde tilgjengelig oksygen kan øke det metabolske vinduet hos zebrafisk (*Danio rerio*). Et større metabolsk vindu ville potensielt tillat et høyere inntak av større måltider, og derfor gitt større vekst sammenlignet med fisk akklimert til normale oksygennivåer. Vi spår samtidig at fisk akklimert til et hypoksisk miljø, vil oppleve et mindre metabolsk vindu sammenlignet med fisk akklimert til normoksi ved samme temperatur.

En gruppe zebrafisk ble akklimert utenfor optimale forhold, med hensyn på temperatur og oksygenmetning. Fiskene ble delt opp i to ulike temperaturer, lavere enn optimal og høyere enn optimal temperatur, 20°C og 34°C, respektivt. Hver av disse temperaturgruppene ble så inndelt i tre oksygengrupper, hypoksisk, normoksisk og hyperoksisk behandling, 50%, 100% og 200% oksygenmettet vann respektivt.

Det ble funnet variasjoner i vekstrate for både lengde og vekt mellom de to temperaturbehandlingene. Det ble også observert ulikheter innad i temperaturbehandlingene mellom de ulike oksygenmetningene. Det metabolske vinduet hos fisk akklimert til hyperoksi, ble vist å krympe når sammenlignet med fisk akklimert til normoksi, begge ved 34°C. Dette viser at andre underliggende fysiologiske mekanismer er med på å senke det metabolske vinduet enn oksygenmetning ved høyere enn optimale temperaturer. Disse observasjonene kan samtidig vise til en potensiell giftig effekt av høyt oksygennivå ved høye temperaturer.

Acknowledgements

Glossary

- % day^{-1} Change in percent of initial length or weight per day
- AS   Aerobic Scope
- ASPH   Aerobic Scope Protection Hypothesis
- DO   Dissolved Oxygen
- MMR   Maximum Metabolic Rate (mg h^{-1}g^{-1})
- OCLTT   Oxygen-Capacity Limited Thermal Tolerance
- PRAS   Post Prandial Residual Aerobic Scope
- RMR   Routine Metabolic Rate (mg h^{-1}g^{-1})

Table of contents

List of Figures

List of Tables

1 Introduction

Understanding the effects of natural factors like water temperature and oxygen saturation on fish growth and development is paramount in the efforts of trying to understand the effects of an environment in change. By now, manmade climate change is proven to cause an increase in the average global temperature, and there is an increase in stochastic heating events during periods of local high temperatures (Seneviratne et al., 2014, Przeslawski et al., 2008, Rahel et al., 2008). Together with the increase in heating events, more and more hypoxic areas are being observed in coastal areas around the world. This is in a large degree caused by global warming together with eutrophication and anthropogenic activities (Breitburg et al., 2018). One solution to this problem for animals is migration, an universal ecological response to battle global warming (Daufresne et al., 2009). However, for many aquatic organisms, migratory possibilities are restricted due to habitat preferences or physical barriers, manmade or natural. Many organisms are therefore forced to stay and endure extreme changes in their local habitat. Its ability to cope with these changes in its surroundings, for longer or shorter periods, is directly connected to its future survival in said location. There are no other options than for individuals to acclimate to the new environment. When acclimating to new surrounding temperatures is the only viable option, ectotherms are forced to adjust their physiology in response to the change in temperature. This allows the organisms to sustain certain biochemical mechanisms and reactions to maintain performance over a range of temperatures. An individual acclimated to a certain environment is given a performance advantage over individuals not acclimated to said environment (Leroi et al., 1994).

Knowledge of how fish and other vertebrates are affected by these changes is necessary to help understand future problems occurring as our climate keeps undergoing changes. This project focuses on the growth and appetite, as well as the metabolism in zebrafish (*Danio rerio*), when exposed to non-optimal growing temperatures in combination with hypoxic, normoxic, and hyperoxic environments.

The effect of acclimation to non-optimal temperatures and oxygen saturations on growth, appetite, and metabolism is not yet fully understood. However, variations in temperature do have a correlation with the growth, metabolism, and food conversion rate of fish (Wurtsbaugh and Cech, 1983, Goolish and Adelman, 1984, Vondracek et al., 1988). When the increase in

temperature is above optimal, growth rate start to decline (Baldwin, 1957). The availability of oxygen and sufficient transport to tissues have been proposed as a physiological mechanism that may limit the performance in fish when exposed to higher temperatures. This effect of oxygen limitation, together with the dependence of temperature on metabolism, has been implemented into the "oxygen-capacity limited thermal tolerance" hypothesis (OCLTT) (Pörtner, 2010).

Higher than optimal temperatures are shown to cause stress and decrease the growth rate in zebrafish (Vergauwen et al., 2010). As temperature increases, the specific dynamic action (SDA), metabolism allocated for digestion of food and assimilation of nutrients, is temporally compressed, increased in amplitude, and will take up a larger part of the fish's aerobic scope (AS). This will reduce the amount of available AS needed for routine metabolic activities. If the peak SDA response exceeds the fish's maximum metabolic rate (MMR), it will acquire an aerobic deficit (Figure 1.1). This in turn might result in death if the "oxygen debt" is not "paid off". In 2021 Jutfelt et. al. proposed the "aerobic scope protection hypothesis". This hypothesis suggests that ectotherms reduce their food intake to protect their oxygen transporting capacity at higher temperatures when oxygen is limited, and limit the processes of digestion and assimilation. Meaning fish exposed to situations where oxygen is a limiting factor, i.e. high temperatures, will reduce their food intake to allow for enough available post prandial residual aerobic scope (PRAS) to maintain critical physiological functions.
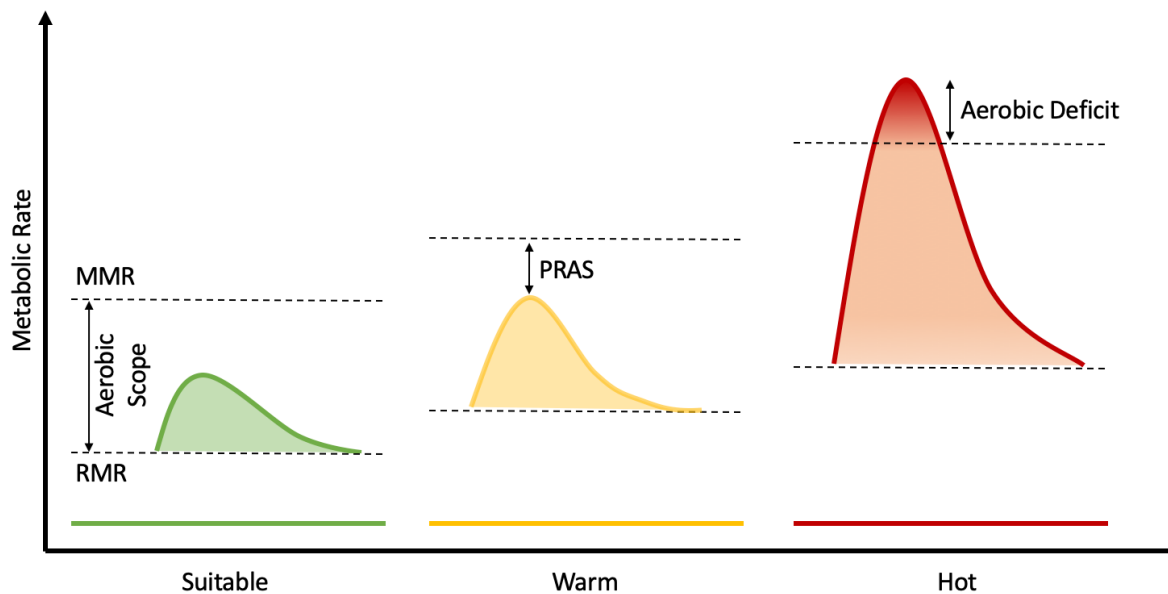

Figure 1.1 Illustrations of the effect of increasing temperatures have on metabolic rate, and therefore aerobic scope (AS), specific dynamic action (SDA), post prandial residual aerobic scope (PRAS), and the aerobic deficit of ectotherms. This figure illustrates temperatures above optimal for any given ectotherm. Modified from Jutfelt et al., 2021.

In this experiment 240 zebrafish were acclimated for approximately 7 weeks at lower than and higher than optimal temperature. Each temperature treatment was further divided into three oxygen treatments – Hypoxia, normoxia, and hyperoxia. Hypoxia being lower than 100% DO, normoxia being 100% DO, and hyperoxia being higher than 100% DO. By measuring the fish's length and weight before, during, and after the acclimation, we were able to calculate the growth rate after the last measurement was performed, and the experiment was ended. The response in growth to acclimation at various extreme environments will help us further understand the effects temperature and oxygen saturation have on fish over a longer period of time. This could help better understand the mitigating effects of climate change, and give us information to make future predictions regarding the survival of several fish populations worldwide.

1.1 Hypothesis

By increasing the amount of dissolved oxygen in the water, we believe we can artificially increase the AS of zebrafish. "Raising the metabolic ceiling", the MMR, and therefore increasing the PRAS. An increased PRAS will in turn allow the fish to eat larger meals, increase their SDA, and therefore grow more compared to fish at lower oxygen saturations, and at the same time maintain crucial physiological and voluntary processes.

