Grad Project – BIOL 7220 Quantitative Methods in Biology (Fall 2017)

Topic: The Response of Mass-Specific Oxygen Consumption of Sablefish (*Anoplopoma fimbria*) and Atlantic Salmon (*Salmo salar*) to an Incremental Temperature Increase

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Background

Sablefish Life History, Markets and Farming Potential

The sablefish (*Anoplopoma fimbria*) (Pallas, 1814), also known as black cod, is a long-lived deep-water species with a broad bathymetric and geographic range. It can be found in surface waters and at depths over 1,500 meters, and is widely distributed along the continental shelf of the Eastern and Western North Pacific [1]. Sablefish has a high market value, is considered a delicacy item in some countries and is the most valuable species on Canada's west coast [2]. Canada's largest export market for sablefish is Japan, with the USA as the second most important market [3]. Because of the decline in wild stocks [2], increasing consumer demands from emerging markets [3], and a high market value [4], sablefish farming is an emerging aquaculture industry. The sablefish has been shown to adapt well to aquaculture rearing [5] and to have one of the fastest recorded growth rates of all teleost species [6]. Sablefish is also considered a good candidate as a fed trophic level for integrated multi-trophic aquaculture, since its premium price enables culture at smaller scales [7]. There is now one major sablefish hatchery (Golden Eagle Sable Fish) on Salt Spring Island in British Columbia (BC), Canada. In addition, Newfoundland Cod Broodstock Corporation is interested in culturing sablefish in Newfoundland for the European market and grow-out farms like Global Blue Technologies in Texas have recently conducted trials with sablefish using a recirculating aquaculture system.

Global Warming and Associated Hypoxia in Coastal Areas

Many coastal ecosystems around the world are experiencing hypoxia, and some are currently within an oxygen minimum zone (OMZ) or are a concern to become one [8]. Global warming due to climate change is an important contributor to the development of hypoxia, as the warming of marine and freshwater systems leads to a decrease in oxygen (O_2) solubility, increased organism metabolism and remineralization rates, and enhanced stratification. In Canada, coastal areas are also affected by hypoxic events which are expected to occur more often in the future [9]. Coastal fish populations, as well as fish farmed at cage-sites, are exposed to these hypoxic conditions as well as elevated temperatures that are both associated to global warming. Given that sablefish farming in is an emerging industry in BC, and that elevated temperatures and chronic hypoxia are a major challenge to cage-site aquaculture operations in many locations in BC (Jamie Gaskill, Marine Harvest Canada; Peter McKenzie, Cermaq, personal communication), it is important to understand the effects of these environmental conditions on sablefish. At the moment, however, there is very little information available on the physiology of sablefish under elevated temperatures and hypoxia.

Upper Thermal and Hypoxia Tolerance and the OCLTT Concept

In fish, there is some evidence that upper thermal tolerance and hypoxia tolerance are related to one another [10-11]. This is likely because they are both determined by limitations in the capacity to deliver O_2 to the tissues, as described in the oxygen and capacity limited thermal tolerance (OCLTT) concept (for a recent review, see [12]). Acute (short-term) upper thermal and hypoxia tolerance of individual fish (and species) are often described and quantified based on the critical maximum temperature (CT_{max}) or critical thermal limit (T_{crit}), and the critical oxygen tension (P_{crit}), respectively (see **Figure 1** for a schematic representation of the OCLTT concept and for more information about CT_{max}, T_{crit} and P_{crit}).

Typically, CT_{max} and T_{crit}, and P_{crit}, are determined in two separate experiments whereby: 1) water temperature is incrementally increased (*i.e.*, by several ºC per hour) until loss of equilibrium (LOE) occurs; and 2) the water oxygen level is slowly decreased over several hours and the water PO_2 whereby the fish can no longer maintain routine metabolic rate (RMR) is defined as P_{crit}, and water O_2 levels are decreased further until LOE is reached (this is the lethal O_2 level). Throughout such experiments, the fish's mass-specific oxygen consumption (MO_2) is measured to study the influence of temperature or oxygen level on the fish's aerobic metabolism.

The OCLTT concept has been supported with empirical evidence by various studies (for example, [10; 13]). On the other hand, it is also a topic of controversy, as the validity of the concept and its ecological relevance has been questioned, and in some cases seriously challenged with experimental findings (for example, [14-15]). To date, the CT_{max}, T_{crit} and P_{crit} of sablefish have not been determined. These metrics may provide important insights in this species' capacity to tolerate hypoxia and elevated temperatures. Further, data on thermal tolerance of sablefish will add to the OCLTT debate about the relationship between MO_2 and upper thermal tolerance.

The Objective of this Report

This report will focus on the data that was obtained from an experiment which was designed to determine CT_{max} and T_{crit} as measures of upper thermal tolerance in sablefish. The experiment also allowed to describe the relationship between MO_2 and incrementally increased temperatures in this species. This report will describe how I have researched this relationship in closer detail, with the objective to confirm that MO_2 changes (increases) with temperature, as predicted by the OCLTT concept.

