4th year

Investigation into how geometric morphologies vary through space and time in Icelandic threespine stickleback

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1) Introduction:

Icelandic freshwater habitats, shaped by the volcanic nature of the island (Einarsson 2004) present heterogeneous habitats for freshwater organisms. Due to their high resource availability and habitat heterogeneity, they provide interesting study systems to assess spatial variation of phenotypic divergence across multiple environments. The subject of the study is an investigation into how geometric morphology vary through space and time in Icelandic threespine stickleback (Gasterosteus aculeatus). The main objective of my internship was to evaluate the difference of morphology of threespine stickleback in different habitats (Cladophorales, Warm, Shore, Mined, Pondweed) and the time (June and August 2009, and June 2010) in the Lake Mývatn

In fact, biological diversity is dynamic, in this project I study the interplay among ecological (ECO), evolutionary (EVO) and developmental (DEVO) processes in eco-evolutionary dynamics in the wild. (Hólar University and College 2017). This project allows to give insight to the processes influencing the evolution of biological diversity as the functioning of natural ecosystems.

“Such process understanding is critical also for our ability to protect ecosystem functioning and services - a major challenge facing societies in the anthropocene.” (Hólar University and College 2017)

When the icecaps retreated at the end of the last glacial epoch some 10 000-15 000 years ago, rivers were formed. In fact, like Iceland, northern freshwater systems are young, and have been available for colonization since the end of the last glacial period (Skúlason, Snorrason, and Jónsson 1999). Understanding the functioning of such ecosystems can be useful to solve some problems related to climate change.

One of the most important problem in ecology is understanding species richness patterns. For that, spatial and temporal can be studied to understand phenotypic variation (White et al. 2010). Spatial refer to space and temporal to time in that case spatio-temporal can be saw like a phenotypic variation of different individuals of the same species, across different locations and different time periods. Few attempts have been made to understand the linkages between the spatial and temporal patterns related to richness (White et al. 2010). However, it is an important subject because measuring variation over space and time, could give different indication of how environment changes could influence phenotypic expression within species due to selection. (Calsbeek, Knouft, and Smith 2006; Swain and Foote 1999). For that different studies are set up to understand the variation of genetic (microsatellites) and phenotypic (body size, defensive structures, and feeding morphology) structures in stickleback in the lake Mývatn in Iceland (Millet et al. 2013).

These systems are still in colonization phase; hence they are usually poor and invading species. Due to the conditions of colonization, there is a huge number of different habitats and food resources. Furthermore, the postglacial diversification occurred independently and repeatedly in different group of fish: stickleback, salmonids, smelt, whitefish, and created a divergence resulting in polymorphism (Skúlason, Snorrason, and Jónsson 1999). Polymorphism, in biology, is a discontinuous genetic variation resulting in the occurrence of several forms or types of individuals among the members of a single species. And a discontinuous genetic variation divides the individuals of a population into two or more sharply distinct forms.(The Editors of Encyclopedia Britannica n.d.)

Different morphs are often related to differential use of resources among groups and is especially prevalent in northern freshwater fishes. Resource-based adaptations commonly occur among lakes in a parallel way. Similar traits can be found in unrelated groups in similar ecological surroundings, showing the importance of natural selection in such divergent evolution (Kristjánsson 2005).

Sympatric morphs that have adapted in a parallel way to the pelagic and benthic habitats are often found among lakes (Robinson and Wilson 1994). Thus, resource polymorphism and natural selection in speciation are responsible for creating new species (Kristjánsson 2005). Freshwater threespine stickleback express phenotypic plasticity in body shape when raised in different salinities (Mazzarella et al. 2015) or on different prey (Skúlason et al. 2019)

One of the long-term projects conducted by the Hólar University aims to provide evidence for the Eco-Evo-Devo Model. In this model Eco stands for ecology, Devo for developmental biology and Evo for evolution. Eco-Evo-Devo have different feedbacks (Figure 1):

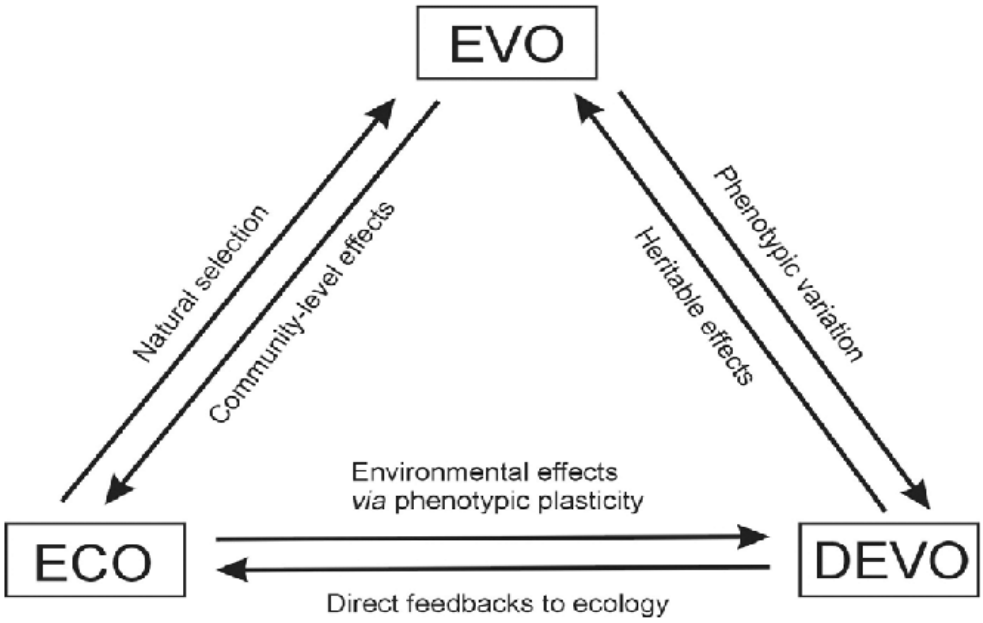

Figure 1: ECO-EVO-DEVO approach (Skúlason et al. 2019)

In ECO-EVO the environment influences the evolution of populations through natural selection and EVO-ECO through communty-level-effects. In ECO-DEVO the environment affects developmental processes of individual organisms. In DEVO-ECO, within-generation developmental responses of individuals influence the responses of populations and thus ecosystems to environmental change. In EVO-DEVO, inherited signals providing by the evolutionary processes across generations influence phenotypic development. And in DEVO-EVO the selection acts on phenotypic variation from development. (Skúlason et al. 2019)

This approach is a powerful conceptual framework understanding the complex integrated processes that ultimately determine the patterns of biological diversity in nature.