The main driver of growth is assumed to be the intake of food. The effect of altering the appetite of fish is therefore thought to result in changes in growth (Figure 1.2). Based on descriptions of thermal performance curves, we predict a loss in appetite and growth rate for both temperature treatments during the acclimation of this experiment due to them both being non-optimal temperatures for zebrafish to thrive (Schulte, 2015). Fish acclimated to lower than optimal temperature, 20°C, is believed to have a greater decline in appetite compared to fish acclimated to higher than optimal temperature, 34°C (Figure 1.2). Due to the predicted effects of an increased or decreased aerobic scope in the various oxygen treatments, a variation within each temperature treatment was also assumed. Here the zebrafish acclimated to hypoxia would have a significantly lower appetite compared to fish acclimated to normoxia. Due to a suppression of the AS and therefore the lowering of PRAS or risk of entering aerobic deficit if meal size is too large. The effects of oxygen treatments are thought to be larger at 34°C compared to 20°C, again due to the overall increase in metabolism and physiological processes at higher temperatures.
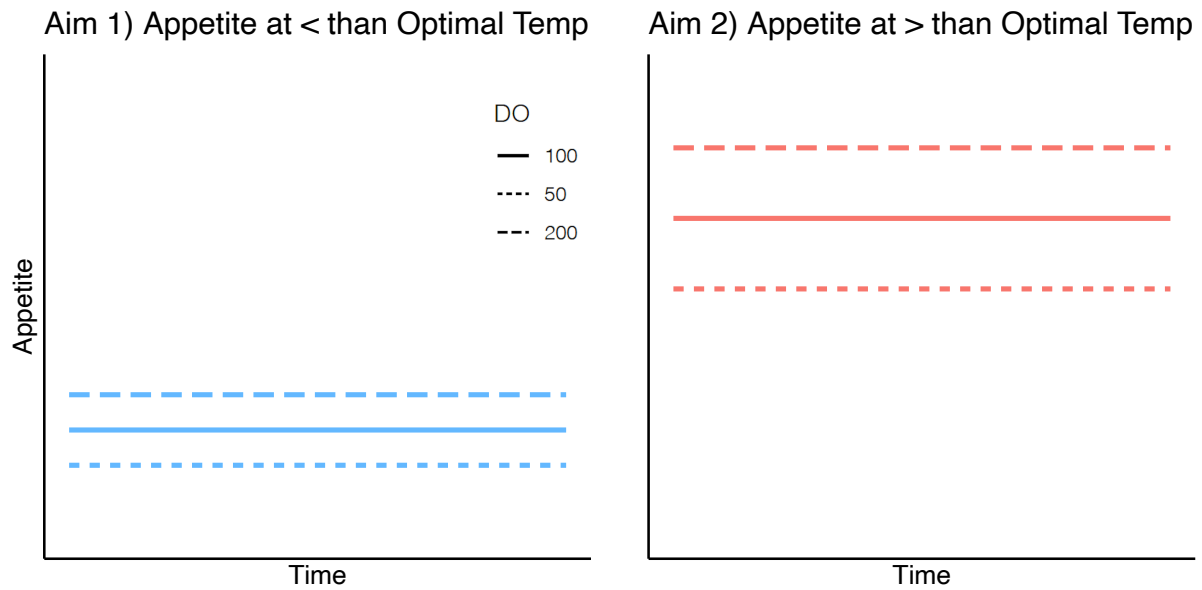
Figure 1.2 Temperatures lower than optimal, 20°C **(Aim 1),** and higher than optimal, 34°C **(Aim 2)**, are both predicted to cause a decreased appetite in zebrafish compared to zebrafish kept at optimal temperature of 28°C. In this figure, appetite represents the amount of food eaten during each measurement trail.

Based on known effects of temperature on growth in zebrafish together with the aerobic scope protection hypothesis presented by Jutfelt et al., 2021, we predict zebrafish acclimated to a higher temperature will have an increased growth compared to fish acclimated to lower temperatures (Figure 1.3). Fish acclimated to 34°C, warmer than optimal temperatures, will experience a reduction in aerobic scope caused by an increase in routine metabolic rate (RMR), this in turn have been suggested to be correlated with a reduction in growth (Jobling, 1996).

We predict increasing the amount of available oxygen in the water to hyperoxic levels will allow for an increase in aerobic scope. The opposite effect is expected when fish are acclimated hypoxia. In other words, we believe fish acclimated to 50% dissolved oxygen (DO) will have a reduced growth for both length and weight compared to fish acclimated to 100% DO. Fish acclimated to 200% DO, will have an increased growth compared to 100% DO, due to a potential increase in their aerobic scope allowing for a higher appetite and therefore an increase in growth (Figure 1.3). Since the experiment is performed at non-optimal growing temperatures for the fish, we except mediocre but still reduced growth for the fish acclimated to 100% DO in both temperature treatments compared to fish acclimated to 28°C, their optimal temperature (Matthews et al., 2002).
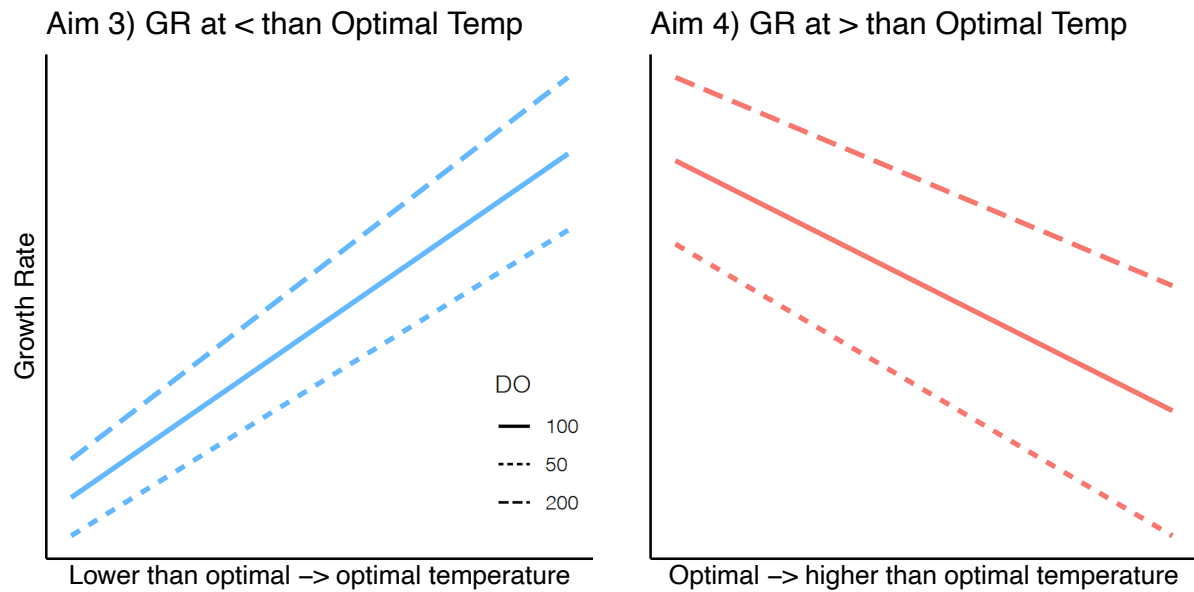
Figure 1.3 Both lower than optimal, 20°C **(Aim 3)**, and higher than optimal, 34°C **(Aim 4),** temperatures are predicted to cause a decreased growth rate in zebrafish. We predict a larger difference between the three oxygen saturations at higher than optimal compared to lower than optimal. Hyperoxia having a higher growth rate, followed by normoxia, and hypoxia lowest growth rate. In this figure, growth rate represents the rate of growth in both length and weight.

2 Methods

2.1 Study species – Zebrafish (*Danio rerio*)

Zebrafish (*Danio rerio*) is well-established as a model organism world-wide within several scientific fields like genetics, biomedicine, physiology and behavior. The zebrafish is a small freshwater species belonging to the minnow family (*Cyprinidae*) (López-Olmeda and Sánchez-Vázquez, 2011). The zebrafish is a tropical species naturally found in the areas of the Brahmaputra and Ganges basin in north-eastern India. Inhabiting these areas comes with thermal challenges both daily and through the seasonal monsoon climate. The zebrafish is found to live in temperatures ranging from 14 to 39°C, making it a eurythermal species (López-Olmeda and Sánchez-Vázquez, 2011). Its thermal tolerance is even larger, allowing for survival in a range from 6.7 to 41.7°C (Cortemeglia and Beitinger, 2005, Schaefer and Ryan, 2006). The thermal biology of the zebrafish has been studied through acclimation as well as through acute testing like CT_{max} and behavior (Vergauwen et al., 2010, Morgan and T., 2019, Roche et al., 2020). Because of their use in a multitude of different experiments within several fields of study, the zebrafish is an excellent proxy for scientific research on vertebrates. This together with its short generation time and the fact that it is easy reproducible, makes it a great study species to keep in laboratories (Spence et al., 2007).

The fish used in this experiment were reproduced from a line of wild caught zebrafish kept at the animal facility of the Norwegian University of Science and Technology (NTNU), Trondheim, Norway. They are reproduced from the F6 random lineage, making them F7. All individuals used for the acclimation experiment were reproduced between the 8th and the 11th December 2020, following the labs standard procedure of reproduction.

2.2 Animal husbandry

The fish were fed TetraPRO energy flakes (Spectrum Brands, Inc.) *ad libitum* three times each day, except when fasting every third day. The fasting was performed to estimate the appetite of the fish the next day with an empty digestion tract. After each appetite measurement, ~10% of each tanks water was removed together with the leftover food. The same amount of water was then added to keep the tanks full. ~75% of the water in each tank was changed once each week. Otherwise, regular cleaning of tanks and filters were performed when found necessary.

Water was prepared using 0.5 dL NaCl and 0.5 dL Aquasafe (Tetra®, Blacksburg, VA, USA) per 100 L. Water barrels (200 L) were kept at the same temperature as the treatments, 20°C and 34°C, to avoid any sudden changes in temperature for the fish when changing water. The temperature of each tank was controlled using thermostats (ITC-310 T, Inkbird, Shenzen, China) and titanium heaters (TH-100, Aqua Medic, Bissendorf, Germany). All water used in the experiment had a conductivity of 800-1200 µS/cm. Each tank was fitted with a pump (EHEIM universal 300, EHEIM®, Deizisau, Germany) with a flow diffuser on the outlet. Each pump was fitted with a cylindrical sponge filter on its inlet. This was to ensure sufficient circulation and filtration of the water in the tanks. The room was on a 12:12 light regime.

The experimental setup consisted of 12 tanks with $N=20$ fish, giving a total of $N=240$ fish at the start of acclimation. At the end of the experiment, one fish had died and nine fish had missing values/missing tags, and were therefore removed from the dataset. Making the final number $N=230$. Duplicates of tanks were adjusted to 6 different treatments for acclimation. Half of the tanks were kept at 20°C, while the other half was kept at 34°C. These two non-optimal temperatures were chosen to be temperature-extremes for zebrafish based on their difference from the most used optimal temperature for zebrafish of 28°C. Three different water oxygen saturations were chosen to create normoxic, hypoxic, and hyperoxic environments. 100%, 50%, and 200%, respectively. Combining the two temperatures and the three oxygen

saturations gave a total of six different treatments (Table 2.1). Duplicates of each treatment were placed in such a way to not be placed next to the same treatment as well as distributed as evenly across the grid as possible (Figure 2.2).

Table 2.1 The six combinations of dissolved oxygen and temperature making up the acclimation treatments for the fish in the experiment.