I made use of automated intermittent-flow respirometry to measure MO_2 (see Material & Methods). In addition, I performed the experiment concurrently on Atlantic salmon (*Salmo salar*), which allowed for a direct comparison of the upper thermal tolerance and the MO_2 response to increasing temperatures in both species. Atlantic salmon is an important aquaculture species in Canada, and like sablefish, is it also experiencing elevated temperatures and hypoxic conditions at BC cage-sites (Jamie Gaskill, Marine Harvest Canada; Peter McKenzie, Cermaq, personal communication). Moreover, environmental tolerance and the metabolism of Atlantic salmon has already been relatively well studied, which makes this species a suitable positive control for the study of sablefish. Because the MO_2 response to incrementally increased temperatures was investigated in two species using the exact same experimental procedure, another objective of this report is to investigate whether this response is any different between the two species.

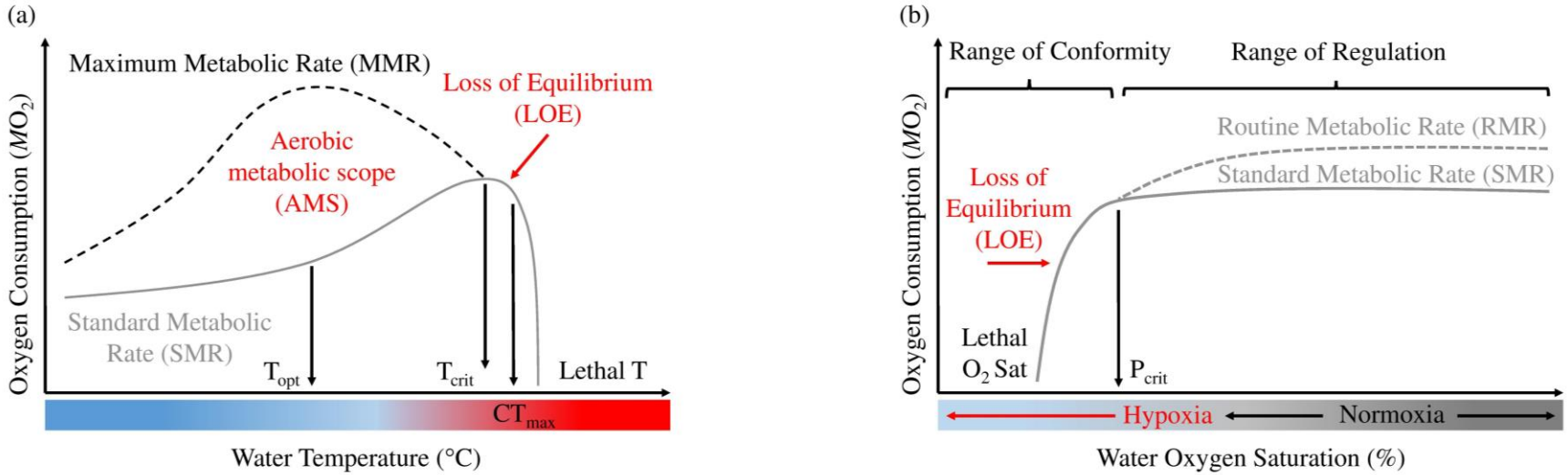

Figure 1. Schematic overview of the oxygen consumption (MO_2) of fish as influenced by (a) temperature or (b) water oxygen (O_2) saturation level, as predicted by the OCLTT concept. **(a)** The MO_2 of a resting, post-prandial fish (*i.e.*, standard metabolic rate, SMR) increases along with temperature, until reaching the critical temperature (T_{crit}). At the optimal temperature (T_{opt}), aerobic metabolic scope (AMS) is at its highest level. At the T_{crit}, maximum metabolic rate (MMR) and SMR are equivalent, and thus, AMS is zero. Beyond this point, survival is time-limited and defined by the ability to utilize anaerobic metabolism and depress metabolism, and eventually the fish loses equilibrium (LOE). The temperature at this point is defined as the fish's critical thermal maximum (CT_{max}). **(b)** The SMR and routine metabolic rate (RMR; which takes into account low levels of random activity) are relatively stable during normoxia and moderate hypoxic conditions. Under severe hypoxia, however, MO_2 rapidly declines. The range of regulation and conformity are when fish MO_2 is independent and dependent of environmental O_2 saturation, respectively. The breakpoint between regulation and conformity is marked by the critical oxygen saturation level (P_{crit}). Below the P_{crit}, water O_2 levels can no longer support tissue O_2 demand, and fish survival time is determined by the capacity for metabolic suppression and anaerobic metabolism. Eventually, the fish loses equilibrium, which is followed shortly thereafter by death. The O_2 saturation level at LOE is normally recorded as the lowest O_2 level that can be tolerated by the fish.

Material & Methods

Animals and Diets

Adult *A. fimbria* of the 2016 year class were transported as fingerlings from the Golden Eagle Sable Fish hatchery on Salt Spring Island (British Columbia, Canada) to the Joe Brown Aquaculture Research Building (JBARB). Adult *S. salar* were obtained from the stock at the JBARB. Both species were held each in a separate tank of 3,000 L (initial stocking density was approximately 0.022 and 0.020 kg L^{-1}, respectively) at 12 °C and on a 12L: 12D photoperiod. Each were fed a commercial diet (Skretting, www.skretting.ca); *A. fimbria* was fed a cod/haddock diet (Europa 15-18 NP, 4-6 mm, 50-55% crude protein, 15-18% crude fat) and *S. salar* was fed a salmonid diet (Optiline microbalance, Summer 500 EP (6 mm), 45% crude protein, 30% crude fat). During the experiment, both species were fed daily at a ratio of 1.00% per body mass. Fish were not fed on the day of sampling for intermittent-flow respirometry. All experimental procedures were approved by the Animal Care Committee (ACC) of the Memorial University of Newfoundland (Protocol #16-92-KG).