2) Presentation of the study

Hólar University:

The internship was conducted in teleworking with different researchers of the university of Hólar and others interns. Hólar University College is located in the Hjaltadalur Valley in the municipality of Skagafjörður in Iceland. This village is about 380 kilometers of the capital of the country (Reykjavik), and the nearest town is Sauðárkrókur. (Figure 1)

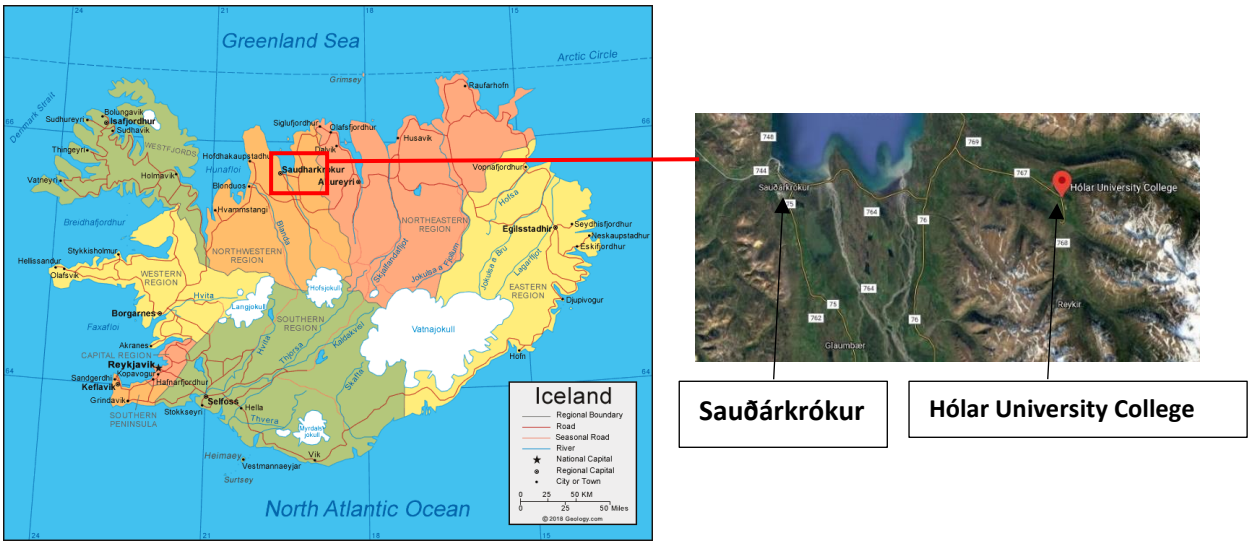

Figure 2: Iceland map (https://geology.com/world/iceland-satellite-image.shtml)

The university of Hólar is divided mainly between three departments: Aquaculture and fish biology, Equine studies and Rural Tourism. I was in relation with the department of Aquaculture and fish biology, different researches are made in this field. “The objective of this department is to gather and disseminate knowledge of aquatic and fish biology and aquaculture. This department is and international centre for research, instruction and continuing education in aquatic biology, fish biology and aquaculture. The department promotes the professional development of aquaculture in the spirit of sustainable development”. (Hólar University and College 2017) It is located in the city of Skagafjörður and has a fish farming facility whose name is Verið. There is a team composed of researchers, doctorates, internships, technicians, administrative employees and international students. There are different studies especially about Arctic Char (*Salvelinus alpinus*) and threespine stickleback. Their research is mainly focused on biological diversity.

3) Material and Methods

Organization:

Due to the COVID-19, all work was done by teleworking and a specific remote organization has been set up:

1) Send a weekly email to update on where I am in my project each Friday
2) A regular weekly Zoom meeting to discuss any problems or questions that I may be facing.
3) A weekly group meeting on Wednesday at 14:30 IS
4) Write a project proposal with bibliography of different article about stickleback.
5) Work on landmarking and after on statistical analysis (R studio, TPS-Dig, TPS-Util)
6) Write an internship report.

Study system:

The project is performed in the lake Mývatn a young only 2300 years old and highly heterogeneous lake. (Figure 4) It measures 37 km^2, and the phenotype of stickleback is studied (body size, defensive, structures and feeding morphology in 5 habitats and two basins (North and South)). Lake Mývatn represents a well suited system to study evolutionary process in natural populations. (Delarue 2017) This young lake promote divergent selection due to strong habitat gradients in temperature, vegetation, invertebrate composition (prey for stickleback) and substrate. (Millet et al. 2013)
The North (N) basin is smaller with 8,5 km^2 than the Sud but deeper (up to 5,5m) and water is warmer (up to 30°C) due to the warm water springs on its East Shore.

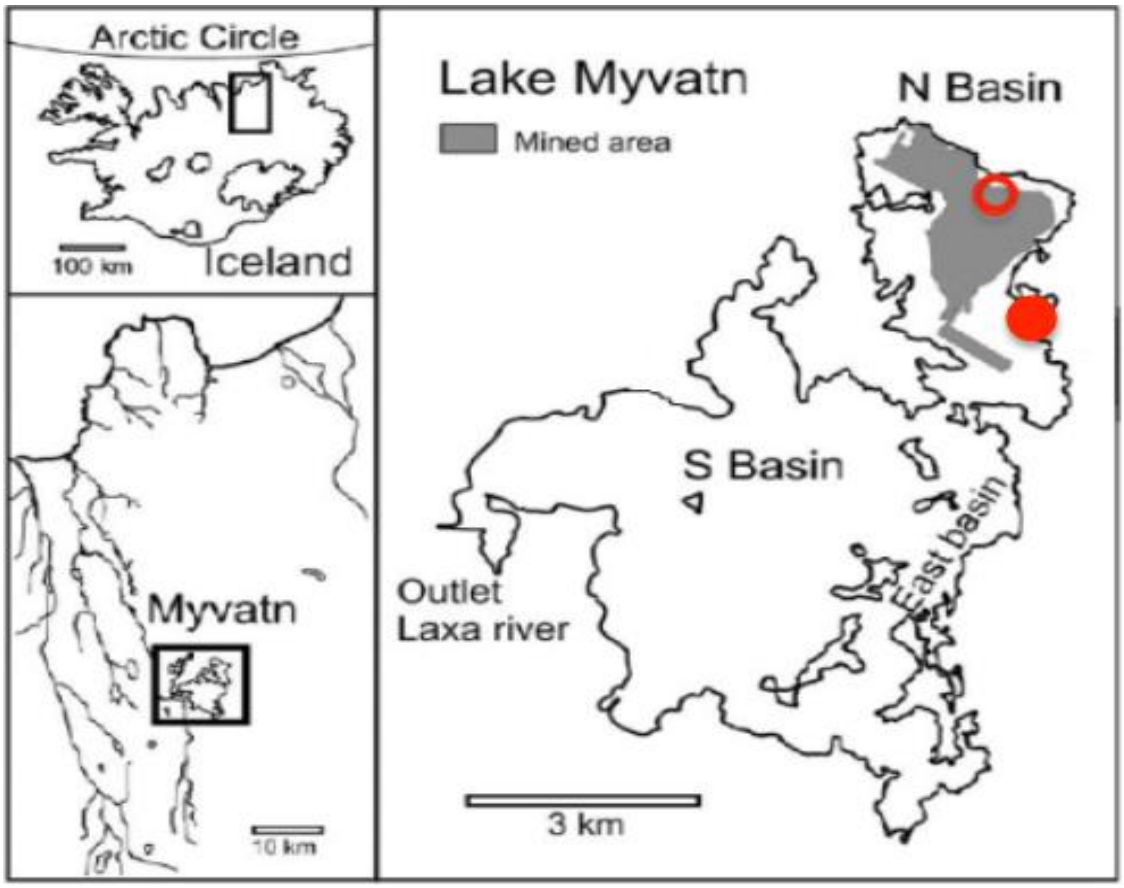

Figure 3: Location of Lake Myvatn and shape (Einarsson 2004)

The South (S) is larger (28,2 km^2) but this area is shallower with a maximum depth of 4m, and it's fed by cold water springs on its East shore.

