Rapport de stage individuel

4ème année

Impact of treated and untreated insect dietary proteins (*Hermetia illucens*) on the behaviour of farmed juvenile Atlantic Salmon (*Salmo salar*)

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List of the Figures

List of the Tables

Summary

Introduction

Global population is expanding, leading to an increased demand for protein. Indeed, the world population reached 3 018,3 million in 1960, and 7349,5 million in 2015 (INSEE, 2018). This represents an average annual growth of the population by 1,6% and this trend is predicted to continue. The scientific literature estimates there will be 9 300 million people by 2050 (Simpson, 2011). There is a need to find a way to meet future food security needs. Moreover, there is a changing mentality and new food production methods need to respect the rules of sustainable development. Indeed, there is collective awareness in institutions like the European Commission, which highlight these changing attitudes by specifically defining sustainability: *"the use of resources at rates which do not exceed the capacity of the Earth to replace them"* (European Commission 2016). In this context, the new method developed for food production must be environmentally and socially sustainable to meet future food security needs.

In answer to this growing demand for protein, the aquaculture production is increasing faster than all other animal food-production sectors (Gatlin et al, 2007). However, farmed fish, require feeding, and the main sources of dietary protein for aquafeed in aquaculture are traditionally marine fish (Fry et al, 2016) or plants like soybean (Gatlin et al, 2007), and the use of this both diet cause too many environmental problem and don't respect the rule of the sustainable development (Hardy, 2010). The main problem of this aquaculture sector is now to find a sustainable protein source for fish diets, this is particularly challenging and urgent for Atlantic salmon which as carnivores requires high protein diets and are popular food fish in Europe (Gatlin et al, 2007). Sustainable fish farming has the potential to be a solution to meet growing food production demands (Sans and Combris, 2015). It may also be a good alternative to the meat consumption as well as reduce the pressure on wild fish stock (Sans and Combris, 2015). For all of these reasons, scientific publications focused on sustainable aquaculture and alternative proteins have increased dramatically in number.

In 2017, a research project was carried out by the Matís ohf aquaculture department entitled: *´Sustainable Novel Feed Ingredients in Aquaculture during a Crucial Growth Period´* (Leeper et al, In preparation). This study investigated the application of insect proteins in the diet of juvenile Atlantic salmon (*Salmo salar*) as a novel sustainable protein source. The final objective was to see if it was feasible to create a sustainable salmon feed where insect protein partially replaced traditional proteins in a formulated diet. Insect protein from black soldier fly larvae (*Hermetia illucens*) was selected for this study since it has an appropriate amino acid profile for salmonis. The use of this insect as a protein source adheres to the definition of sustainable development, because it's possible to rear these insects on a wide range of organic waste material, produce them in controlled environment at high density and with low water input. For this reason, it's possible to integrate this insect in a sustainable fish farm, by reducing, reusing and recycling, to limit the production of waste (Sheposh and Richard, 2017), however this research also showed that insect protein needed optimising.

This report will present one part of a much larger research project and it will investigate the impact on the treated and untreated black soldier fly larvae protein on key aspects of behaviour of juvenile Atlantic salmon compared with traditional proteins. Firstly the host company and the background of this study will be introduced. Followed by a detailed investigation of behaviours indicating fish boldness under different protein feed treatments. The report will conclude with a detailed discussion of findings and their importance for aquaculture research.

1. Presentation of the host company and the study subject

1.1 Matis

Matís ohf. Food and Biotechnology is a non-profit independent, partially government funded research company, created in 2007 and based in Iceland. It's specialises in food research and development (Matis). The company was created through the fusion of three former public research institutes and it is now divided into four divisions: Implementation and Impact, Research and Innovation, Analysis and Infrastructure and Operations (Annex 1).