	50% dissolved oxygen	100% dissolved oxygen	200% dissolved oxygen
20°C	20°C 50%	20°C 100%	20°C 200%
34°C	34°C 50%	34°C 100%	34°C 200%

To control the amount of DO in each tank separately, a gas flow system allowing either to add gaseous nitrogen (N_2), gaseous oxygen (O_2), or compressed air to the tanks was used. This system was controlled using precise air flow meters (Brooks instruments Hatfield, PA, USA) allowing the DO to be finely adjust by the amount of N_2 or O_2 to add to the mixture together with compressed air. The percentage of dissolved oxygen in the tanks containing hypoxic or hyperoxic water was continuously monitored. This system was manually controlled, keeping each tank within a margin of ±5% DO for the 50% DO treatment and ±10% DO for the 200% DO treatment. The ability to control the inflow of gas together with a series of variable valves and continuous monitoring of the DO values for all hypoxic and hyperoxic tanks, enabled full control of the oxygen saturation of each individual tank in the experiment.

To achieve 50% oxygen saturation, N_2 gas was forced into the tanks expelling dissolved O_2 from the tanks. This hypoxic environment (50% ± 5% DO) was kept under control using a ratio of 1:1 compressed air and N_2 gas. To achieve 200% oxygen saturation, O_2 gas was forced into the tanks. This hyperoxic environment (200% ± 10% DO) was kept under control using a ratio of 2:1 compressed air and O_2 gas. To maintain a 100% oxygen saturation, a normoxic environment, the tanks were simply aerated using the labs built in compressed air system. All gas mixtures were forced into the individual tanks through a common aquarium air stone to maximize surface area. The gas supply to every tank was made from the same length of acryl tubing, making the amount of air in the system as close to each other as possible.

Collection of data from the habituation tanks was done using two separate systems – one for oxygen saturation and one for temperature. The % DO in tanks with hypo- and hyperoxic

treatment was monitored using optical probes (FireSting®-O2, Pyroscience, Aachen, Germany). To record and monitor the temperatures of the various tanks, a TC-08 thermocouple data logger (10 m, Pico Technology®, Cambridgeshire, UK) was connected to each tank.

Figure 2.1 The experimental set up for the acclimation project. To the left, there are one tank of N_2 and one tank of O_2 used for adjusting the amount of dissolved oxygen in each tank. To the right, we see half of the Inkbird thermostats controlling the temperature of individual tanks. On the top, one laptop was used to monitor oxygen saturation of the 4 hypoxic and the 4 hyperoxic tanks, as well as temperature for all 12 tanks.
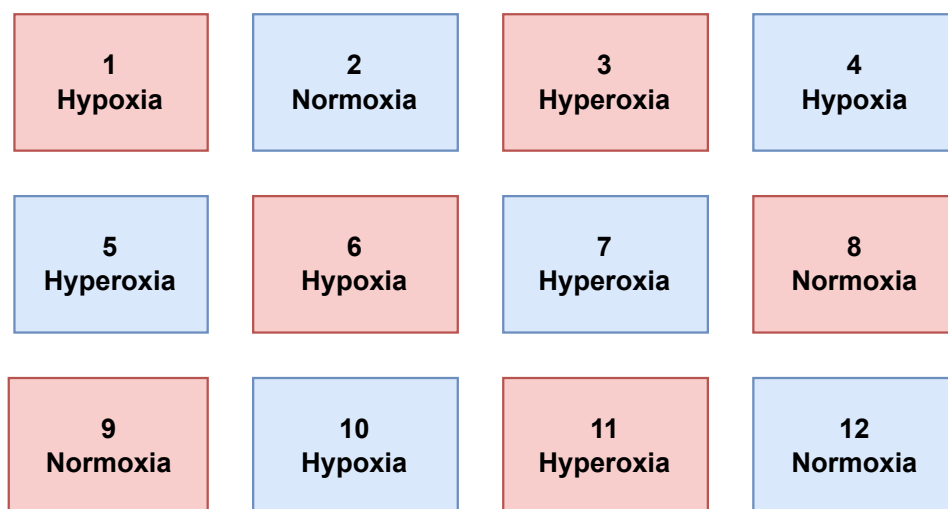
Figure 2.2 Chart showing the distribution of temperatures and oxygen saturation. Tanks acclimated to 20°C are shown as blue and 34°C are shown as red. The placement of the treatment tanks was deliberately stratified to minimize the effect of tank location.

2.3 Experimental procedures

All fish used in the experiment were individually tagged using plastic elastomers (VIE, Northwest Marine Technologies, Shaw Island, WA, USA) subcutaneous on each side just in front of the dorsal fin (Hohn and Petrie-Hanson, 2013). Using two tags per fish, one on each side of the dorsal fin, and five different colors, 20 fish were uniquely tagged in each tank. This made it possible to gather length- and weight-data on an individual level.

Acclimation process

After all fish had been tagged and allocated to their respective treatment tank, the acclimation phase started. The temperature of all tanks to be kept at 20°C was decreased by 4°C per day for two days. The temperature of all tanks to be kept at 34°C was increased by 3°C per day for two days. After all tanks had reached their predetermined acclimation temperature, the oxygen saturation in the water for hypoxic and hyperoxic treatments was either increased or decreased to reach their predetermined dissolved oxygen content of 50% DO and 200% DO. This change in oxygen saturation was performed over the course of six hours (Figure 2.3).
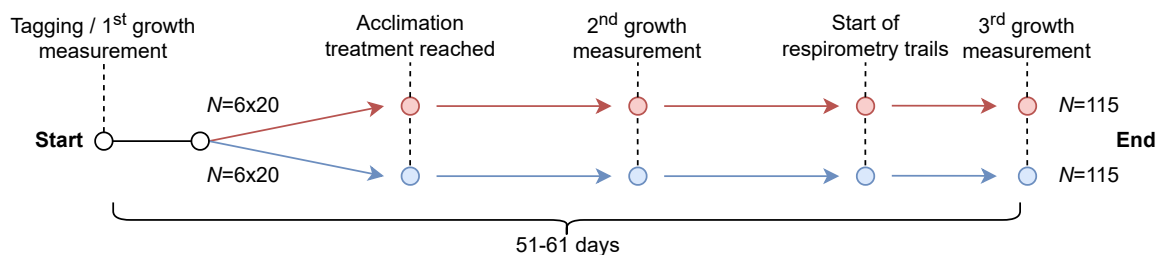
Figure 2.3 Timeline of the complete experiment from tagging of fish until the end of the experiment after respirometry trails were completed. Red and blue lines and points represent 34°C and 20°C, respectively.

Growth

Before tagging, each fish was individually anesthetized using buffered tricaine methanesulfonate (MS-222), before standard length (the length of the fish measured form the tip of the snout to the posterior end before the start of the caudal fin) and mass were measured. The measuring routine was repeated 21-25 days after tagging, and at the end of the experiment directly after metabolic rate measurements 51-61 days after tagging (Figure 2.3). This resulted in a total of three length and three mass measurements for each individual fish. All fish were euthanized before the 3rd growth measurement. These measurements gave an estimate of the fish's growth over the course of the acclimation. The reason for the large time span within the

last measurements is due to the RMR/MMR trail. This trail was time consuming, much due to the amount of fish *N*=230, but also restrictions posed by the number of respirometers, only allowing for running a group of five fish each trail at the same time. This created a bottle neck in the experimental setup.

Appetite

Appetite was measured every third day. To allow for more accurate results, all fish had been starved 24 hours before appetite measurements. Each tank was fed 10 Tetramin flakes at the time. The fish were then left alone in the room allowing them to eat undisturbed. After 10 minutes, the remaining flakes in each tank were counted, before the procedure was repeated until all tanks had been fed a total of 50 flakes. To prevent buildup of food in the tanks, all remaining flakes were removed from the tanks the following day.

Respirometry

All respirometry was performed using a respirometer constructed out of an IKEA 0.4 L circular glass container. This container was kept submerged in a larger tank to enable us to maintain the correct acclimation treatment during the respirometry trail. Each trail included five fish, giving us a total of 48 respirometry groups in total. The container was connected to a flush pump (Eheim CompactON 300, Deizisau, Germany) to allow for intermittent-flow respirometry. The cycles for the flushing was controlled using an external timer. The oxygen saturation was monitored using an optical Firesting probe. To exercise fish for measuring MMR, a magnetic stirrer placed under a plastic mesh on the floor of the respirometry chamber was used. This allowed us to manually control the swimming speed during each MMR trail. Due to computer difficulties, 4 respirometry groups were lost. Two groups were lost from 20°C 200% DO, one group was lost from 34°C 50%, and one group was lost from 34°C 200%.

2.4 Statistical Analysis

All statistical analysis was performed in R version 4.0.2 (2020-06-22), using R Studio. Results were considered statistically significant with a 95% CI. All data was assumed to be a normal distribution around the mean. All results originate from the linear models performed on the data.

The growth of the individual fish was calculated to percentage per day growth (%day^{-1}) using equation consisting of the initial and final length and mass (1). This model assumes a linear growth rate in juvenile zebrafish.

$$\left(\frac{(W_{time} - W_{initial})}{W_{initial}} \times 100\right) / (T_{time} - T_{initial}) = \% \, day^{-1} \qquad (1)$$

Only individuals that survived the whole experiment were included in the analysis of the data. The %day^{-1} was calculated for the growth in length/mass from start to end. All length measurements were standard length in millimeter. All weight measurements were body weight in gram. %day^{-1} was calculated from measurements before the acclimation started, once at 21-25 days after tagging, and after the fish were euthanized at 51-61 days (Figure 2.3).

%day^{-1} was chosen as the measure of the fish's growth during the experiment due to both the zebrafish's linear growth early in life and the simplicity and ease of understanding of equation 1. A specific growth rate assuming an asymptotic growth was first considered, but left unused due to the fish's linear growth.

3 Results

3.1 Growth

The growth rate from fish acclimated to 34°C have a good fit to a linear model for both length and weight with adjusted R^2 values of 0.97 and 0.95, respectively. The growth rate from fish acclimated to 20°C have a poorer fit for a linear model for both length and weight, with adjusted R^2 values of 0.70 and 0.55, respectively (Figure 3.1). Both fish acclimated to 20°C and fish acclimated to 34°C are considered to have a linear growth in both length and weight.