With these differences we have different biotics characteristics between North and South. (Figure 5) Such as vegetation type, zooplankton, phytoplankton, chironomid midge densities and community composition like bird and stickleback densities. (Millet et al. 2013)

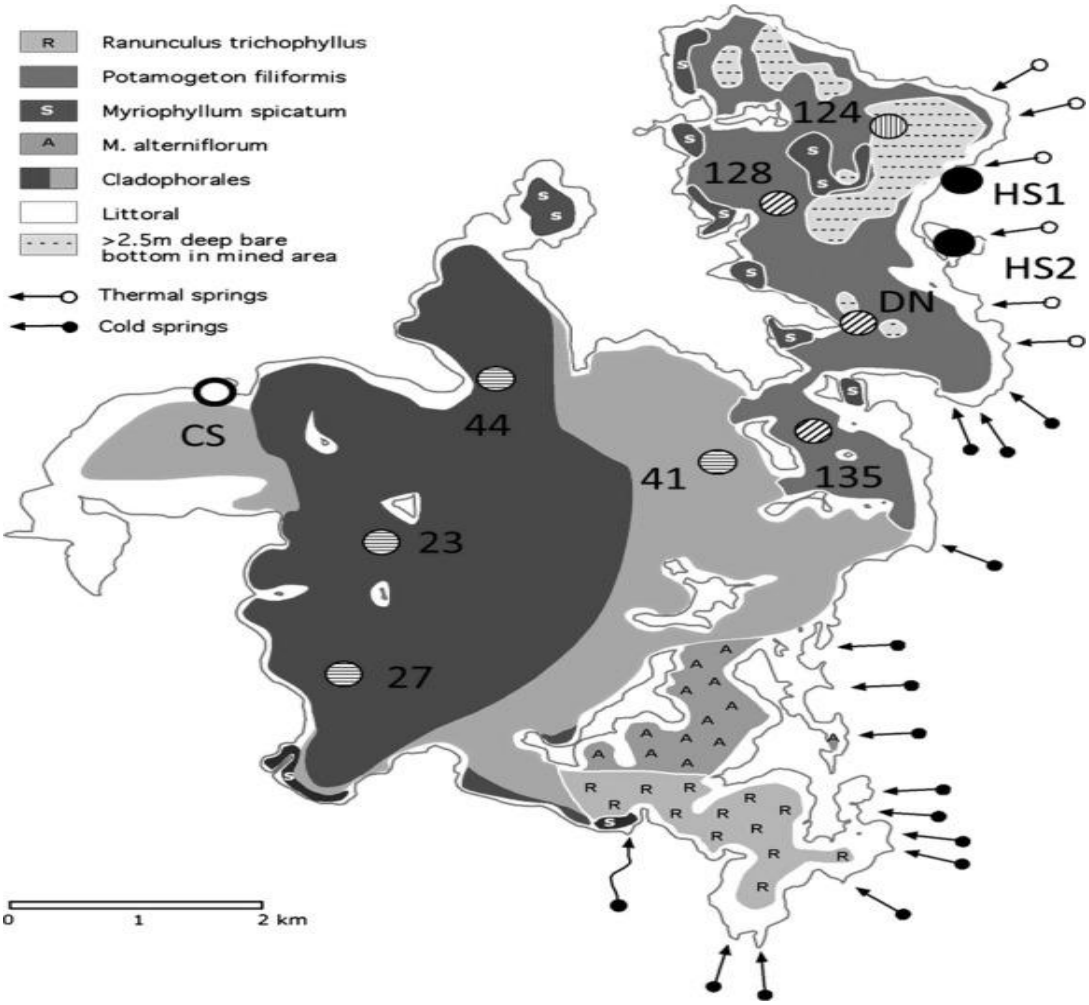

Figure 4 : Lake Myvatn and its 5 habitats (Millet et al. 2013)

Thus, the lake is divided into five different habitats, based on water temperature and vegetation cover, to facilitate predictions about environmental drivers of diversification:

Table 1: Habitats and their characteristics

Habitats	**Location**	**Characteristics**
Warm = HS2, HS1	North basin	characterized with warm water inflow, lacks vegetatuibs, lava rocks, silica mud substrate with spare pondweed. (*Potomogeton filiformis*)
Mined = 124, 128	North basin	The deepest part of the lake without vegetation, and partly anaerobic (mining activites) and with a muddy substrate.
Pondweed = DN (North), 135 (South)	Most of North basin and North end of South basin	it's a shallow area with pondweed (*Potamogeton spp.)*
Cladophorales = 41, 44, 23, 27	South basin	it's a large area with bare mud interrupted by patches of mat-forming Cladophorales algae.
Shore = CS	South basin	1m deep shoreline with sparse rocks and vegetation.

The different habitats are much colder (average range during summer 11-13°C) than warm habitat (20-23°C).

About Threespine Stickleback (Gasterosteus aculeatus):

This is a small fish with a size between 3 and 8 cm. It can live in freshwater, salt and brackish waters. These fish is easily recognizable because of its three dorsal spines (the third is spaced and short from the other two), one anal spine and two pelvic spines.("Threespine Stickleback | UNB" n.d.). Threespine stickleback have bony lateral plates and the number of plates is related of the environment of the fish and generally, saltwater fish having more. (Figure 3)

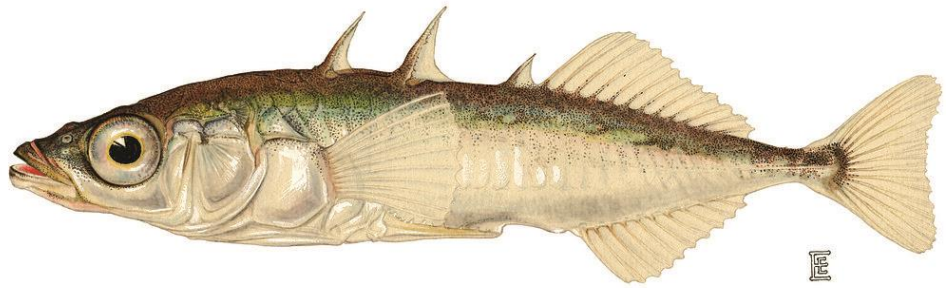

Figure 5 : Threespine stickleback (https://www.flickr.com/photos/nysdec/29865050421)

It is a fish that shows high propensity for colonization and adaptation to different aquatic environments. In Iceland, marine threespine stickleback has colonized freshwater habitats and have more rapid individual growth. Due to low salinity we can see a phenotypic plasticity (Millet et al. 2013), furthermore colonization of freshwater by marine stickleback has given rise to repeated parallel divergence and ecological speciation (Robinson 2013).
This fish feeds on worms, larvae, crustaceans, small fish, eggs, aquatic insects as well as eggs and fry of its own species. (Corolla, Fey, and Kupfer 2019)
During the breeding season (March to July), females are slightly pink on the throat and belly and males have an orange-red belly with a bright blue black and have blue eyes. (Luna 2019)

Fish sampling:

Stickleback were sampled during breeding season in June and all stickleback were frozen and transferred for analyses.
Each year, around the 20th of June and the 20th of August, 2 samplings sessions are organized.
And in these type of habitats, different samples are posed to catch stickleback in the different basins. Each time, a site is sampled twice, once for night catch and one for day catch and five unbait minnow per site were set out overnight for approximately 12 hours/site in an approximate distance of five meters.