The activities of the company are mainly focused on food science and safety. For example, food production is explored from a wide range of angles like production methods, processing, safety, and packaging. Matís expertise includes microbiology, biochemistry and aquaculture. The company it's also frequently engages in partnerships with universities, foreign research institutes, along with private and public companies.

In recent years Matís has increased research effort in the field of sustainable aquaculture, having identified fish farm production as a future priority in Europe. Aquaculture background recreated in Reykjavík, a work on the salmon alimentation is currently realised. This project is a one year project focused on zero waste and called Metamorphosis. It's in the continuation a previous Nordic Innovation project called Proffaqua, based also on the juvenile salmon and the use of insect protein of Black Soldier fly (*Hermetia illucens).*

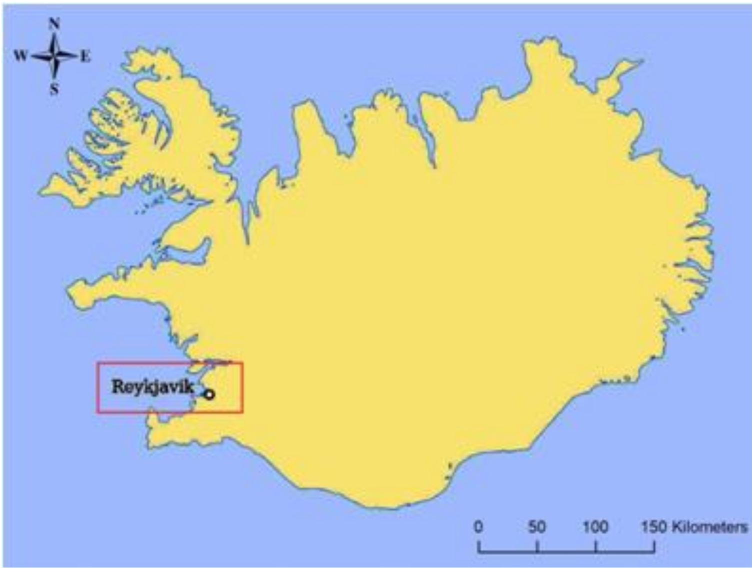

Figure 1 : Localisation map of Reykjavik (Personal realization)

1.2 The current state of fish farming

Aquaculture production has been identified as a possible solution for future sustainable food production (Gilbert et al, 1974). Indeed, with good practice, fish farming promises to be a good alternative to meat consumption, which is increasingly important (Sans and Combris, 2015), and, they can reduce pressure on wild fishery. However, farmed fish are still associated with several environmental problems that must be addressed. The stocking density of fish in this intensive aquaculture results in high concentrations of particulate waste product in the surrounding area which cause environmental problem (Backman et al, 2009). Fish frequently escape from the aquaculture cages (Izquierdo-Gomez et al, 2016), and can transfer diseases between the farmed fish and the wild fish (Morton et al, 2017).

Among these problems associated with aquaculture are sustainability challenges associated with traditional feed for farmed fish. This is particularly problematic for carnivorous fish species like Atlantic salmon (*Salmo salar)*. For aquaculture to produce carnivorous fish in an environmentally sustainable way feed production need to be solved. This study will focus on the need for sustainable aquafeed protein sources in particular for Atlantic salmon *(Salmo Salar),* one of the most commonly farmed fish in Europe (Tacon and Metian 2015). Indeed, this study will focus on salmon because it is economically important and popular with consumers in Europe (Tacon and Metian 2015). Also, this fish it's rich in protein and omega-3 which is beneficial for healthy development in humans (Calder, 2014).

1.3 Traditional feed for farmed Atlantic salmon

Salmonid aquaculture has historically fed fish using proteins sourced from wild fish populations (Fry et al, 2016). In 1990 in Norwegian aquaculture, 90% of the ingredients used to feed *Salmo Salar* were derived from marine origin (Ytrestøyl, 2015). Indeed, use of marine fish provides both protein and lipids needed for good growth performance, supporting the popularity of fish meal and fish oil in aquafeeds (Tacon and Metian, 2008).