Intermittent-Flow Respirometry

The upper thermal tolerance and oxygen consumption response to increasing temperatures of adult *A. fimbria* and *S. salar* were compared experiments of 3 subsequent days using intermittent-flow respirometry. In each experiment, one fish from each species was briefly anaesthetized with 0.1 g L^{-1} tricaine methanesulphonate (TMS) (AquaLife TMS, Syndel Laboratories Ltd, www.syndel.com) and body mass and fin length were recorded. The two fish were placed into cylindrical plexiglass respirometry chambers and allowed to acclimate for 24 h (day 1). The respirometry chambers were submersed in a 274.37 L ambient table with seawater that was replaced with a flow rate of 0.1875 L s^{-1}. Throughout each experiment, fish were kept at 12 °C, at 100-110% air saturation and on a 12L: 12D photoperiod unless mentioned otherwise. The O_2 level was regulated by a controller system (OXY-REG, Loligo Systems, www.loligosystems.com) that monitored the O_2 level with a galvanic cell O_2 probe (model MINI-DO) and made adjustments by either releasing O_2 or N_2 from a reservoir tank using a solenoid valve. Although *A. fimbria* and *S. salar* were both in the adult life stage, they had a significantly different average body mass (679 ± 21 g and 1134 ± 57 g, respectively), therefore, two different sizes of respirometry chambers were used to better match the size of the species. Thus, for *A. fimbria*, a 14.07 L chamber of 17.15 cm in diameter × 60.96 cm long was used, and for *S. salar*, a 19.77 L chamber of 20.32 cm in diameter × 60.96 cm long was used. The position of the respirometry chambers in the ambient table (*i.e.*, in the front of table, or in the back) during each experiment was randomized.

On day 2, the mass-specific O_2 consumption (MO_2) (mg O_2 kg^{-1} h^{-1}) was measured continuously (every 20 min) for 24 h and used to determine SMR as the mean of the 10% lowest metabolic rates measured, and RMR as the mean of all metabolic rates measured (this data is not analysed in this report). On day 3, MO_2 was measured continuously again, while the temperature of the water was gradually increased at a constant rate of 2 °C h^{-1}. The CT_{max} was determined as the temperature whereby the fish shows loss of equilibrium (LOE) and was used as a measure for (acute) upper thermal tolerance (this data is not analysed in this report). This procedure of 72 h was repeated for a total of 10 times (each set of measurements treated as a block in the statistical analysis) and required a total of 30 days to complete. The mean ± S.E. of the *A. fimbria* and *S. salar* used in the experiment were 607 ± 21 g and 1010 ± 71 g, respectively.

The MO_2 was measured with a computer running AutoResp software 2.2.2 (Loligo Systems, www.loligosystems.com) that was interfaced with a fibre-optic oxygen meter (model OXY-4 mini) fitted with pre-calibrated dipping probes (PreSens, www.presens.de), and Loligo DAQ-4 and TEMP-4 modules. The dipping probes were inserted into each of the respirometry chambers. To make MO_2 measurements using intermittent-flow respirometry, the AutoResp software switched between "flushing" and "recirculating" (*i.e.*, making the chamber a closed circuit) submersible pumps (Eheim GmBh & Co., www.eiheim.com). The MO_2 measurements were taken in cycles consisting of three components: (1) a flushing period, (2) a wait period whereby the system was recirculating (closed), and (3) a measurement period whereby the system was recirculating and measurements of O_2 levels (mg L^{-1}) in the chambers were made. Because MO_2 varied greatly at different temperatures and O_2 saturation levels, it was required to adjust the length of each component of the cycle throughout the experiments to achieve a $R^2 > 0.90$ for each measurement. The AutoResp software automatically calculated the MO_2 of the fish at each time point using the slope of the relationship between time and the O_2 level, and taking into account the volume that the fish occupied in the respirometry chamber (*i.e.* assuming 1 g = 1 mL of seawater). Only MO_2 measurements with a $R^2 > 0.90$ were selected for further analysis. Chambers were cleaned after every 3-day experiment to minimize background respiration. Background respiration was quantified overnight using chambers without animals, and was considered as negligible as O_2 saturation did not change more than 1% during a measurement cycle.

During each experiment, one fish from each species was tested in parallel, however *A. fimbria* and *S. salar* had a different CT_{max} and P_{crit}. To avoid unnecessary suffering, the first fish that showed LOE was euthanized immediately by injecting 0.2 g L^{-1} TMS into the chamber. The flush pump of the chamber was unplugged to ensure that TMS could not spill out of the chamber and enter the other chamber. Injection of TMS into the chamber allowed to leave the remaining fish undisturbed. When the remaining fish showed LOE, it was taken out from the chamber and euthanized in 0.2 g L^{-1} TMS. To remove TMS from the other chamber, it was flushed for 15 min before being reconnected to the rest of the system.

Results

For my data analysis, I followed the generic, 10 step recipe for the general linear model (GLM).

1. Construct model.

First, I placed the data in a graph (**Figure 2**) to help with visualization. The oxygen consumption seems to increase linearly along with temperature.