When there is too much fish, some of them can be released on site. For each habitat, fish were kept for body size analyses and up to 50 large (> 50mm) stickleback/site were selected for further genetic and phenotypic analysis. The minimum numbers of fish needed is 150 small fish (<45/50mm), 250 big fish (> 45/50 mm)

If the number of sticklebacks is not enough, some stickleback with a size > 35 mm are added to reach 50 individual/site.
After that, fish are transferred in the lab of the University of Hólar. All fish from each trap and station have to be counted, classified and arranged according to the number of individuals desired per station. Fish are put in bags and are labelled (site, year, season and date) and after that frozen.

Different biometric measurements are performed like total body length (with a ruler), weight and sex status (male, female, immature). The length of spine and the number of lateral armor plates on the left side of the fish were also measured with a stereomicroscope. And for a certain amount of fish per trap, the right pectoral fin is cut and put in a tube with 95% ethanol for genetic analyses.

Landmarking method:

This part was the most time-consuming part of my internship.
Landmarks are used to quantify the morphology of threespine stickleback. There are different steps for this method:
After the different measurements in the laboratory, a picture of the left side of each fish is taken with spines and fins in open positions. After download TPS UTILITY and TPS DIG software, all digital photographs are transferred and classified in a file with their Year-Month-Habitat. For each samples a TPS file from images is created thanks to the TPS Utility software. In fact, this TPS file is used after on the software TPS-DIG to place our points (landmarks). Furthermore, the landmarked coordinates on fixed morphological features have been chosen on the basis of earlier research by Kristjánsson. (Figure 6).

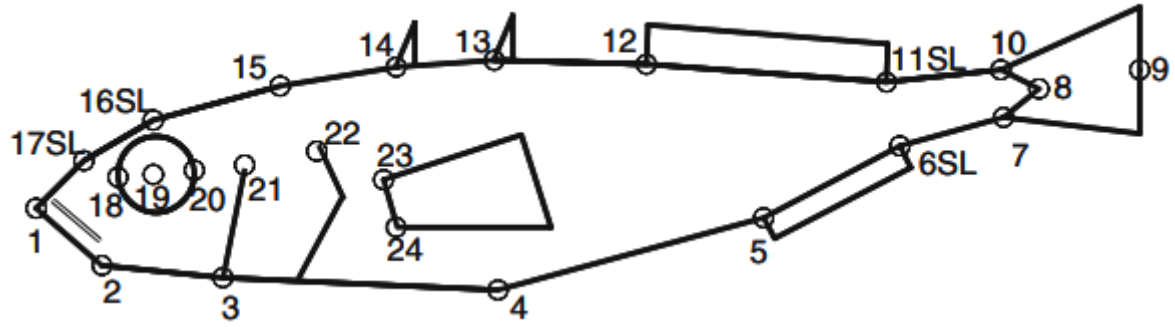

Figure 6 : Landmarking method (Kristjánsson 2005)

Figure 7 : Landmarking method example.

Before starting landmarks, we set scale for each sample. After that, there is a digitalization of 24 landmarks on each fish depending on the model of Kristjánsson. (Figure 7).
I did this method for the year 2019 and 2020 and for the month June and August. In total 2486 samples are digitalized. Figure 7 represents one of the sampling examples. Thus, an average shape of all the fish is computed. And for each sample we have coordinates for Y axis and X axis, in this way we can see if there are changes in morphology between samples. With that, the morphology of fish can be compared with each other.

Data set up

With the collected data from landmark measurements, different statistical analysis is set up to observe if there are differences of morphology between habitats and years. For that, the software R studio is used with a α-value of 0.05 as the margin of significance.

First of all, the data of our different landmarks are reorganised on Excel to be compatible with R studio. It's essential to remember that with landmarking method there is some variation in orientation, position and size in the different samples, thus to remove this errors General Procrustes Superimposition is used. (Rohlf 2015) Which then gives Procrustes Coordinates (PC). Here, PC1 represent x coordinates and PC2 y coordinates of individual landmarks. Variation due to detected allometry was removed by doing a regression of each coordinate against fish size. (Lundsgaard-Hansen et al. 2013). Principal component analysis on residuals was then carried out to produce shape variables (PC axes) to display the main shape variance axes. (Annex 1)
variance axes

Shape variable, time, and habitat are the principal components for geomorphometric variation so this is why this data is used.

PC1 and PC2 are dependent variable and are continuous quantitative variable. They represent the geometric morphology. And Habitat and Date are independent variable and used as factors.
For PC1 and PC2 an ANOVA (Analysis of variance) test will be performed for each variable (habitat and date) and in interaction (Habitat * Date).
Here, ANOVA test is used because different variables are testing. It's a statistical technique used to test if the means of two or more groups are significantly different from each other. It tests the impact of one or more factors by comparing the means of different samples. ("What Is ANOVA (Analysis of Variance) and What It's Used For" n.d.)

Furthermore, a multivariate analysis of variance (MANOVA) were conducted on PC1 and PC2 as a function of Habitat and time. This method uses the variance-covariance between variables to test for the statistical significance of the mean differences.(Delarue 2017)

4) Results

If the result of the ANOVA test is statistically significant (P < 0,05) it represents that the means of the different groups are unequal.("What Is ANOVA (Analysis of Variance) and What It's Used For" n.d.) Before to use ANOVA test, it's necessary to see if our data is normally distributed. A histogram is used to visualize that. To better see the normal distribution, the logarithm is used.