The mean length for each treatment at start was between 17.63 ± 0.30 mm and 18.31 ± 0.3 mm (Table 3.1). The mean weight for each treatment at start was all between 0.0929 ± 0.005 g and 0.1153 ± 0.005 g (Table 3.2).
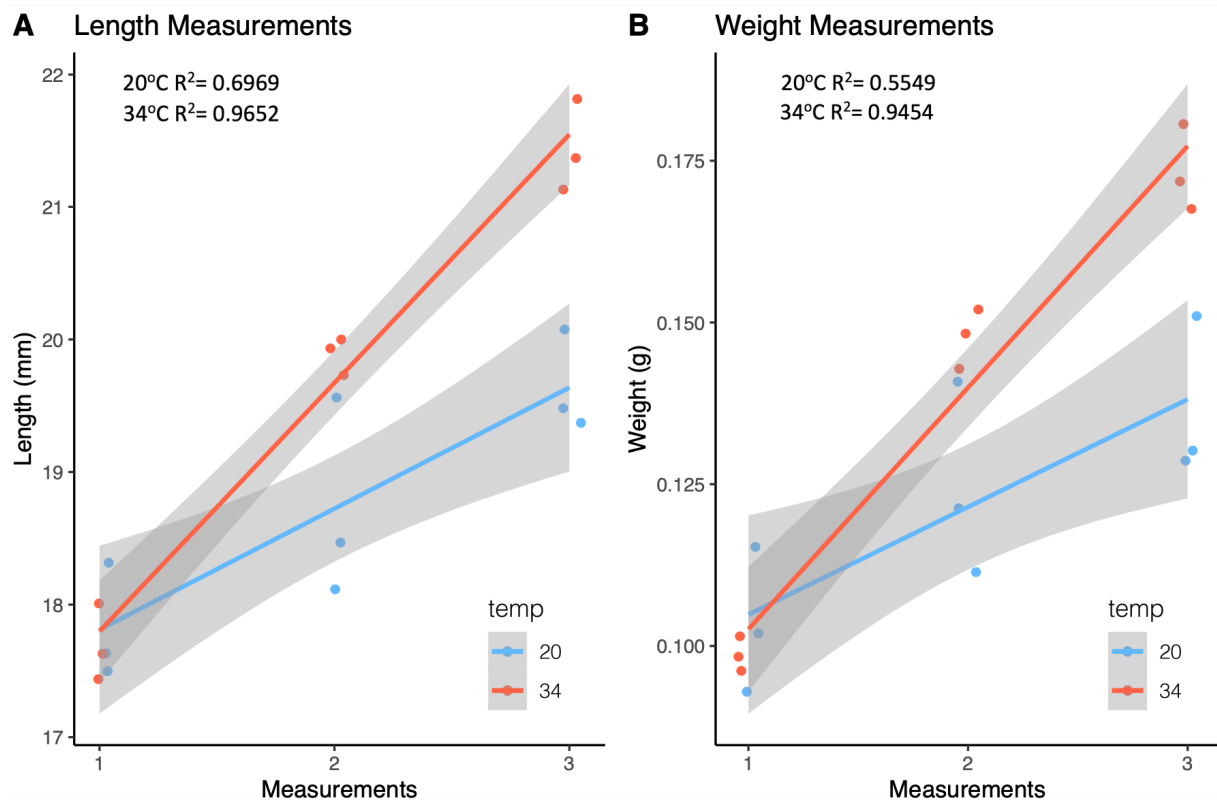
Figure 3.1 Linear models showing the linear growth of the zebrafish in both temperature treatments for **(A)** length (mm) and **(B)** weight (g) over the course of the three measurements. Each point represents the mean of each treatments. The fit of the regression line is shown by citing the adjusted R-squared value from the data to the linear model for both parameters. The gray area illustrates the 95% CI.

The acclimation temperature of the treatments had a significant effect on growth rate. Fish growth increased by 0.27 ± 0.04 %day^{-1} ($\beta \pm$ SE) in length and 1.17 ± 0.23 %day^{-1} in weight when acclimated to 34°C (t=6.637, p<0.001 and t=5.021, p<0.001, respectively) compared to the fish acclimated to 20°C (Figure 3.2 A and 3.2 B).
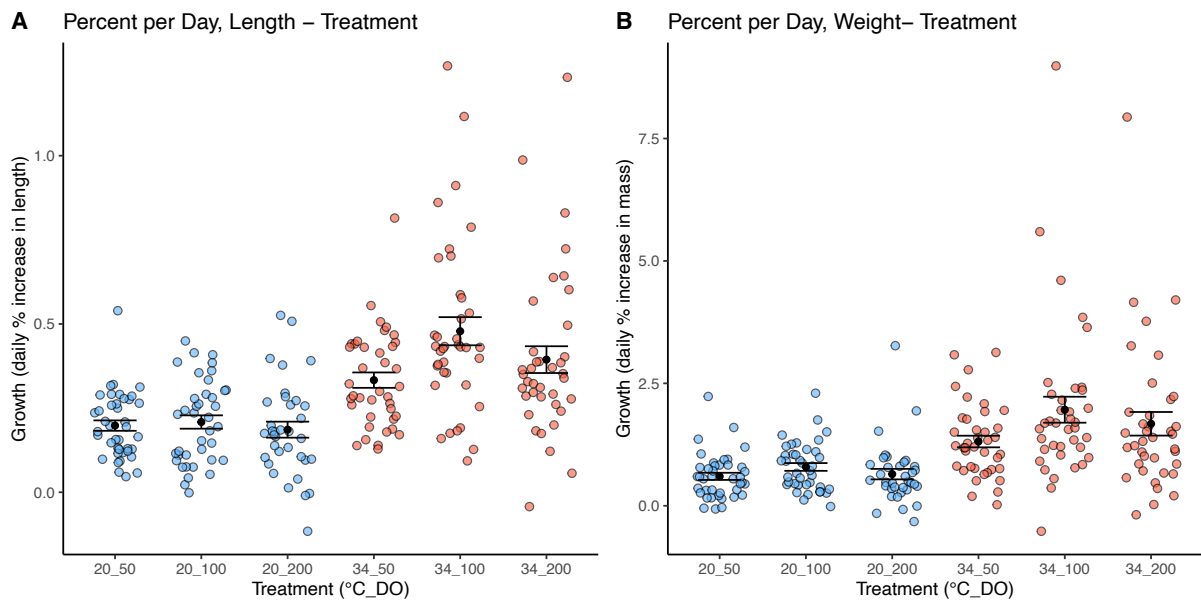
Figure 3.2 Growth, in percentage of initial length and weight per day (%day^{-1}) per treatment. Each treatment is composed of a temperature of either 20°C or 34°C combined with a water oxygen saturation (DO) of either 50%, 100%, or 200%. Error bars centered at mean showing standard error within each treatment. **A)** Change in growth rate in standard length of zebrafish, and **B)** Change in growth rate in weight of zebrafish.

Within each acclimated temperature, there were minor variations between the three oxygen treatments in the growth rate of both length and weight, only some of these tendencies were statistically significant. The variations between oxygen treatments within temperature treatments were larger for fish acclimated to 34°C. None of the fish exposed to either hypoxia or hyperoxia had an increase in growth rate compared to fish exposed to normoxia (Figure 3.2). All fish subjected to non-normoxic oxygen treatment had lower growth rate than fish subjected to normoxic treatment at their same temperature. At 34 °C this decrease in growth rate was found to be statistically significant. Compared to fish acclimated to normoxia, fish acclimated to hypoxia and hyperoxia grew -0.15 ± 0.03 %day^{-1} (t=-3.576, p<0.001) and -0.08 ± 0.03 %day^{-1} (t=-2.063, p<0.05) less in length, respectively (Figure 3.3 A & Table 3.1). Both hypoxia and hyperoxia did reduce the growth in weight in fish acclimated to 34°C as well, although this change in growth was only statistically significant for fish acclimated to hypoxia, growing -0.65 ± 0.23 %day^{-1} (t=-2.789, p<0.01) less (Figure 3.2 B & Table 3.2).

Table 3.1 Mean of initial length, final length and the total length gained during the acclimation experiment, as well as the growth during the experiment measured in percent of initial length per day. All fish were measured in millimeters. Δ% represents the % difference between hypoxic and hyperoxic treatments compared normoxic treatment. SE for mean of each treatment. Significant results highlighted in bold font. t and p values for significant data mentioned in text and appendix.

Treatment	Initial length (mm fish^{-1})	Final length (mm fish^{-1})	Length gain (mm fish^{-1})	Growth rate (%day^{-1})	Δ% from 100%
20°C 50%	17.63 ± 0.30	19.48 ± 0.25	1.84 ± 0.21	0.198 ± 0.03	-5
20°C 100%	17.49 ± 0.30	19.36 ± 0.25	1.93 ± 0.21	0.208 ± 0.03	---
20°C 200%	18.31 ± 0.30	20.07 ± 0.26	1.79 ± 0.22	0.185 ± 0.03	-11
34°C 50%	18.00 ± 0.30	21.36 ± 0.25	3.30 ± 0.21	0.333 ± 0.03	**-30**
34°C 100%	17.43 ± 0.30	21.81 ± 0.26	4.31 ± 0.21	0.478 ± 0.03	---
34°C 200%	17.62 ± 0.30	21.12 ± 0.26	3.56 ± 0.21	0.394 ± 0.03	**-18**

Table 3.2 Mean of initial weight, final weight and the total weight gained during the acclimation experiment, as well as the growth during the experiment measured in percent of initial weight per day. All fish were measured in grams. Δ% represents the % difference between hypoxic and hyperoxic treatments compared normoxic treatment. SE for mean of each treatment. Significant results highlighted in bold font. t and p values for significant data mentioned in text and appendix

Treatment	Initial weight (g fish^{-1})	Final weight (g fish^{-1})	Weight gain (g fish^{-1})	Growth rate (%day^{-1})	Δ% from 100%
20°C 50%	0.1018 ± 0.005	0.1301 ± 0.006	0.0282 ± 0.005	0.60 ± 0.16	-24
20°C 100%	0.0929 ± 0.005	0.1286 ± 0.006	0.0364 ± 0.005	0.79 ± 0.16	---
20°C 200%	0.1153 ± 0.005	0.1510 ± 0.007	0.0357 ± 0.005	0.64 ± 0.17	-18
34°C 50%	0.1015 ± 0.005	0.1718 ± 0.006	0.0694 ± 0.005	1.31 ± 0.16	**-33**
34°C 100%	0.0962 ± 0.005	0.1806 ± 0.006	0.0832 ± 0.005	1.96 ± 0.16	---
34°C 200%	0.0983 ± 0.005	0.1675 ± 0.006	0.0702 ± 0.005	1.67 ± 0.16	-15

3.2 Appetite

From the first appetite measurement was performed after the fish had reached their acclimation temperature and oxygen saturation, the effects of acclimation temperature were already apparent. These recorded differences in appetite between the two temperatures were maintained throughout the experiment. At the end of the experiment, the fish acclimated to 20°C consumed on average 8.5 ± 7.5, 12 ± 0.5, and 6.5 ± 0.5 flakes, acclimated to hypoxia, normoxia, and hyperoxia, respectively. Fish acclimated to 34°C consumed on average 22.5 ± 1.0, 30.5 ± 1.5, and 24.5 ± 1.5 flakes acclimated to hypoxia, normoxia, and hyperoxia, respectively (Table 3.3). In other words, fish acclimated to 34°C ate 165%, 154%, and 277% more than the fish acclimated to 20°C, hypoxia, normoxia, and hyperoxia, respectively, at the end of the experiment

Table 3.3 Mean ± SE number of flakes consumed by each treatment for the first and last appetite measurement as well as the change in percentage between first and last appetite measurement.