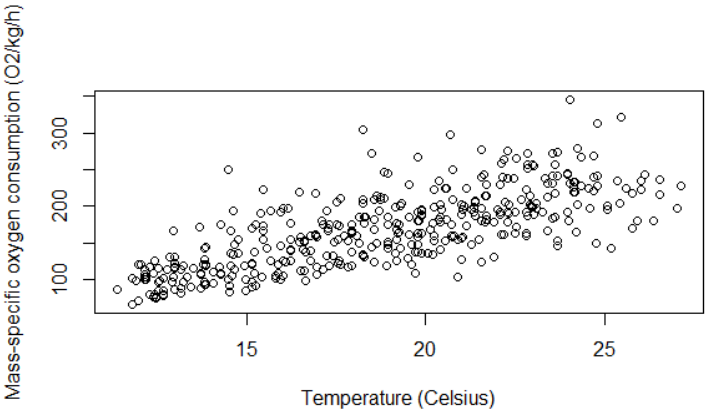

Figure 2. Mass-specific oxygen consumption (mg O_2/kg/h) at increasing temperatures (°C) in two species of fish, sablefish and Atlantic salmon.

Then, I defined and characterized the response and explanatory variables.

Response variable

- Mass-specific oxygen consumption
- Symbol = MOC
- Unit = mg O_2/kg/h
- Scale = ratio

Explanatory variable 1

- Species
- Symbol = Sp
- Scale = nominal
- Levels = 2, 1 for sablefish and 1 for Atlantic salmon
- Fixed variable
- Design: crossed, there are MOC measurements for each species at each temperature

Table 1 was used to confirm the crossed design as there are measurements (X) in all cells.

Table 1. Crossed design between species and temperature.

	Species	
Temperature	Sablefish	Atlantic salmon
12	X	X
13	X	X
...	X	X
27	X	X

Explanatory variable 2

- Temperature
- Symbol = T
- Unit = °C
- Scale = ratio
- Fixed variable
- Design = crossed, there are MOC measurements at each temperature in each fish

Table 2 was used to confirm the crossed design as there are measurements (X) in all cells (with a few exceptions; at high temperatures some values are missing).

Table 2. Crossed design between temperature and fish.

	Fish			
Temperature	1	2	...	20
12	X	X	X	X
13	X	X	X	X
...	X	X	X	X
27	X	X	X	X

Explanatory variable 3

- Fish
- Symbol = F
- Scale = nominal
- Levels = 20, as 20 individual fish were tested (10 of each species)
- Random variable, because the variation from this variable is beyond control of the experimenter
- Design = nested, as the MOC of each fish could not be measured for each species (after all, 1 fish cannot be 2 species at the same time). In other words, fish is a variable *nested within* the variable species.

Table 3 was used to confirm the nested design as there are measurements (X) missing in the cells.

Table 3. Nested design between fish and species.

	Fish							
Species	1	2	...	10	11	12	...	20
Sablefish	X	X	X	X				
Atlantic salmon					X	X	X	X

Because there are both fixed as random variables, this will be a *mixed* model (**Table 4**, **Table 5**). Because there is also a nested variable, the amount of terms in the model will be reduced, as there are less interactions that can be tested for:

- I cannot test for the interaction between fish and species, because fish is nested within species;
- I cannot test for the interactions between fish and temperature (F*T), and fish, species and temperature (F*Sp*T); instead, I can only test for the interaction between species and temperature, with fish nested within species (F(Sp*T)).

See **Table 4** for a hypothetical overview of the terms in the model (7 in total), shown as the "sources" in an ANOVA table, if all variables were in a *crossed* design. In **Table 5**, an overview of the actual, reduced amount of terms in the model is provided (5 in total), taking into account that there are variables in a *nested* design. The reduced amount of terms given in **Table 5** is what I will use to write the model.

Table 4. Overview of model terms and type of variables in an ANOVA table format, assuming all variables are crossed.

Source	*Type of variable*
Sp	Fixed
T	Fixed
F	Random
Sp*T	Fixed*fixed = fixed
Sp*F	Fixed*random = mixed
T*F	Fixed*random = mixed
Sp*T*F	Fixed*fixed*random = mixed
Residual	
Total	

Table 5. Overview of reduced amount of model terms and type of variables in an ANOVA table format, taking into account nested variables.

Source	*Type of variable*
Sp	Fixed
T	Fixed
F(Sp)	Random
Sp*T	Fixed*fixed = fixed
F(Sp*T)	Random*fixed = mixed
Residual	
Total	

Now, I can verbally formulate and formally write the model:

Verbal: the mass-specific oxygen consumption of fish changes with temperature, depending on the species, controlled for the random variation between fish.

Formal:
$$MOC = \beta_0 + \beta_{Sp} \cdot Sp + \beta_T \cdot T + \beta_{F(Sp)} \cdot F(Sp) + \beta_{Sp \cdot T} \cdot Sp \cdot T + \beta_{F(Sp \cdot T)} \cdot F(Sp \cdot T) + \varepsilon$$

With β_0 as the intercept and ε as the residuals (normal error), β_{Sp} as the contrast between species and β_T as the regression slope that depends on the temperature. The difference in the response to temperature between species is $\beta_{Sp \cdot T}$. The random effects that need to be controlled for are $\beta_{F(Sp)}$ and $\beta_{F(Sp \cdot T)}$.

2. Execute model.

I placed my data in model format (**Table 6**). I prepared 1 column for the response variable MOC, followed by 3 columns of explanatory variables (Sp, T, F). The species were numbered

as 1-2 with 1 for sablefish and 2 for Atlantic salmon. The temperature ranged from the lowest temperature of ~12 °C to the highest temperature of ~27 °C. The fish were numbered as 1-10 for each species.