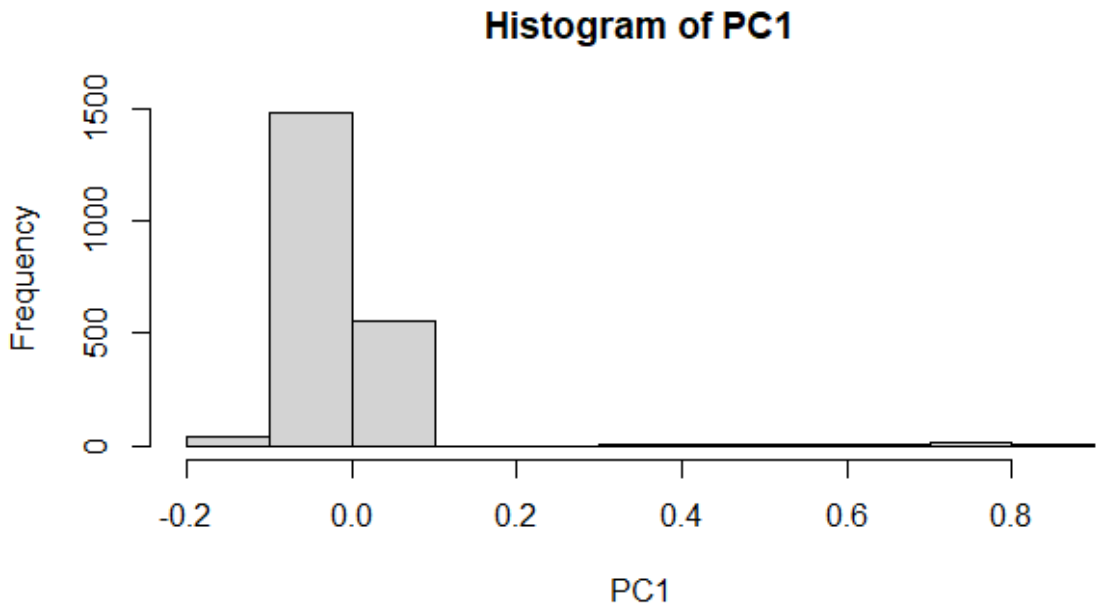

Figure 8 : Normality diagram for PC1

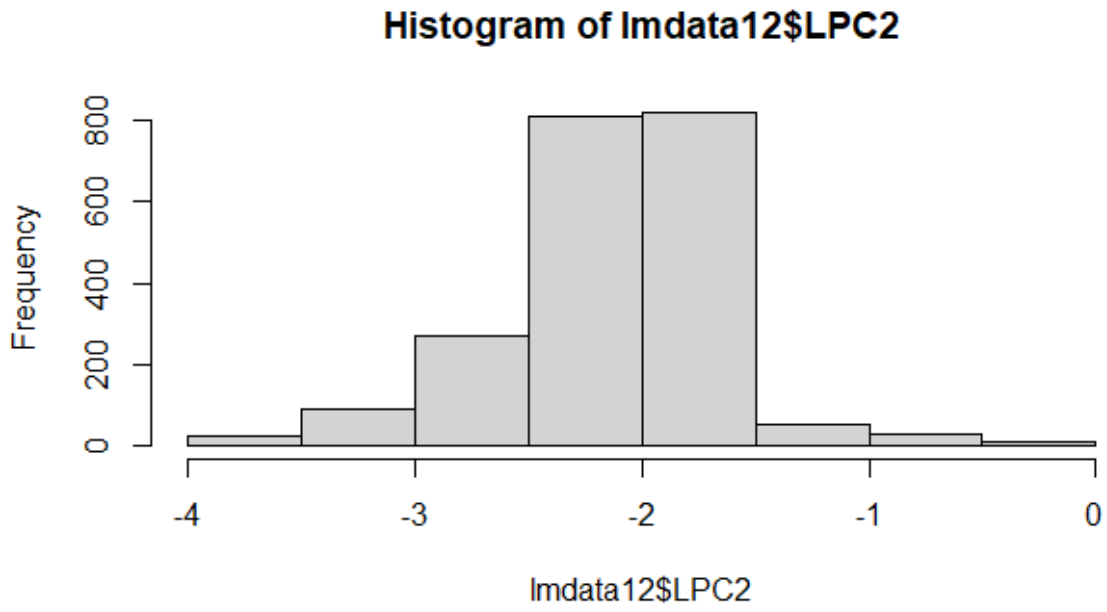

Figure 9 : Normality diagram for PC2

The data is normally distributed, so we can use ANOVA test and setting null hypothesis.

Null hypothesis (hypothesis of equal means = H0) for PC1 in ANOVA test are:

Habitat: H0 = The morphology of individuals doesn't depend on the habitat.
Time (Date): H0 = The morphology of individuals doesn't depend on the time.
Habitat*Date: H0 = The interaction between habitat and time don't have a link on the morphology of individuals.

Null hypotheses for PC2 in ANOVA test are:

Habitat: H0 = The morphology of individuals doesn't depend on the habitat.
Time (Date) : H0 = The morphology of individuals doesn't depend on the time.
Habitat*Date: H0 = The interaction between habitat and time don't have a link on the morphology of individuals.

Null hypotheses for PC1 and PC2 in MANOVA test are the same as precedently.
So we have 9 null hypotheses in total.

After this step I wrote the script for the ANOVA test and manova on R. (Annex 2)
The ANOVA test is used for PC1 and then PC2 and to finish we all variables is combined with MANOVA test (PC1 and PC2). So three tests are performed (2 ANOVA and 1 MANOVA). All results is summarized in the table below (Table 2) :

Table 2 : Results of the p-value with Anova and Manova for PC1 and PC2.

Variables/ Factors	P-value (Anova PC1)	P-value (Anova PC2)	P-value (Manova PC1 and PC2)
Habitat	2^{e-16}	0.04312	2^{e-16}
Time (Date)	2^{e-16}	0.00449	2^{e-16}
Habitat x Time	2^{e-16}	5.42^{e-08}	2^{e-16}

As we can see, all the P-value for every factor (Habitat and Time) is inferior to 0.05, so all results are significantly different we can reject our 9 null hypothesis.

All results of R are put in Annex. (Annex 3)

For PC1 :

We can see our results with a graph for the 5 different habitats with PC1 (Figure 10) :

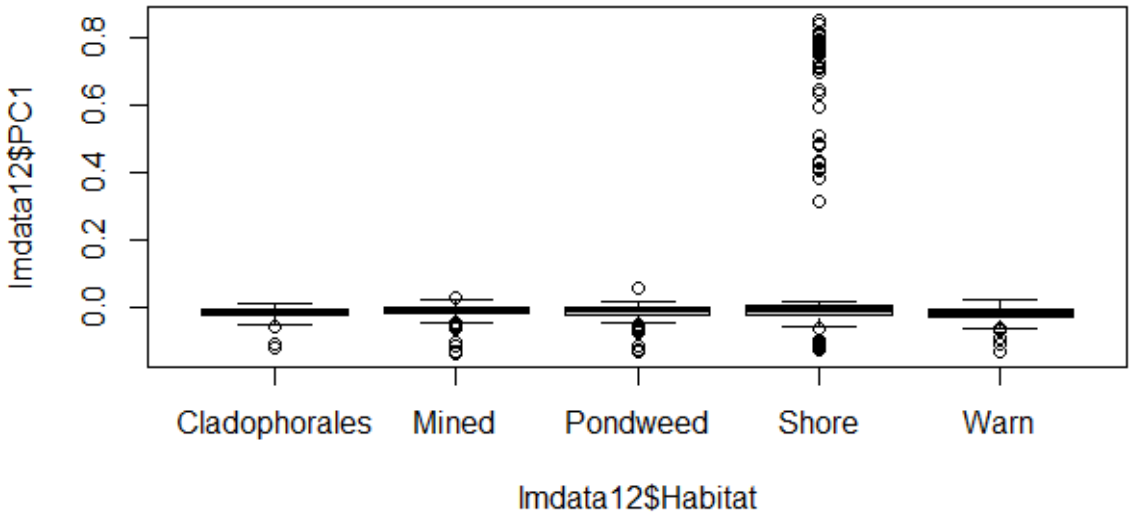

Figure 10 : PC1 as function of different habitats.