The popularity of wild capture fish in aquafeeds has contributed significantly to major losses in the population of fish like *Engraulis ringens* or *Sardina pilchardus* (Laubier and Lucien, 2003), which are regularly included in farm diets. Long term over-exploitation and decline of this resource along with an increase in the price of wild caught fish for aquaculture has made continued sole reliance on this resource impractical (Naylor et al., 2000).

Recent research has focused on the need to develop alternative protein sources for inclusion in salmonid diets. Numerous alternatives have been investigated, with the objective to maintain high growth performance and profit required for the aquaculture industry (Gatlin et al, 2007). A change in the composition of diets as already resulted from research into alternative proteins. In 2013, on average salmonids diets now include only 30% from a marine origin, against 90% in 1990 (Ytrestøyl, 2015). The most successful alternative protein, that has been widely adopted by the industry are these from plant-based sources (Fry et al, 2016; Hardy, 2010).

Plants like soybean, barley, canola, corn, lupin, and wheat are now used in commercially sold aquaculture feeds (Gatlin et al, 2007). The most used is currently soybean. However, the use of this plant in aquaculture is not optimal for two important reasons. Firstly, while the amino acid profile is suitable for Atlantic salmon, soy protein also contains anti-nutritional factors (ANFs). These are substances which interfere with health and limit growth of the fish directly or via their metabolic products (Francis et al 2001). For example, these ANFs can affect nutrient uptake, act as antivitamins substances or damage the salmon gut. Different species of fish have different tolerance to these ANFs, Atlantic salmon are highly sensitive compared with other species (Francis et al 2001).

Secondly, production of soybeans does not adhere to the principles of sustainable development and can cause many environmental problems. Soy is grown outside of Europe and is imported long distances for the large-scale Atlantic salmon aquaculture feed industry in Europe (Gatlin et al, 2007). The increase in soy production causes loss of native ecosystems and this trend is in constant growth. During the period 2007 to 2012 compared with 2000 to 2006, 29% more rainforest habitat was cleared for soy production. These regions also correspond with high biodiversity, making them desirable to protect (Eisner et al, 2016). Soy production requires large quantities of water and pesticides (Mekonnen and Hoekstra, 2014). Finally, use of soy protein in aquaculture feeds is in direct competition with the human food market (Hardy, 2010).

For all of this reason, even if many plants are used in salmon aquaculture, they are not optimially sustainable alternative. They are not the most environmentally or nutritionally beneficial alternatives.

1.4 Insects as alternatives to fish and soy meal in *Salmo Salar* aquaculture

The problems associated with traditional proteins in aquafeeds have driven the need to find new options for and expanding Atlantic salmon farm industry (Gatlin and al, 2007). Recent research has highlighted the potential of insect proteins as new alternatives for the diet of salmonids.

Insects are rich in protein, energy and lipids, and are not known to contain anti-nutritional factors (ANFs) like many plant species (Tran and et al, 2015). Also, they are found naturally in the diet of wild salmonids, especially during the juvenile life stages (Tran and al, 2015). For optimal nutrition benefit insects have been suggested as the best candidates for partial or complete substitution of traditional proteins in salmonid feed. Different studies have assessed a range of species for example larvae of black soldier fly (*Hermetia illucens*) has shown potential for inclusion in salmon diet up to 100% (Lock and al, 2015).

Figure 2 : Black soldier fly larvae at harvestable size and developmental stage for aquafeed inclusion (Stamer 2015)

Insect proteins can be integrated into the principle of circular economy, which is beneficial to sustainable development. The circular economy is an economic system where the objective is to reduce, reuse and recycle, to limit the production of waste (Sheposh and Richard, 2017). Insects can be integrated into this system because they can be produced, from example near to farm sites and reared on organic wastes.

To conclude, aquaculture needs develop more sustainable feed practices through the modification of protein content.