Table 6. Sample of data placed in model format.

MOC	*Species*	*T*	*Fish*
104.5	1	12.15	1
143.7	1	13.83	1
148.4	1	14.67	1
...	...	...	...

I coded my general linear mixed model (GLMM) in R using the following command:

```
GLMM <- lm(MOC ~ factor(Species) + T + factor(Species)/factor(Fish)
     + factor(Species) * T + (factor(Species) * T)/factor(Fish),
     data = MOC_data)
```

Subsequently, I computed the residuals and fitted values to use for the next step, the evaluation of the model.

3. Evaluate model.

Is a straight line appropriate?

Testing for this assumption is relevant, because there is a regression component in the model (the effect of T on MOC). I will use the residuals vs. fit plot (**Figure 3**), which shows no bow or arch shape, so there is no indication that the straight line assumption is inappropriate.

Because I am evaluating a general linear model (GLM), I need to test for the assumptions of homogeneity, normality and independence of the residuals.

Are residuals homogeneous?

I am using the residual vs. fit plot (**Figure 3**) again to test for this assumption. The plot shows a considerable fan-shape, as the residuals increase and become more variable when fitted values increase (*i.e.*, there is a wider band of residuals on the right side of the graph than on the left side). So, the residuals seem to be heterogeneous.

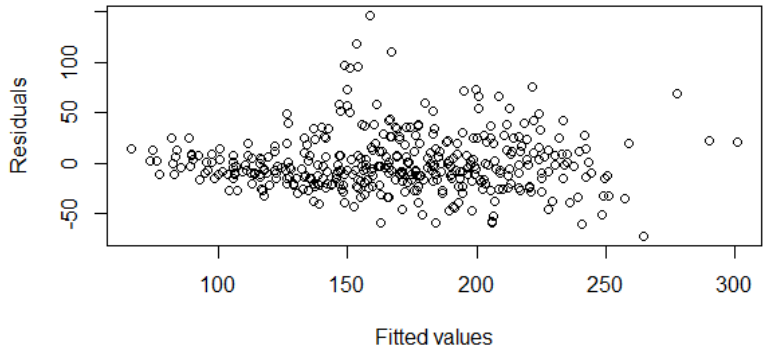

Figure 3. Residual vs. fit plot for the MOC data analysed with a GLMM.

Are residuals normal?

The assumption of normality is tested with a residuals frequency histogram (**Figure 4**) and a normal probability (Q-Q) plot (**Figure 5**). The histogram has a bell shape with a slightly longer right tail compared to the left tail, but apart from this tail asymmetry, it shows no major deviations from normality. The Q-Q plot shows a generally straight, diagonally rising line, with the line being very slightly less steep in the middle (around zero). Overall, there is no evidence that the residuals strongly deviate from normality.

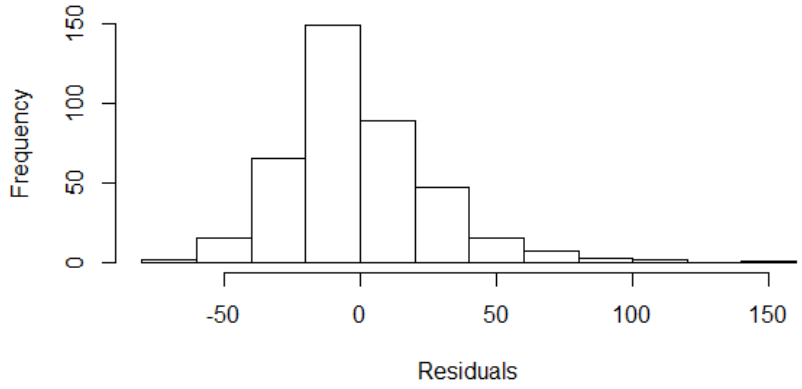

Figure 4. Residual frequency histogram for the MOC data analysed with a GLMM.

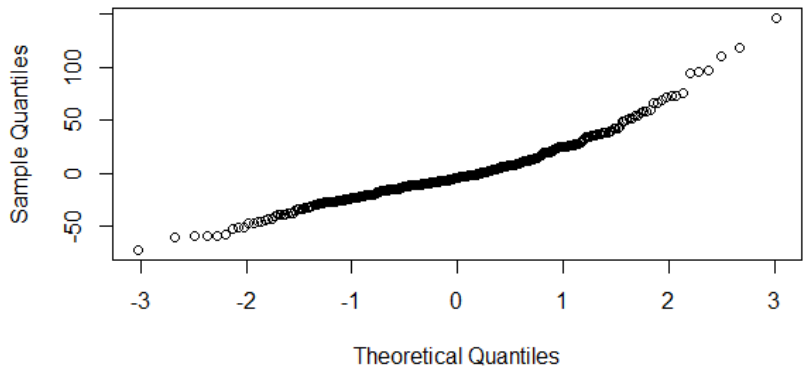

Figure 5. Normal probability (Q-Q) plot for the residuals of the MOC data analysed by a GLMM.

Are residuals independent?

The assumption of independence is tested with a lag plot, whereby residuals are plotted against their neighbouring value (**Figure 6**). The residuals do not show any obvious pattern. There are a few residuals that are flaring out into the right upper corner of the plot, however this does not result in any clear pattern.