And for the time (Figure 11) :

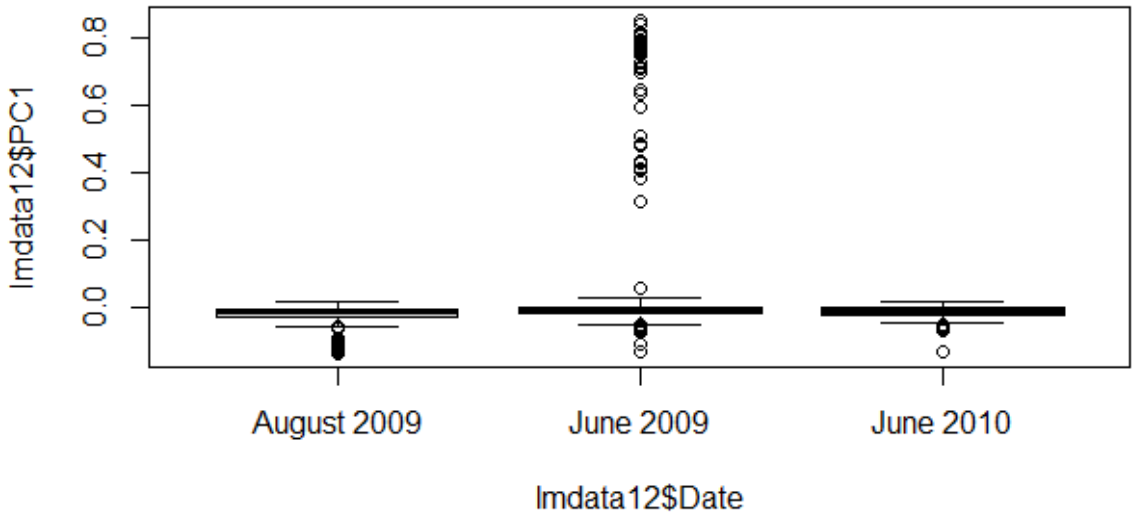

Figure 11 : PC2 as function of the Time (Date)

For PC2 :

Here is the result of PC2 for the 5 habitats and the time (Figure 12 and 13) :

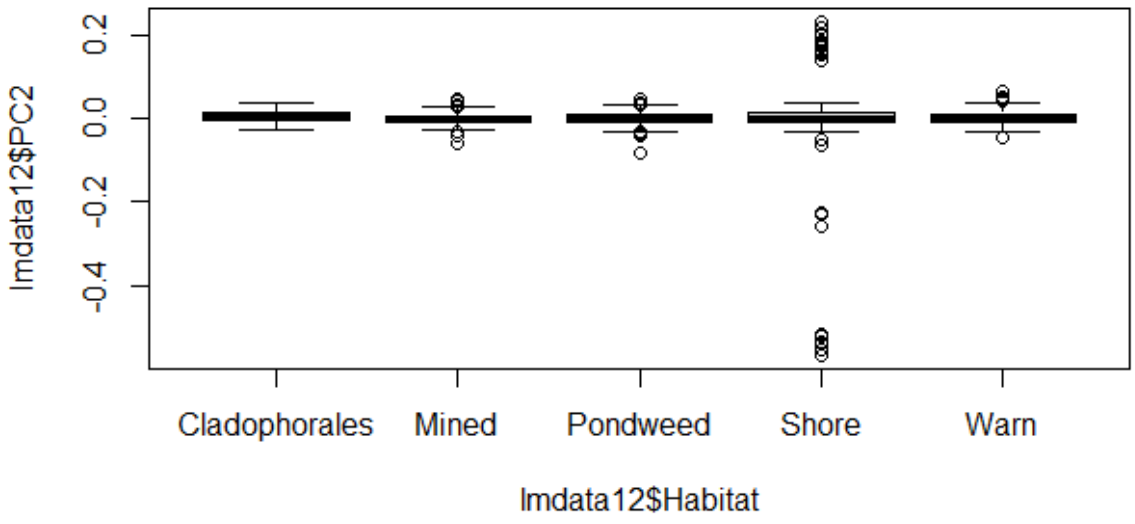

Figure 12 : PC2 as function of habitats.

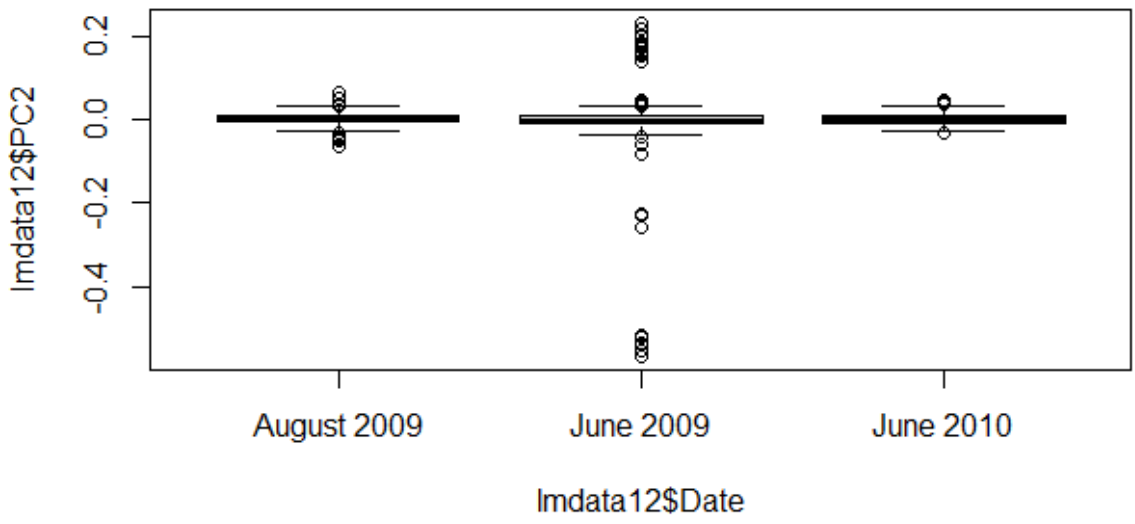

Figure 13 : PC2 as function of the Time (Date)

MANOVA : PC1 and PC2 together:

Multivariate analysis of variance (MANOVAs) were conducted on Habitat, Time and Habitat in interaction with the Time for variables PC1 and PC2 in the same time.
This method uses the variance-covariance between variables to test for the statistical significance of the mean differences.