1.4 Animal welfare and behaviours

In the context of sustainable development other elements need to be considered. Animal welfare is an important consideration in fish farm. Welfare is a new and complex subject, and, in recent years lots of scientific articles were published about this field. The majority of these papers concern mammals, which have a high degree of public concern, but few studies are about fish welfare in aquaculture (Walker, 2014). For this reason, there is a need for more studies on fish welfare in intensive farms.

Understanding welfare is the key because poor welfare can increase the risk of catching diseases, limit growth, a factor which will reduce the profitability for the farm owner. In this context it is better for the fish farmer to avoid poor fish welfare (Castanheira et al, 2017). Moreover, there is a strong inter-relationship between behaviour and welfare of fish. For this reason the studying of the fish behaviour can indicate welfare state.

It is possible to select for specific fish behavioural types, which may experience better welfare in aquaculture conditions. Behavioural type can be dependent on many parameters. This is especially interesting for aquaculture but also for the restocking and brood-stock selection programs, with the study of behaviours it is possible to select fish which have the highest chance to survive (Benhaïm et al, 2012). For example, with the selection of the fish which have faster reflex to respond to a simulated attack by a piscivorous bird for example (Huntingford and Adams, 2005; Benhaïm et al, 2012). This selection of behaviours may vary between farmed and wild fish. It's the farming condition which will introduce in part a selection of the fish and a standardization of the fish behaviours (Benhaïm et al, 2012). Indeed, in farming it's the men which will achieve the selection of the salmon, by selecting the fish which present the better performance, growth, and with this choice a selection of the behaviours will also realised.

In this context, the behaviour of fish can result in accidental selection and domestication over long time periods but behaviour can also be influenced by many parameters, like the fish density in the tank, the environment of the tank, the frequency of feeding and the type of food (Huntingford and Adams, 2005; Ferrari et al, 2014). Recent research has shown that the dietary protein fish are fed can have an important influence on behaviour. For this reason, it is possible to modulate fish behaviour through diet (Ibarra-Zatarain et al, 2015). This is especially interesting for the aquaculture. Indeed, this element opens the possibility to improve the fish welfare in the aquaculture, that it's positive to improve the productivity (Ibarra-Zatarain et al, 2015).

1.5 Fish different coping style

It may be is possible to alter some fish behaviours through modification of the dietary protein from traditional to insect proteins. This knowledge could also improving overall aquaculture performance. Existing research has identified two different coping styles: the reactive and the proactive fish. It has been suggested that proactive fish may be better suited for intensive aquaculture experience better welfare and perhaps productivity (Leeper et al., InPreparation).

Proactive fish present characteristic behaviours like high feed efficiency or growth (Mas-Munoz et al., 2011) and physiological characteristics such as low cortisol responses to stress (Øverli et al., 2007). These coping style are the same for the different fish species but particularly important to study in popular and intensively farmed species like salmon. Indeed, the identification of this two coping style was realised with studies on *Salmo salar* (Leeper et al., InPreparation) but also with other species of fish, for example with *Solea solea* (Mas-Munoz et al., 2011) or with *Cyprinus carpio* (Øverli et al., 2007).

A list with all the characterises of proactive and reactive strategies based on many different studies, which is available in the figure 3, a recap graph has been realised (Castanheira et al, 2017). This list suggests proactive fish have more characteristics that may translate to better fish welfare and they could have a better productivity in farming condition. This may result in improved disease resistance compared with other fish, which is an important cause of mortality in the fish farm. Also, there could be better growth than for the reactive fish, because they will be more competitive for food and more adaptive to feed