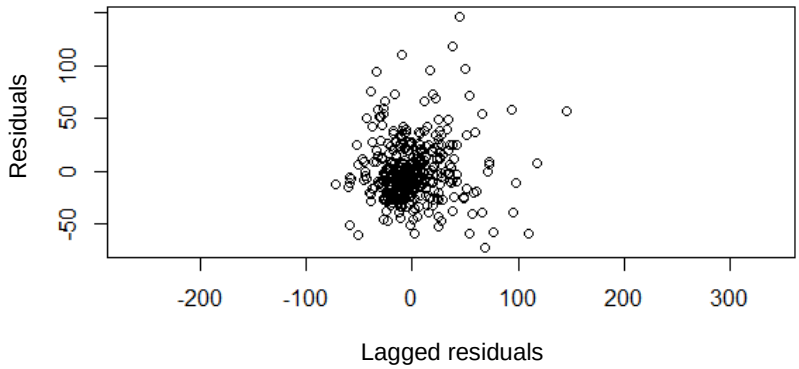

Figure 6. Lag plot for residuals of the MOC data analysed by a GLMM.

4. State population.

The fish (10 sablefish and 10 Atlantic salmon) used in this experiment were randomly sampled from two tanks (one tank for each species) which contained 80 fish each. The 80 sablefish were transported to the aquaculture facility at the Ocean Sciences Centre a year before as fingerlings (small juveniles, year class 2016) from one hatchery in Salt Spring Island (British Columbia, Canada) and since then kept under the same conditions. The 80 Atlantic salmon originated from the stock kept at the aquaculture facility of the Ocean Sciences Centre (year class 2016) and have also been kept under the same conditions. I think that it is reasonable to say that the random sample of fish used in the experiment, is representative for the population of the same species of the same year class at the aquaculture facility at the Ocean Sciences Centre. I will *not* infer to any broader population than this, like the overall population of farmed sablefish and Atlantic salmon in Canada, because fish in a broader population will likely have differences in genetic background and rearing conditions.

5. Decide on mode of inference. Is hypothesis testing appropriate?

Hypothesis testing in appropriate in this data situation, because I don't have any previous knowledge about whether the MOC response to T will be any different between species.

6. State H_0/H_A pair and tolerance for Type I error.

I am only formulating hypotheses for the fixed terms (Sp, T and Sp*T); I am not testing for the effects of the random and mixed terms (instead, I just want to control for their effects).

I will start with formulating a hypothesis for the interaction term.

H_0: $var(\beta_{Sp \cdot T}) = 0$ or $\beta_{Sp \cdot T} = 0$
H_A: $var(\beta_{Sp \cdot T}) > 0$ or $\beta_{Sp \cdot T} \neq 0$

Only when the interaction term is not significant, hypotheses for the other terms (Sp and T) in the model become relevant, because only then I can interpret the effects of each factor regardless of the effects of the other factor.

The hypothesis for the species term:

H_0: $var(\beta_{Sp}) = 0$ or $\beta_{Sp} = 0$

H_A: $var(\beta_{Sp}) > 0$ or $\beta_{Sp} \neq 0$

The hypothesis for the temperature term:

H_0: $var(\beta_T) = 0$ or $\beta_T = 0$
H_A: $var(\beta_T) > 0$ or $\beta_T \neq 0$

The test statistic is the F-ratio and the distribution of the test statistic is the F-distribution. The tolerance for Type I error (α) is 5%, as this is the conventional criterion for hypothesis testing in biology.

7. ANOVA table.

Obtain SS and df from ANOVA table.

The ANOVA table (Type III, for adjusted SS) that I obtained with R is shown below (**Table 7**). The sums of squares (SS) and degrees of freedom (df) are correct, however, the F-values and p-values cannot be interpreted and will be recalculated later (this will be explained below).

Table 7. ANOVA table (Type III) obtained with R, with F-values still to be recalculated.

Response: MOC				
	Sum Sq	Df	F value	Pr(>F)
(Intercept)	23	1	0.0255	0.873148
factor(Species)	364	1	0.4111	0.521823
T	20180	1	22.8142	2.62E-06
factor(Species):factor(Fish)	39691	18	2.4929	0.000752
factor(Species):T	10	1	0.0111	0.916174
factor(Species):T:factor(Fish)	45296	18	2.8449	0.000111
Residuals	314014	355		

Check for correct partitioning of df.

But first, this is how I checked whether the partitioning of the degrees of freedom (df) in the ANOVA table is correct:

- The total df is the total sample size (n), minus 1 — $df_{Total} = n - 1 = 396 - 1 = 395$
- There are 2 species, so 2-1 = 1 df — $df_{Sp} = 1$
- Temperature is on a ratio scale, so 1 df — $df_T = 1$
- There are 10 fish nested within each species, and there are 2 species — $df_{F(Sp)} = (10 - 1)*2 = 18$
- The df for the interactions is calculated as the product of the df of each term — $df_{Sp*T} = 1*1 = 1$; $df_{F(Sp*T)} = 18 * 1 = 18$
- The df for the residuals are what is left — $df_{Res} = 395 - 1 - 1 - 18 - 1 - 18 = 355$

Determine correct denominator for F-ratio using EMS.