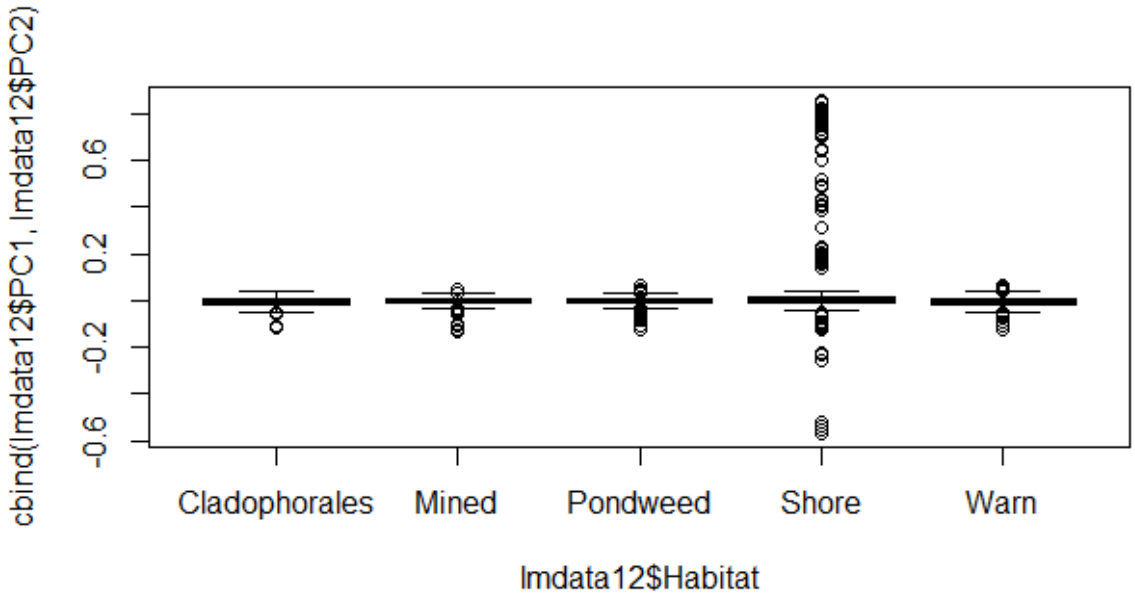

Figure 15 : PC1 and PC2 as function of habitats.

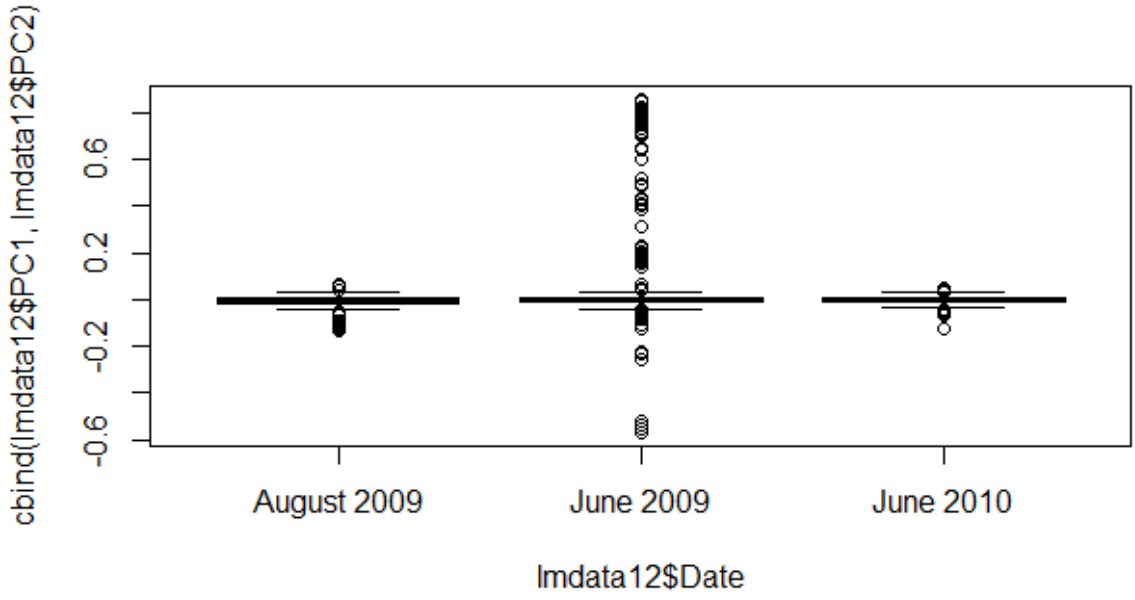

Figure 14 : PC1 and PC2 as function of Time

5) Discussion

H0 is rejected because p-value is inferior to 0.05, so all the results are significantly different for our three tests. So, there are differences in the morphology of stickleback as function of habitats and time.

In the table 2, for example for PC1 and habitat an analysis of the dependence of the quantitative variable PC1 on the habitat factor is made. Here the result is 2e-16 so the interaction is very important, so there is an important difference in the morphology of stickleback between habitats. The same thing for the time can be said.

Results on this graph below (Figure 10 and 12 and 14) shows an important difference in the morphology between habitats. For example, for the "Shore" habitat there are some huge differences with others habitats. So according to the habitat we can have different morphology of fish. We have huge heterogeneity in the morphology for the Shore habitat compared to others habitat that why our p-value is very low for PC1 and PC2.

For the time, we can see an important difference between June 2009 and August and June 2010. So according to the date of the years, there are differences in the morphology. In June 2009, there is the Shore habitat and it has an important heterogeneity with others data, it can explain the difference between June 2009, August 2009 and June 2010. (Figure 11, 13, 15).

In fact, environmental temperature has major effects on growth and metabolism but it's difficult to know how much it can affect fish because threespine stickleback have been found in different temperatures of waters (Guderley, Leroy, and Gagné 2001). A wide range of temperatures is tolerated for Stickleback but the growth is more important in warm water. A graph (Figure 16) is made to see if the lengths of the fish are different according to the habitat can explain our statistical differences. But in this graph, we can't see a huge gape between our different habitat in the length of fish. So, we can think that our statistical differences between habitat can come of a variation of the very special trait in the morphology. For example, some stickleback can have differences in gill raker and head shape morphology, and especially in Shore habitat where gill raker is longer than others habitats.(Delarue 2017).