Treatment	Mean first measurement	Mean last measurement	Δ% first to last
20°C 50%	8 ± 4	8.5 ± 7.5	+6
20°C 100%	19 ± 2	12 ± 0.5	-36
20°C 200%	13 ± 4	6.5 ± 0.5	-50
34°C 50%	25 ± 4	22.5 ± 1.0	-10
34°C 100%	27 ± 4	30.5 ± 1.5	+13
34°C 200%	28 ± 6	24.5 ± 1.5	-13

An effect of acclimation was observed for both temperatures as well as for all oxygen saturations within each temperature treatment, none of these effects were found to be statistically significant except the difference in trend lines, linear model of change in appetite, between hyperoxia compared to normoxia at 34°C (Figure 3.3). The trend in appetite for fish acclimated to hyperoxia was found to be -2.61 ± 1.45 flakes per measurement lower ($t=-1.806$, $p<0.1$) compared to trend of appetite in fish acclimated to normoxia at the same temperature.

All over, the appetite of fish acclimated to 34°C was higher compared to the appetite of the fish acclimated to 20°C. The relationship of the trend lines within both temperatures are similar. Most treatments showing a decline in appetite over the course of the acclimation period. Normoxic treatment had the highest appetite at the end of the experiment in both temperatures, followed by the hypoxic then the hyperoxic treatment.
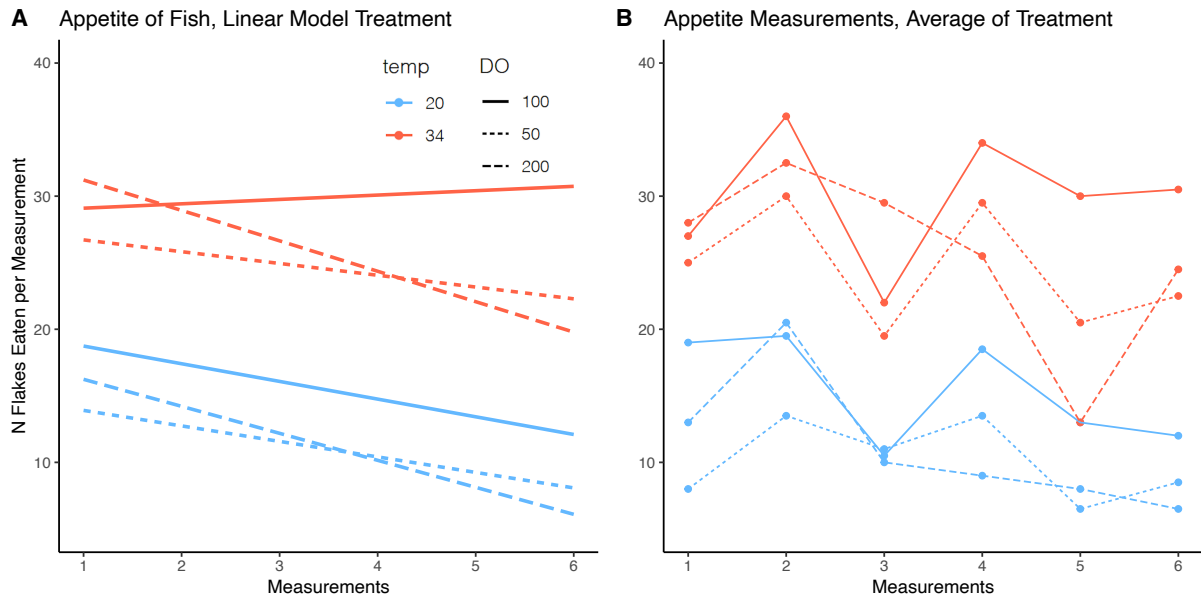
Figure 3.3 A) Linear models comparing the number of Tetramin flakes eaten by each temperature and oxygen treatment in zebrafish over the course of the acclimation project. **B)** Graph showing the recorded appetite data. The mean of the number of flakes eaten by each treatment per measurement. The two temperatures are illustrated by colors, blue representing 20°C and red representing 34°C.

3.3 Metabolism

Temperature affects both the routine metabolic rate (RMR) as well as the maximum metabolic rate (MMR), and thus the AS of the fish. These factors are in turn affected by the oxygen saturation, and at various amounts within the two temperatures.

Fish acclimated to normoxia at 20°C had a RMR of -0.72 ± 0.10 ($\beta \pm SE$) mg/g/h (t=-7.066, p<0.005) compared to fish acclimated to normoxia at 34°C. The differences in MMR were also found to be statistically significant, fish acclimated to normoxia at 20°C had a lower MMR of -1.82 ± 0.25 mg/g/h (t=-7.419, p<0.005) compared to the fish acclimated to normoxia at 34°C. Since the AS is the result of the difference between MMR and RMR, the AS of fish acclimated to normoxia at 20°C had a -1.10 ± 0.22 mg/g/h decrease (t=-5.03, p<0.005) compared to the fish acclimated to normoxia at 34°C (Figure 3.4 and Table 3.4).

The variations between oxygen treatment within each temperature treatment are different. In fish kept at 20°C, fish acclimated to hypoxia had a RMR of -0.2075 ± 0.10 mg/g/h less (t = -2.051, p < 0.05) compared to the fish acclimated to normoxia. The RMR of fish acclimated to hyperoxia did not statistically differ from normoxia. On the other hand, the same fish had a MMR of 0.595 ± 0.26 mg/g/h more (t = 2.249, p < 0.05) compared to the fish acclimated to

normoxia. The MMR of fish acclimated to hypoxia did not statistically differ from fish acclimated to normoxia. The AS of fish acclimated to hyperoxia did have an increase of 0.5642 ± 0.24 mg/g/h (t = 2.38, p < 0.05) compared to the fish acclimated to normoxia (Figure 3.4 C and Table 3.4).

At 34°C, fish acclimated to hyperoxia had a RMR of 0.3011 ± 0.10 mg/g/h more (t = 2.875, p < 0.01) compared to the fish acclimated to normoxia at the same temperature. The RMR of fish acclimated to hypoxia did not statistically differ from normoxia. On the other hand, fish acclimated to hypoxia had a MMR of -0.7461 ± 0.25 mg/g/h less (t = 2.942, p < 0.01) compared to the fish acclimated to normoxia. The MMR of fish acclimated to hyperoxia did not statistically differ from fish acclimated to normoxia. The AS of fish acclimated to hypoxia did have a decrease of -0.5812 ± 0.23 mg/g/h (t = -2.559, p < 0.05) compared to the fish acclimated to normoxia (Figure 3.4 C and Table 3.4).

The variations in RMR, MMR, and AS between temperatures and oxygen saturations are illustrated in figure 3.4, where the large differences between the two temperatures in all three measurements are clearly illustrated. The variations in RMR, MMR, and AS for the treatments can be found summarized in table 3.4.
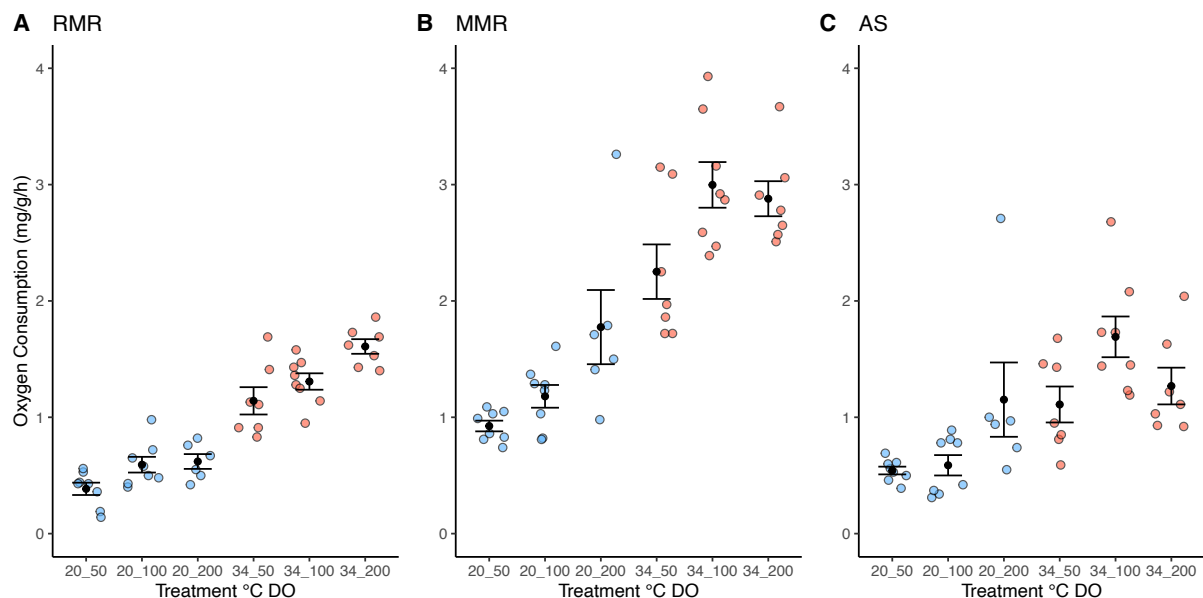
Figure 3.4 Scatterplot illustrating the variations of **A)** routine metabolic rate (RMR), **B)** maximum metabolic rate (MMR), and **C)** aerobic scope (AS) for temperature and oxygen treatments in zebrafish. Red representing 34°C and blue representing 20°C. Error bars centered at mean showing standard error within each treatment. Each treatment was divided into 8 respirometry groups, except for 20°C 200 DO having 6 groups, 34°C 50 DO having 7 groups, and 34°C 200 DO having 7 groups. The 4 missing groups were lost due to computer difficulties.