In R, the F-ratio of a term is automatically calculated by dividing the means of squares (MS) of the term (nominator) over the MS of the residuals (denominator). However, in the presence of nested, random terms, the F-ratio sometimes needs to be calculated using a different denominator. I used a table with SS vs. expected means of squares (EMS) to determine which denominator needs to be used (**Table 8**). I followed 3 steps:

1. First, I asked myself for each term in the SS column: where does the variance come from? For each term, I filled in the components of the variation in the corresponding row. I wrote each component in such a way, that the term was always written last.
2. Then, I used the rule *Random(fixed)* to eliminate options in each row that did not have this format (eliminations are indicated with strikethrough). This means that a random variable needed to be nested within a fixed variable, and not, for example, the other way around. For instance, I eliminated the component T(F(Sp)) because it is fixed(random(fixed)), and not random(fixed).
3. Finally, I used the rule *F-ratio* = 1 to identify the denominator (indicated with shading) from the remaining options. This rule can be best explained using an example:

F-ratio $Sp = \frac{MS\ Sp + MS\ F(Sp) + MS\ \varepsilon}{MS\ F(Sp) + MS\ \varepsilon}$

This F-ratio equation is written in such a way, that if MS Sp = 0, then F = 1. The MS F(Sp) and MS ε are both in the nominator and denominator, so cancel each other out. In other words, we are isolating the MS of the term of interest, by having the nominator be equal to the denominator. If there is a denominator MS component other than MS ε, then we are selecting this component as the denominator for recalculating the F-ratio.

Table 8. Sums of squares (SS) vs. expected means of squares (EMS) to determine the denominator for the F-ratio.

	Expected means of squares (EMS)					
Sums of squares (SS)	Sp	T	F(Sp)	Sp*T	F(Sp*T)	ε
Sp	Sp		F(Sp)	~~T(Sp)~~	~~F(T(Sp))~~	ε
T		T		~~Sp(T)~~	~~F(Sp(T))~~	ε
F(Sp)			F(Sp)		~~T(F(Sp))~~	ε
Sp*T				Sp*T	F(Sp*T)	ε
F(Sp*T)					F(Sp*T)	ε
ε						

Based on **Table 8**, this is how the F-ratio will be recalculated for the fixed terms:

F-ratio $Sp = \frac{MS\ Sp}{MS\ F(Sp)}$

F-ratio $T = \frac{MS\ T}{MS\ \varepsilon}$

F-ratio $Sp * T = \frac{MS\ Sp*T}{MS\ F(Sp*T)}$

Because I am not testing for the random effects, I will not be recalculating the F-ratio for the random terms.

Recalculation of F-ratio and obtain p-value.

The recalculated F-ratios are shown in **Table 9**. The MS are calculated as the SS divided by the df. The p-values were obtained in Excel using the FDIST(F-ratio, df nominator, df denominator) function.

Table 9. ANOVA table (Type III) with recalculated F-ratios and corresponding p-values.

Source	Df	SS	MS	F-ratio	p-value
Species	1	364	364	0.1651	0.689316
T	1	20180	20180	22.8140	0.000003
Fish(Species)	18	39691	2205		
Species*T	1	10	10	0.0040	0.950431
Fish(Species*T)	18	45296	2516		
Residuals	355	314014	885		

8. Decide whether to recomputed p-value through randomization.

The residuals did not strongly deviate from normality and were independent; however, there was evidence that they were not homogeneous. Because the residuals do not meet all assumptions for a general linear model, the recomputation of p-values through randomization might be required. On the other hand, none of the p-values in **Table 9** were close to α of 5%; they were either well above or well below it. For example, the p-value for the species term is 0.689316 which is far above 0.05. A p-value recomputed through randomization almost never changes more than a factor 5, which in this case will not affect the decision to reject or accept the null hypothesis. Therefore, I will continue to declare a decision based on the p-values of **Table 9**.

9. Declare decision about terms.

First, I will look at the interaction term:

F = 0.0040 df = 18, 18 p = 0.950431 α = 0.05

There is no significant interaction term (0.950431 > 0.05), so I will accept the H_0 that the effect of the T on the MOC does not depend on the species ($var(\beta_{Sp \cdot T}) = 0$). This means that the individual effect of the T and the species can now be interpreted.

The effect of the species:
F = 0.1651 df = 1, 18 p = 0.689316 α = 0.05

There is also no significant species effect (0.689316 > 0.05), so I will accept H_0 that the MOC does not depend on the species ($var(\beta_{Sp}) = 0$).

Finally, the effect of the temperature:
F = 22.8140 df = 1, 355 p = 0.000003 α = 0.05

There is a significant temperature effect (0.000003 > 0.05), therefore, I will accept the H_A that the MOC depends on the temperature ($var(\beta_T) > 0$).

10. Report and interpret effect sizes of biological interest.

Only the fixed terms in this model are of interest. The fish are random factors that vary within the species, and were merely enclosed in the model to account and control for their variation. The analysis confirms that, in accordance with the OCLTT concept, mass-specific oxygen consumption (MOC) changes along with temperature. The mean MOC was not different among the two species. The MOC of sablefish and Atlantic salmon also responded in the same way to the temperature, as there was no interaction effect between species and temperature.

With the `Summary(GLMM)` command in R, I obtained the estimate and standard error (S.E.) for the coefficient of the temperature term (β_T), which was 10.097635 ± 2.114059 mg O_2/kg/h per °C. This coefficient estimate (β_T = 10.1 ± 2.1) can be used to interpret the effect size of the temperature on the MOC. For each temperature increment of 1 °C, the MOC increases by approximately 10 mg O_2/kg/h.