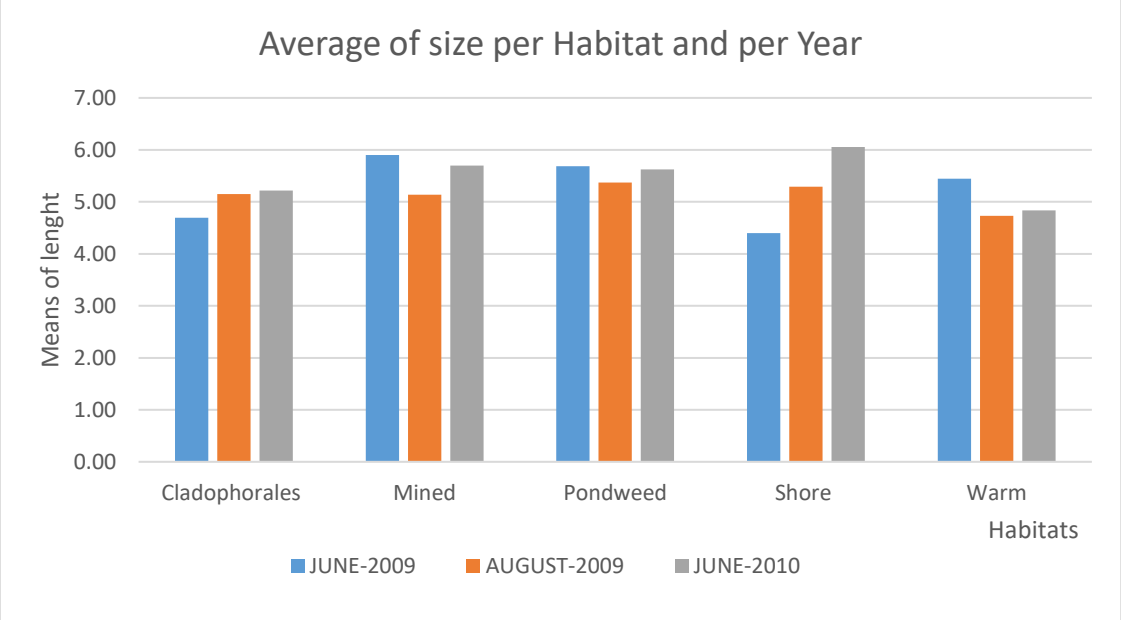

Figure 16 : Means of lenght per Habiat and per Year

Lake Myvatn have a huge heterogeneity in habitats and temperature (Table 1), and likely cause variation in the magnitude of resource competition, which may lead to large size differences of stickleback over space and time. (Delarue 2017). Poor growth conditions (low temperatures, poor diet), caused by important competition or resource limitation can lead to a reduction in body size. In fact in Lake Mývatn, divergence in body size among freshwater populations of thereespine stickleback has been suggested to be mainly caused by their resource environment, while resource abundance or large gape limited predators may favour larger fish (Palkovacs 2003). Furthermore, some habitats like lava in Lake Mývatn can be complex and may select for smaller body size because of narrow spaces and/or differences in food availability, which can lead to competition and an adaptation of stickleback in each part of the Lake.

So all these contrasting selection factors (temperatures, food, competition, predation…) that differ in space and time in this such heterogeneity lake promote plasticity for body size and so differences in morphology over space and time. (Sharpe et al. 2008)

6) Conclusion

Through this study we could see that there are differences in morphology in the different habitats of the lake and in terms of time. Indeed, these results were expected because the stickleback has an important phenotypic plasticity which has already been expressed in several scientific articles (salinity, habitat, temperature, predators ...).
So, through this study, results have shown that there are morphological differences depending on the habitat and the time.

But it's pretty difficult to say exactly which factor is responsible for this difference of morphology due the complexity and numerous factors involved. Moreover, due to the high number of data it's possible that there were errors during the landmarking, which would explain the great difference in values with the Shore habitat.

This internship was very interesting for me because I experienced my first remote intersnship and I have particularly appreciated all the studies on fish. This internship allowed to improve my skills on the R software and to learn many new technics for example the PC (Procrustes Coordinates) and landmarking methods. And it allows me to better learn English thanks to the different interactions with Kasha and Thomas.

Just the critical look is about the remote internship, due to the distance the internship was less interesting because I don't have participate in the study for example to catch some stickleback and to live the real experiences.

My goal after the internship is to always stay in the study of fish and aquatic environments because that's what fascinates me. My future real objective is to study a larger fish, for example trout or salmon, which are fish that I particularly appreciate, and to live this experience with researchers.

Acknowledgement

I would like to thank my supervisor Kasha Strickland and Thomas Weisbeek for their help during my internship. Working on this stickleback project was an excellent opportunity to improve my knowledge on biology and to discover the research world.

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7) Annex :

Annex 1 :

```
##read in your data
lmdata<-read.csv("lmdat.csv")
##reorganise the data to correct format
lmarray<-cbind(lmdata$X.Axis,lmdata$Y.Axis)
lmarray2<-arrayspecs(lmarray,21,2)
##convert coordinates to procrustes coordinates
Y.gpa <- gpagen(lmarray2)
```

Annex 2 :

```
aov(lmdata12$PC1 ~ lmdata12$Habitat*lmdata12$Date)
summary(aov(lmdata12$PC1 ~ lmdata12$Habitat*lmdata12$Date))
#ANOVA TEST FOR PC1
plot(lmdata12$PC1 ~ lmdata12$Habitat*lmdata12$Date)

aov(lmdata12$PC2 ~ lmdata12$Habitat*lmdata12$Date)
summary(aov(lmdata12$PC2 ~ lmdata12$Habitat*lmdata12$Date))
plot(lmdata12$PC2 ~ lmdata12$Habitat*lmdata12$Date)
#ANOVA TEST FOR PC2
manova(cbind(lmdata12$PC1,lmdata12$PC2)~lmdata12$Habitat*lmdata12$Date)
summary(manova(cbind(lmdata12$PC1,lmdata12$PC2)~lmdata12$Habitat*lmdata12$Date))
plot(cbind(lmdata12$PC1,lmdata12$PC2)~lmdata12$Habitat*lmdata12$Date)
#MANOVA TEST.
```

Annex 3 : Results of R :

```
> summary(aov(lmdata12$PC1 ~ lmdata12$Habitat*lmdata12$Date))
                               Df Sum Sq Mean Sq F value Pr(>F)
lmdata12$Habitat                4  2.164  0.5411   430.8 <2e-16 ***
lmdata12$Date                   2  1.153  0.5766   459.1 <2e-16 ***
lmdata12$Habitat:lmdata12$Date  7 12.708  1.8155  1445.5 <2e-16 ***
Residuals                    2083  2.616  0.0013
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
> summary(aov(lmdata12$PC2 ~ lmdata12$Habitat*lmdata12$Date))
                               Df Sum Sq  Mean Sq F value   Pr(>F)
lmdata12$Habitat                4  0.019 0.004870   2.466  0.04312 *
lmdata12$Date                   2  0.021 0.010704   5.421  0.00449 **
lmdata12$Habitat:lmdata12$Date  7  0.094 0.013412   6.792 5.42e-08 ***
Residuals                    2083  4.113 0.001975
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
> summary(manova(cbind(lmdata12$PC1,lmdata12$PC2)~lmdata12$Habitat*lmdata12$Date))
                               Df  Pillai approx F num Df den Df    Pr(>F)
lmdata12$Habitat                4 0.54257   193.87      8   4166 < 2.2e-16 ***
lmdata12$Date                   2 0.39597   257.11      4   4166 < 2.2e-16 ***
lmdata12$Habitat:lmdata12$Date  7 0.87490   231.40     14   4166 < 2.2e-16 ***
Residuals                    2083
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```