Table 3.4 Variations in routine metabolic rate (RMR), maximum metabolic rate (MMR), and aerobic scope (AS) for temperature and oxygen treatments in zebrafish in addition to percentage change from normoxic treatment within both temperatures. All metabolic measurements are nominated in mg/g/h. Significant % changes in values are highlighted in bold. t and p values for significant data mentioned in text and appendix

Treatment	RMR mg/g/h	Δ% from 100%	MMR mg/g/h	Δ% from 100%	AS mg/g/h	Δ% from 100%
20°C 50%	0.38 ± 0.07	**-35**	0.92 ± 0.17	-22	0.54 ± 0.15	-6
20°C 100%	0.59 ± 0.07	---	1.18 ± 0.17	---	0.58 ± 0.15	---
20°C 200%	0.62 ± 0.08	+5	1.77 ± 0.20	**+50**	1.15 ± 0.17	**+98**
34°C 50%	1.14 ± 0.07	-12	2.25 ± 0.18	**-25**	1.11 ± 0.16	**-34**
34°C 100%	1.30 ± 0.07	---	2.99 ± 0.17	---	1.69 ± 0.15	---
34°C 200%	1.60 ± 0.07	**23**	2.87 ± 0.18	-4	1.26 ± 0.16	-25

4 Discussion

In this study, the aim was to test for difference in growth, appetite and metabolic rates between lower than optimal and higher than optimal temperature, combined with hypoxic, normoxic, and hyperoxic treatments. The ideas tested and discussed are derived from a smaller part of the proposed hypotheses from Jutfelt et al., 2021.

4.1 Growth

There was a difference in growth between the treatments. This difference was expected based on earlier research. The aerobic metabolic activity in ectotherms is closely linked to the temperatures of their surroundings (Schulte, 2015). Somatic growth in zebrafish varies with its acclimation temperature (Vergauwen et al., 2010). Compared to the growth rate of zebrafish at optimal temperatures of 28°C (Westerfield, 2000), both the fish acclimated to lower than optimal and higher than optimal temperatures are reduced in growth.
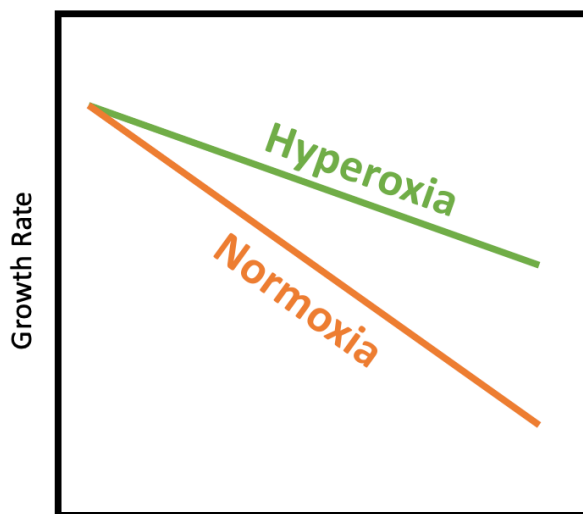
Figure 4.1 Illustration of proposed hypothesis by Jutfelt et. al. Depicts the decline in growth rate for Hyperoxia and Normoxia at a gradient from optimal towards higher than optimal temperatures for ectotherms. Modified from (Jutfelt et al., 2021).

In both temperatures, fish acclimated to hypoxia and hyperoxia grew less compared to the fish acclimated to normoxia. The variations between the oxygen treatments were larger in fish acclimated to 34°C compared to the fish acclimated to 20°C, meaning the oxygen saturation plays a larger role as temperatures become higher. This contradicts the hypothesis presented in Jutfelt et. al. 2021 where the growth rate of fish acclimated to a hyperoxic environment were predicted to have lower decrease in growth rate at higher than optimal temperatures compared to fish acclimated to a normoxic environment (Figure 4.1). The opposite was observed. This in turn is closely related to the food intake of the fish.

The common idea is that a heated environment allow for a faster pace of life and a higher metabolic rate (Fry and Hart, 1948). This increased metabolism at higher temperatures do have a cost. An increase in metabolism requires more energy, and therefore more nutrients through food. An increase in food consumption will again reduce the fish's PRAS. If fish eat larger meals at higher temperatures the SDA curve might exceed the MMR and cause an oxygen deficit. In this experiment, a higher intake of food was observed in fish acclimated to higher temperatures compared to fish acclimated to a lower temperature. This was expected due to their increased metabolic rate. The effect of a decrease in AS and therefore PRAS was thought to be extra prominent at higher temperatures together with lower saturations of oxygen in the water, i.e. our 34°C 50% DO treatment. Growth in length and weight as well as AS was significantly lower in fish acclimated to hypoxia at 34°C compared to fish acclimated to normoxia at the same temperature. This reduction in growth might be due to the limits posed by the higher than optimal temperature on the AS, lowering the meal size and appetite in order to not exceed the MMR and enter anaerobe metabolism to perform vital functions. The lower intake of food therefore stagger the growth of these individuals exposed to hypoxia at higher than optimal temperatures. A stark reduction of appetite at hypoxia at warmer temperatures compared to a low reduction in appetite when exposed to hypoxia at lower colder temperatures has been observed in channel catfish (*Ictalurus punctatus*) (Buentello et al., 2000). The same dependency of oxygen saturation at higher than optimal temperatures on appetite has been observed in Atlantic salmon (*Salmo salar*) (Remen et al., 2016). These previous findings strengthen our observations of a strong negative effect of hypoxia on growth at higher than optimal temperatures. This is not shown clearly in the results from the appetite measurements, but the effect is prominent when comparing the difference in growth rate between hypoxia and normoxia at both lower than and higher than optimal temperatures.

Earlier in the introduction and discussion (Figure 1.3 & Figure 4.1), we predicted an increase in growth rate for fish acclimated to hyperoxia compared to normoxia in fish acclimated to higher than optimal temperatures. This was also predicted in Jutfelt et al., 2021. In this study, a negative effect of hyperoxia on growth rate at 34°C was found. This is believed to be correlated to the observed reduction in AS for the same treatment. A decrease in AS might be the reason for the reduction in growth. Facilitating a higher AS at higher than optimal temperatures does not seem to be feasible, not via hyperoxia at least. These finding are contradictive to this study's predictions, and indicates some other mechanisms playing out at higher than optimal temperatures together with hyperoxia. Not many studies have looked at

both higher than optimal temperatures together with hyperoxia, other results on this specific topic is therefore missing to some degree.

The effects on performance of acclimation to 34°C are larger than acclimation to 20°C when investigating thermal performance curves of zebrafish (Morgan et al., 2019). Changes in environmental factors like oxygen saturation do affect the measured parameters more at higher temperatures, indicating higher stress and more vulnerable fish to changes in oxygen saturation at higher temperatures compared to lower temperatures (Seebacher et al., 2015).

High growth rate is not necessarily optimal. Vergauwen et al., 2010 illustrated this in their 2010 study where zebrafish acclimated to high temperatures, 34°C, became longer and skinnier due to reduced fat storage. Both temperature treatments in this acclimation experiment are non-optimal in regard to growth. Warmer than optimal temperature is believed to be more stressful compared to colder than optimal temperatures when diverging the same amount of degrees from the optimal temperature (Vergauwen et al., 2010). Thermal performance curves illustrate this with the sudden and steep decline in metabolic rate and growth at critical temperature maximums. Therefore, even though fish acclimated to 34°C had a higher growth rate compared to the fish acclimated to 20°C, fast growth is not necessary a result of good fitness (Vergauwen et al., 2010). Since zebrafish exposed to 20°C and 34°C are outside of their thermal optimal zone, both non-optimal temperatures lead to reduction in growth (Morgan and T., 2019)

Since higher temperatures increase the reaction rate of chemical reactions, reactions causing damage to fish will also increase. This might cause accumulation of metabolites together with an increased amount of reactive oxygen species (ROS), causing a toxic build-up facilitated by an abundance of oxygen (Jamieson et al., 1986). These effects might be key factors helping us understand the negative effect of hyperoxic treatment observed in this study.

4.2 Appetite

Appetite measurements were compromised due to a drift in temperature in two tanks of the same treatment, 34°C 100% DO. Due to this anomaly in the data, only the three first and the three last appetite measurements were used. Unfortunately, there is no continuous record of temperature for the course of the whole experiment. There are, however, records of tank 9 drifting as far down as 24°C and tank 8 as far down as 30°C. This drift in temperature caused a significant dip in appetite measurements for both these tanks, and the five appetite measurements in the middle of the experiment were therefore removed for all tanks. It should not be without mention that this event might have affected the growth in fish acclimated to 34°C 100% DO, giving the fish in the treatment a somewhat lower growth rate.

The variations in appetite between the two acclimation temperatures were expected to be different from each other due to the increase in metabolic activity at higher than optimal temperature and the decrease in metabolic activity at lower than optimal temperatures. An increase in temperature will increase the fish's metabolism requiring more nutrition and increased appetite. The opposite is true for a decrease in temperature.

Over the course of the acclimation period, fish acclimated to hypoxia and hyperoxia had a decline in appetite compared to fish acclimated to normoxia. This decline in number of flakes eaten per measurement could be observed within both temperature treatments. The decrease of appetite over time might be an indication of just how stressful hypoxic and hyperoxic treatments are. The decline in appetite in fish acclimated to hyperoxia contradicts the hypothesis presented by Jutfelt et al. 2021, where it is predicted that the food intake of fish acclimated to hyperoxia would be higher for fish acclimated to hyperoxia compared to normoxia, due to the potential increase in AS and therefore room for a larger SDA curve (Figure 4.2). Figure 4.2 also illustrates the potential change in appetite between hypoxia, normoxia, and hyperoxia at a temperature gradient. Where the differences in appetite were expected to be smaller between the oxygen treatments at lower than optimal temperatures compared to higher than optimal temperatures.
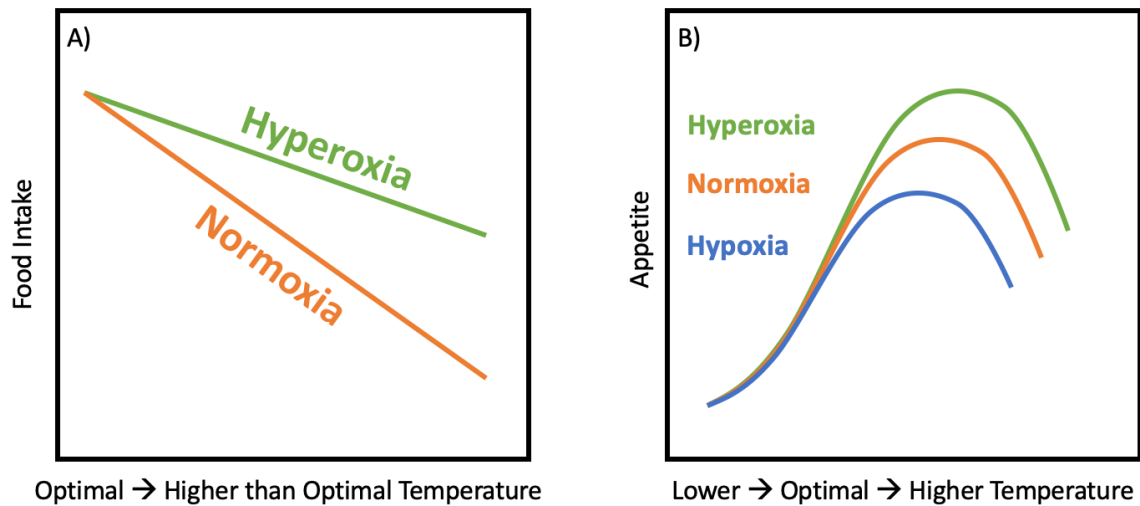
Figure 4.2 Illustration of proposed hypothesis by Jutfelt et. al. **A)** Depicts the decline in food intake for Hyperoxia and Normoxia at a temperature gradient from optimal towards higher than optimal temperatures for ectotherms. **B)** Depicts a peak temperature centered change in appetite for hypoxic, normoxic, and hyperoxic oxygen levels. Predicting smaller to no difference between oxygen saturations at colder than optimal temperatures but an increase in difference at higher than optimal temperatures. Modified from (Jutfelt et al., 2021).