	Proactive	Reactive	References
Behavioural characteristics			
Actively escape to stressor	High	Low	Silva et al. (2010), Martins et al. (2011), Brelin et al. (2005), Laursen et al. (2011)
Boldness	High	Low	(Sneddon 2003)
Feeding motivation	High	Low	Øverli et al. (2007), Kristiansen and Fernö (2007)
Risk taking and exploration	High	Low	Huntingford et al. (2010), Øverli et al. (2006), MacKenzie et al. (2009), Millot et al. (2009)
Aggressiveness	High	Low	Øverli et al. (2004, 2005), Castanheira et al. (2013)
Social influence	Low	High	Magnhagen (2007), Magnhagen and Staffan (2005), Magnhagen and Bunnefeld (2009)
Sensitive to environmental stressors	Low	High	Höglund et al. (2008)
Plasticity/Flexibility/Routine formation	Low	High	Chapman et al. (2010), Ruiz-Gomez et al. (2011)
Physiological characteristics			
HPI reactivity	Low	High	Castanheira et al. (2013), Øverli et al. (2006), Trenzado et al. (2003)
Sympathetic reactivity	High	Low	Schjolden et al. (2006), Verbeek et al. (2008), Barreto and Volpato (2011)
Parasympathetic reactivity	Low	High	Verbeek et al. (2008), Barreto and Volpato (2011)
Hormonal modulation	Low	High	LeBlanc et al. (2012)
Oxygen consumption	High	Low	Herrera et al. (2014), Killen et al. (2011), Martins et al. (2011)
Myocardial dysfunction	Low	High	Johansen et al. (2011)
Neural plasticity	Low	High	Johansen et al. (2012)
Immunity	High	Low	Kittilsen et al. (2012)

Figure 3 : Adapted from Castanheira et al by Leeper, 2017. Table summarising the behavioural and physiological characteristics of proactive compared with reactive fish with their sources in the literature

The determination of these two coping styles have been realised by the creation of different experimentation all based on the observation on the fish reaction, with a stimuli, a stress, or interaction with a new environment. An experiment called "Open Field Test" is one such test recent research has used to assess behaviour related to welfare (Benhaïm and al, 2016; Ferrari and al, 2014; Leeper et al., InPreparation). The objective of this is to see the behaviour of the fish in a new environment, which can contain a shelter, a place for fish to hide. Coping style can only be assessed when the same fish is followed across different time periods and contexts, but single assessments can be useful to quickly indicate behaviour. There are additional ways to assess coping style, for example by measuring characteristic behaviours like fish mobility or by using physiological characteristics like the fish oxygen consumption (figure 3).

1.6 The study outline

In 2017, a research project was carried out by the Matís ohf aquaculture department entitled: ´*Sustainable Novel Feed Ingredients in Aquaculture during a Crucial Growth Period*´ (Leeper, in preparation). The objective of this study was to investigate the application of insect proteins from larvae of black soldier fly (*Hermetia illucens*) in the diet of juvenile Atlantic salmon (*Salmo salar*) as a new sustainable protein source.

This work will also be carried out using juvenile Atlantic salmon because the juvenile development stage is period of crucial development for the fish growth and for fish behaviour. Moreover, it's the time where the fish growth fastest allowing rapid results for protein impact on growth and behavioural. Finally, it's during this period that the salmon need the highest quantity of protein to achieve is development potential. For this reason, the impact of the diet will be more important for a juvenile salmon than for an adult.

The overall project is a year-long study and the final objective it's to see the different impact of using this new diet on early developmental stages of farmed Atlantic salmon. The wide study considers growth, behaviour and gut microbiome of experimental fish under different feed conditions and the link between all these aspects. For the full behaviour section, three tests will be used to assess for coping strategy of fish fed traditional and insect proteins.

The final objective of the overall study is to assess the impact insect proteins would have in commercial farm conditions and if they a feasible replacement. In this context the diet based on black soldier fly will be compared with other diet currently use in aquaculture. The first diet will be a commercially sold standard diet from an international company in the production of food for Salmon's aquaculture. The second will be realised with Soy protein concentrate. These two diet will be use to see the difference between traditional diet based on fish protein and soy protein. These traditional diets will then be compared with those fed treated and untreated Black Soldier Fly larvae protein. The treated Black Soldier Fly larva was treated enzymatically to remove chitin. Due to time limitation of this report only result for the untreated insect protein will be presented.