The findings of this experiment are representative for the population of sablefish and Atlantic salmon of the same year class (2016) that are kept at the aquaculture facility at the Ocean Sciences Centre.

Discussion

I will summarize my major results here. I constructed a model to analyse the data from an experiment that I completed in May 2017. The experiment's objective was to determine the upper thermal limits of sablefish (*Anoplopoma fimbria*) and to look at the effects of incrementally increased temperatures on the mass-specific oxygen consumption (MOC). The experiment was performed simultaneously on Atlantic salmon to allow for comparisons of the results between the species.

For this grad project, I decided to investigate (1) whether the MOC indeed changes with temperature (T), as predicted by the OCLTT concept (**Figure 1**); (2) whether the average MOC is any different between the two species (Sp); (3) and whether these two factors interact with one another, while *controlling* for any random variation between fish (F). This was captured in the following formal model:

$$MOC = \beta_0 + \beta_{Sp} \cdot Sp + \beta_T \cdot T + \beta_{F(Sp)} \cdot F(Sp) + \beta_{Sp \cdot T} \cdot Sp \cdot T + \beta_{F(Sp \cdot T)} \cdot F(Sp \cdot T) + \varepsilon$$

This is a general linear *mixed* model (GLMM) as it contained both fixed (Sp and T) and random (F) variables. Among the variables, one was *nested*, F(Sp) (**Table 3**), while the other variables were crossed (Sp and T) (**Table 1**, **Table 2**). Due to the presence of a nested variable, the amount of testable interactions was *reduced*, resulting into the model that is presented above (**Table 4**, **Table 5**).

After I placed my data in a model format (**Table 6**), I evaluated the residuals of the model to check whether these meet the assumptions of the general linear model (straight line, homogeneity, normality, and independence). The evaluation revealed that the residuals were heterogeneous (**Figure 3**), however the straight line assumption was met and there were no strong deviations from normality (**Figure 4**, **Figure 5**) and independence (**Figure 6**).

Because hypothesis testing was appropriate in this data situation, the following hypotheses were formulated for the *interaction* term:

H_0: $var(\beta_{Sp \cdot T}) = 0$
H_A: $var(\beta_{Sp \cdot T}) > 0$

Only when the interaction term (Sp*T) is not significant, the effects of the individual factors (Sp and T) can be interpreted. The hypotheses for the individual factors were as follows:

For the species term:
H_0: $var(\beta_{Sp}) = 0$
H_A: $var(\beta_{Sp}) > 0$

For the temperature term:
H_0: $var(\beta_T) = 0$
H_A: $var(\beta_T) > 0$

The test statistic and its distribution were the F-ratio and the F-distribution, the tolerance for Type I error (α) was set at the conventional level of 5%.

When an ANOVA table (Type III for adjusted SS) was obtained in R (**Table 7**), the sums of squares (SS) and degrees of freedom (df) were used to *recalculate* the F-ratio and the corresponding p-values. This was necessary as R by default uses the means of squares (MS) of the residuals as the denominator to compute the F-ratio for each term, however this is not

always the correct denominator in the presence of a nested term. To determine the correct *denominator* of the F-ratio, the Expected Means of Squares (EMS) were used (**Table 8**). Below is summarized how the F-ratio of each fixed term was recalculated:

F-ratio $Sp = \frac{MS\ Sp}{MS\ F(Sp)}$
F-ratio $T = \frac{MS\ T}{MS\ \varepsilon}$
F-ratio $Sp * T = \frac{MS\ Sp*T}{MS\ F(Sp*T)}$

Using the recomputed F-ratios, the corresponding p-values could be recalculated (**Table 9**). The F-ratios and p-values were not recalculated for the random variables, as the random terms were merely incorporated in the model so that their random effects could be controlled for. Below is a summary of the recomputed F-ratios and p-values for the fixed variables, starting with the interaction term.

Interaction term: $F_{18, 18} = 0.0040$ $p = 0.950431$
Species term: $F_{1, 18} = 0.1651$ $p = 0.689316$
Temperature term: $F_{1, 355} = 22.8140$ $p = 0.000003$

Based on the recomputed F-ratios and p-values, the following decisions were made. First, there was no significant interaction effect (0.950431 > 0.05), so I accepted the H_0 that the effect of the T on the MOC does not depend on the species. Now the individual terms could be interpreted. There was no significant species effect either (0.689316 > 0.05), so I also accepted the H_0 that the MOC does not depend on the species. The temperature, however, had a significant effect on the MOC (0.000003 > 0.05) and so I accepted the H_A that the MOC depends on the temperature.

This GLMM analysis confirmed that MOC (or MO_2) changes along with temperature, which is in line with the OCLTT concept (**Figure 1**). The coefficient β_T was estimated as 10.1 ± 2.1 mg O_2/kg/h per °C (estimate ± S.E.), which can be used to interpret the effect size of the temperature on the MOC. For each temperature increment of 1 °C, the MOC increased by approximately 10 mg O_2/kg/h.

The GLMM analysis also showed that the MO_2 response to temperature was not statistically different between two species of fish, sablefish and Atlantic salmon. There was no difference between the average MO_2 levels of the two species either. The sablefish and Atlantic salmon used in this experiment were representative for a population of the year class 2016 that is kept at the aquaculture facility of the Ocean Sciences Centre.

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