The difficulties of performing appetite measurements on Zebrafish

Whether zebrafish is the most suitable fish to perform appetite measurements on is a though question, there are several problems occurring from theses trails. The size of the zebrafish makes it hard to divide meals at a high enough accuracy between each trail and tank. The Tetramin flakes used for this experiment are highly variable in size, this in combination with the fact that the fish "nibble" on pieces rather than eating the whole flake makes it hard to determine the amount of food that is ingested by the fish. The flakes do also dissolve after a certain time in the water. Performing appetite measurements on such small fish and with so small margins made the appetite measurements challenging. In addition, the subjective measurements of counting remaining flakes in each tank added to the inaccuracy.

The possibility of calculating food conversion to somatic growth in zebrafish

Firstly, due to difficulties concerning accuracy in measuring appetite (Figure 3.5) in zebrafish mentioned above, a reasonable food conversion rate is not possible to calculate. And If calculated, these estimates would highly inaccurate. Having a more precise way of measuring the appetite of these fish would help significantly. Secondly this estimated food conversion rate would only be on a tank level. This is not inherently bad, but would be inaccurate when considering the variations in somatic growth within each tank, especially since the sex distribution within and between each tank was not considered.

4.3 Metabolism

An increase in AS is only possible if the maximum metabolic rate (MMR) increase more than the routine metabolic rate (RMR). An increase in temperature causes an increase in both MMR and RMR (Fry and Hart, 1948). The mission of this study was to investigate if an increase in oxygen saturation in the fish's surroundings, i.e. more available oxygen for metabolic activity, could help increase the MMR in a larger degree than the RMR, in order to increase the fish's AS. The effect of increased oxygen saturation might have allowed for an increased AS which in turn could have allowed for more room for a larger SDA curve, meaning higher food intake and therefore a higher growth rate (Jutfelt et al., 2021). This effect was not observed in this experiment. Fish acclimated to hyperoxia at 34°C had both lower growth rate and appetite compared to fish acclimated to normoxia at the same temperature. In fact, the AS of fish acclimated to hyperoxia at 34°C was lower compared to fish acclimated to normoxia at the same temperature. This indicates other underlying effects decreasing the physiological performance of zebrafish when exposed to chronic hyperoxia at higher than optimal temperatures.

In fish acclimated to lower than optimal temperature, an immense increase in aerobic scope when the fish were exposed to chronic hyperoxia was observed. The AS of fish acclimated to hyperoxia was 98% larger than in fish acclimated to normoxia at the same temperature. When seeing the increase in AS alone, one would believe that fish acclimated to hyperoxia at 20°C would have an increased somatic growth due to the room for a larger SDA response. This was not the case, indicating that a direct connection between appetite/growth and AS is more vague than expected. Variations in growth between temperatures are simply not controlled by the AS of the fish alone. This contradicts what presented in (Jutfelt et al., 2021), and shows that fish will not necessarily fill their AS with a larger SDA if possible.

In fish acclimated to lower than optimal temperatures, an increase in the aerobic scope of fish kept at hyperoxic oxygen levels compared to normoxic levels was recorded. This shows that MMR at lower temperatures is not restricted by the same limitations MMR at higher temperatures are constrained by, possibly due to the overall lowered metabolism. This correspond with the findings presented by Sandblom et al., 2016, where the metabolic ceiling is presented as concrete.

4.6 Mishaps during the experiment

During the seven weeks of acclimation there were a few incidents affecting our experiment as well as our amount of collected data.

On April 22nd there was a power outage most likely caused by all heating elements turning on simultaneously overloading the circuit. This power outage affected the room temperature causing tanks kept at 20°C to increase slightly. This power outage also turned off the circulation pumps in all tanks.

During the experiment, several of the Inkbird thermostats were drifting, some more than others. This was usually not a problem. The variations were usually not more than the natural fluctuation in the tanks. On one occasion two tanks drifted more than 10°C causing both tanks within the 34°C 100% treatments to stay at ~24°C. This did of course affect the appetite of this group significantly, and resulted in the removal of 5 appetite measurements in the middle of the dataset, leaving only the three first and three last measurements, compromising the appetite data.

During the whole experiment, we continuously logged the temperature of each of the individual tanks. This data was to be used to illustrate the initial acclimation steps for the treatments and as a proof of acclimated temperature. Unfortunately, the temperature log only stored the last 48 hours of data. All previous data was being overwritten. This error was not detected before the 15th of May, one month after the acclimation was started, leaving us without a complete digital log over the exposure temperature of each individual tank.

4.6 Conclusion

In conclusion, temperature acclimation to 34°C and 20°C affected both length and weight growth, as well as appetite. A higher than optimal temperature allowed for an all over larger somatic growth compared to a lower than optimal temperature. When compared to normal growth of zebrafish at its optimal temperature of 28°C, both temperatures in this experiment caused a retardation in growth.

Both acclimation to hypoxia and hyperoxia had a negative effect on the growth rate of length and weight. The effects of both acclimation to hypoxia and hyperoxia on growth, appetite and metabolism are all significantly larger at 34°C than at 20°C.

It is hard to derive any conclusive results from the appetite measurements, but simple non-significant trends were observed. As predicted, fish acclimated to 20°C had a lower appetite than fish acclimated to 34°C. There was an observed a negative trend in appetite for both hypoxic and hyperoxic treatments compared to normoxic treatments at the same temperature, although these trends could not be proven statistically. However, a possible reduction in appetite for said treatments do correspond to the decrease in growth rate that was observed.

The reduction in growth at hyperoxic environments stands in stark contrast to the AS protection hypothesis, which predicted an increase in growth in hyperoxia compared to normoxia at higher temperatures. Neither the AS nor the somatic growth of zebrafish were increased at 34°C simply by increasing the oxygen saturation of the water to hyperoxic levels. Even though an increase in AS in fish acclimated to lower than optimal temperatures and hyperoxia compared to normoxia at the same temperature was observed, no significant increase in growth was observed in hyperoxia. This corresponds with Auer et al., 2015, saying AS is not directly linked to growth, only positively correlated. Allowing other mechanisms, such as oxygen saturation or other environmental factors, to suppress growth when fish are exposed to lower than optimal temperatures.

The fact that hyperoxia in warmer than optimal temperatures do not increase the growth compared to fish at lower oxygen saturation, shows the existence of negative effects of hypoxia and hyperoxia. Exactly what underlying mechanisms affected cannot be answered by this study and will need further research.

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6 Appendix

Table A.1 R-output, Linear Model percent growth per day in *Length* by treatment. Intercept is DO100:temp20

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.2087	0.02855	7.31	4.73E-12 ***
DO50	-0.01065	0.04012	-0.265	0.7909
DO200	-0.02272	0.04151	-0.547	0.5847
temp34	0.26974	0.04064	6.637	2.40E-10 ***
DO50:temp34	-0.13468	0.05711	-2.358	0.0192 *
DO200:temp34	-0.06165	0.05828	-1.058	0.2913

Table A.2 R-output, Linear Model percent growth per day in *Length* by treatment. Intercept is DO100:temp34

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.47844	0.02892	16.541	< 2e-16 ***
DO50	-0.14533	0.04064	-3.576	0.000428 ***
DO200	-0.08437	0.0409	-2.063	0.040299 *
temp20	-0.26974	0.04064	-6.637	2.40E-10 ***
DO50:temp20	0.13468	0.05711	2.358	0.019227 *
DO200:temp20	0.06165	0.05828	1.058	0.291281

Table A.3 R-output, Linear Model percent growth per day in *Weight*, treatment. Intercept is DO100:temp20

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.7927	0.1636	4.845	2.37E-06 ***
DO50	-0.1914	0.2299	-0.833	0.406
DO200	-0.146	0.2379	-0.614	0.54
temp34	1.1692	0.2329	5.021	1.05E-06 ***
DO50:temp34	-0.4581	0.3272	-1.4	0.163
DO200:temp34	-0.1413	0.3339	-0.423	0.673

Table A.4 R-output, Linear Model percent growth per day in *Weight*, treatment. Intercept is DO100:temp34

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.9619	0.1657	11.838	< 2e-16 ***
DO50	-0.6496	0.2329	-2.789	0.00574 **
DO200	-0.2873	0.2344	-1.226	0.22159
temp20	-1.1692	0.2329	-5.021	1.05E-06 ***
DO50:temp20	0.4581	0.3272	1.4	0.1629
DO200:temp20	0.1413	0.3339	0.423	0.67255

Table A.5 R-output, Linear Model final *Length* measurements, treatment. Intercept is DO100:temp20

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	19.3697	0.2583	74.998	< 2e-16 ***
DO50	0.1115	0.363	0.307	0.75897
DO200	0.7064	0.3728	1.895	0.0594 .
temp34	2.4476	0.3676	6.658	2.12E-10 ***
DO50:temp34	-0.5604	0.5166	-1.085	0.2792
DO200:temp34	-1.394	0.5252	-2.654	0.00853 **

Table A.6 R-output, Linear Model final *Length* measurements, treatment. Intercept is DO100:temp34

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	21.8174	0.2616	83.384	< 2e-16 ***
DO50	-0.4489	0.3676	-1.221	0.22336
DO200	-0.6876	0.37	-1.858	0.06443 .
temp20	-2.4476	0.3676	-6.658	2.12E-10 ***
DO50:temp20	0.5604	0.5166	1.085	0.2792
DO200:temp20	1.394	0.5252	2.654	0.00853 **

Table A.7 R-output, Linear Model final *Weight* measurements, treatment. intercept is DO100:temp20

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.128672	0.006337	20.304	< 2e-16 ***
DO50	0.001458	0.008906	0.164	0.87009
DO200	0.022353	0.009147	2.444	0.01531 *
temp34	0.052026	0.009021	5.767	2.66E-08 ***
DO50:temp34	-0.010294	0.012677	-0.812	0.41762
DO200:temp34	-0.035485	0.012888	-2.753	0.00638 **

Table A.8 R-output, Linear Model final *Weight* measurements, treatment. intercept is DO100:temp34

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.180697	0.00642	28.146	< 2e-16 ***
DO50	-0.008836	0.009021	-0.979	0.3284
DO200	-0.013132	0.009079	-1.446	0.14949
temp20	-0.052026	0.009021	-5.767	2.66E-08 ***
DO50:temp20	0.010294	0.012677	0.812	0.41762
DO200:temp20	0.035485	0.012888	2.753	0.00638 **

Table A.9 R-output, Linear model, Appetite measurements for fish acclimated to 20°C

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	19.8667	2.5181	7.89	5.30E-09 ***
variable	-1.2714	0.5495	-2.314	0.0273 *
DO50	-5.25	2.2987	-2.284	0.0292 *
DO200	-4.25	2.2987	-1.849	7.37E-02 .

Table A.10 R-output, Random effect linear model, Appetite measurements for fish acclimated to 34°C

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	28.7667	4.3364	19.6505	6.63E+00	2.02E-06 ***
variable	0.3286	1.0234	27	0.321	0.751
DO50	-1.1667	6.1326	19.6505	-0.19	0.851
DO200	4.7333	6.1326	19.6505	7.72E-01	0.449
variable:DO50	-1.2143	1.4473	27	-0.839	0.409
variable:DO200	-2.6143	1.4473	27	-1.806	0.082

Table A.11 R-output, Linear model, Routine Metabolic Rate, treatment. Intercept is DO100:temp20

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.5925	0.07155	8.281	4.89E-10 ***
DO50	0.715	0.10119	7.066	2.00E-08 ***
DO200	-0.2075	0.10119	-2.051	0.0472 *
temp34	0.0275	0.10929	0.252	0.8027
DO50:temp34	0.04143	0.14563	0.284	0.7776
DO200:temp34	0.27357	0.15138	1.807	0.0787 .

Table A.12 R-output, Linear model, Routine Metabolic Rate, treatment. Intercept is DO100:temp34

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.3075	0.07155	18.274	< 2e-16 ***
Temp20	-0.715	0.10119	-7.066	2.00E-08 ***
O250	-0.16607	0.10474	-1.586	0.12112
O2200	0.30107	0.10474	2.875	0.00659 **
Temp20:O250	-0.04143	0.14563	-0.284	0.77759
Temp20:O2200	-0.27357	0.15138	-1.807	0.07865 .

Table A.13 R-output, Linear model, Maximum Metabolic Rate, treatment. Intercept is DO100:temp20

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.18	0.1732	6.812	4.42E-08 ***
Temp34	1.8175	0.245	7.419	6.72E-09 ***
O250	-0.255	0.245	-1.041	0.3045
O2200	0.595	0.2646	2.249	0.0304 *
Temp34:O250	-0.4911	0.3526	-1.393	0.1718
Temp34:O2200	-0.7139	0.3665	-1.948	0.0588 .

Table A.14 R-output, Linear model, Maximum Metabolic Rate, treatment. Intercept is DO100:temp34

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.9975	0.1732	17.304	< 2e-16 ***
Temp20	-1.8175	0.245	-7.419	6.72E-09 ***
O250	-0.7461	0.2536	-2.942	0.00553 **
O2200	-0.1189	0.2536	-0.469	0.64175
Temp20:O250	0.4911	0.3526	1.393	0.17179
Temp20:O2200	0.7139	0.3665	1.948	0.05883 .

Table A.15 R-output, Linear model, Aerobic Scope, treatment. Intercept is DO100:temp20

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.5875	0.1552	3.787	0.000529 ***
Temp34	1.1038	0.2194	5.03	1.21E-05 ***
O250	-0.045	0.2194	-0.205	0.838601
O2200	0.5642	0.237	2.38	0.022412 *
Temp34:O250	-0.5363	0.3158	-1.698	0.097672 .
Temp34:O2200	-0.9869	0.3283	-3.006	0.004668 **

Table A.16 R-output, Linear model, Aerobic Scope, treatment. Intercept is DO100:temp34

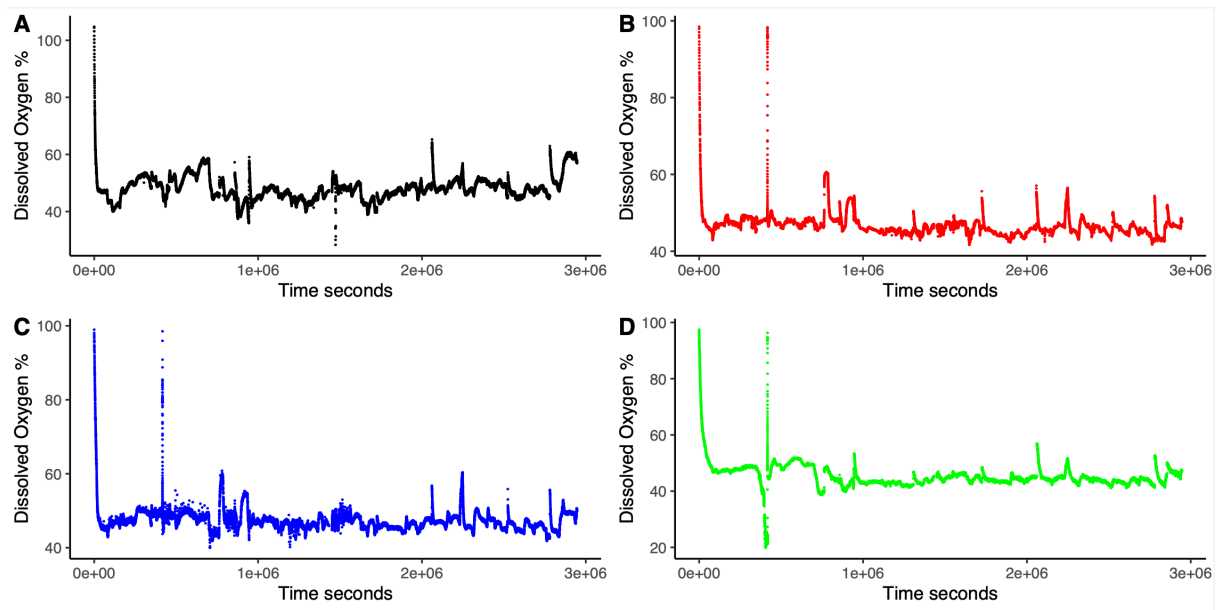|	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.6913	0.1552	10.9	2.95E-13 ***
Temp20	-1.1038	0.2194	-5.03	1.21E-05 ***
O250	-0.5812	0.2271	-2.559	0.0146 *
O2200	-0.4227	0.2271	-1.861	0.07049 .
Temp20:O250	0.5363	0.3158	1.698	0.09767 .
Temp20:O2200	0.9868	0.3283	3.006	0.00467 **

Figure A.1 Oxygen saturation for all tanks treated with hypoxia. **A)** tank 1 (34°C 50%), **B)** tank 4 (20°C 50%), **C)** tank 6 (34°C 50%), and **D)** tank 10 (20°C 50%). The large sudden spike in B, C, and D are caused by the probes being removed.
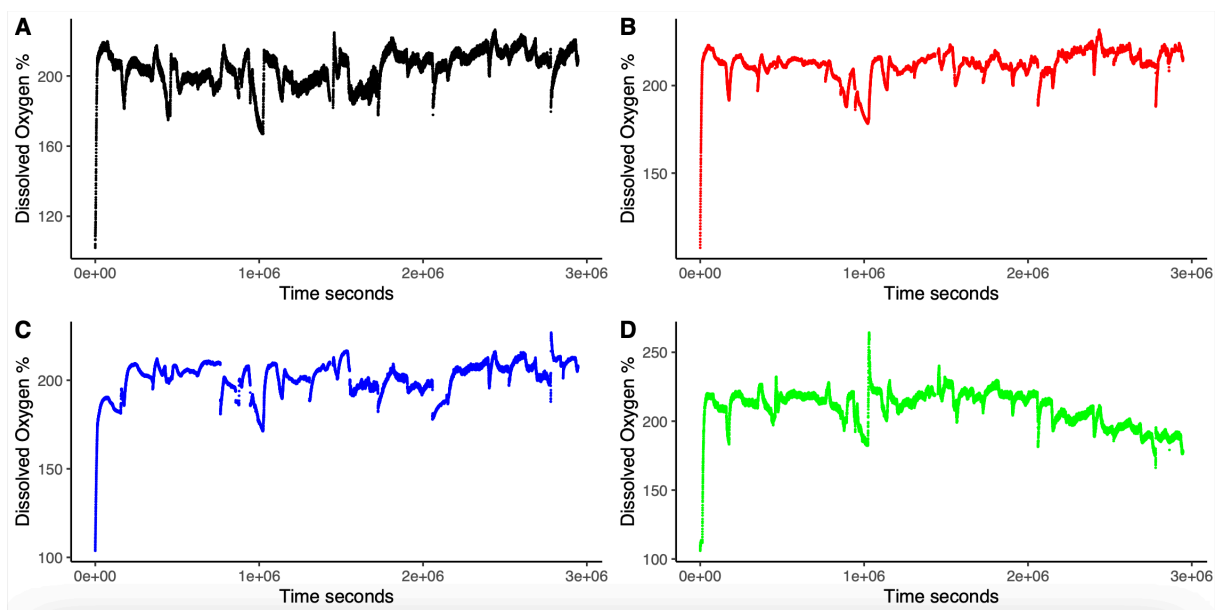

Figure A.2 Oxygen saturation for all tanks treated with hyperoxia. **A)** tank 3 (34°C 200%), **B)** tank 5 (20°C 200%), **C)** tank 7 (20°C 200%), and **D)** tank 11 (34°C 200%).