However this report will only be focus on the study on the modification of the salmon the behaviours. The objective it's to answer of this problematic:

Will there be a difference in behaviour of farmed juvenile Atlantic Salmon (Salmo salar) fed traditional protein and those fed on treated and untreated of black soldier fly larvae (Hermetia illucens) protein?

The objective of this study is to develop a better understanding of how insect proteins affect specific behavioural indicators of coping strategy compared with traditional proteins.

2. Materials and methods

2.1 Fish aquaculture condition

2.1.1 Study location and condition

This study was carried out in a research building of the Matís company, where an experimental aquaculture facility has been installed. In this place the objective is to recreate the same condition as those found in commercial farms. All the parameters are standardised to achieve this objective (figure 4).

The temperature in each tank was 9,5 ± 0,5°C throughout the study. The light conditions are also stable and always keep on lux ± 250, because it's common in the industry to use long hours of light to increase the feeding time and growth of the fish. The oxygen level is always above 90% saturation. Indeed, these parameters are essential to keep good welfare of the fish and optimize their growth. The experiment was carried out in freshwater.

The experiment started in February and is ongoing at the end of this internship at the end of July. At the start of the experimentation 30 fish were placed in each tank. Each tank presents exactly the same structure, a cylindrical shape diameter 31,5 centimeters and 34 centimeters height. The water level in each tank is stay at 19 centimeters height (figure 5). The water renewal is assured by a pipe on the overhead of the tank which supplied new water in continue, and by a filter on the middle of the tank which drains water and maintains water level and exchange. This filter is cleaned every two days with the same methods for all tanks.

Figure 4 : Picture of the experimental fish farm (Leeper)

Figure 5 : Picture of one tank where the fish are farm (Leeper)

Finally, all the fish experience has the same environmental conditions. The only parameter which will change between the tanks is the diet to be sure this is the only parameter which changes the growth, welfare and behaviours of the fish. For this report and the behavioural section of the overall study, fish number was reduced to 8 individuals per replicate tank (N=32 per feed treatment)

2.1.2 Diet use and feeding methods

For this experimentation, there are four different diets test. The first two diets are based on traditional feed used in farmed of Atlantic salmon. One is a commercial classic food used for this developmental stage on commercial farms and is based on marine proteins, it is an extruded feed. The other is a baked diet where the protein is from soy protein concentrate. These two diet with traditional proteins will be used to compare with the result obtain with the insect dietary protein.

Two diets with black solider fly larvae were formulated, where 10% of soy protein concentrate is replaced by the two types of the Black soldier fly larvae prepare. However, the food prepared from black solid fly content chitin which is suspected to have bad effect in the fish growth and welfare. For this reasons the first diet based on insect protein will contain chitin, but the second will be treated to enzymatically remove the chitin. Due to time limitations only the two traditional protein diets and the untreated Black solider fly diet were assessed in this report. Since this study is part of a larger European study which is still ongoing, it is not possible to provide the exact dietary composition of these feeds.

For each feed treatment there were four replicates. . The conditions of feeding are the same for all the tank and feed volume was identical following standard farm feed volume recommendation. The fish are fed by automatic feeders which drops feed to each tank fifteen times per day in a continuous feeding method. Also, the fish were slightly overfed to be sure all the fish of each tank were assessed on different proteins and not feed availability.

2.2 Behaviours experiments: "the Open Field Test" with shelter

2.2.1 Description of the experiment

The key research question of this study was:

Will there be a difference in behaviour of farmed juvenile Atlantic Salmon (Salmo salar) fed traditional protein and those fed on treated and untreated of black soldier fly larvae (Hermetia illucens) protein?

In this context the test called "Open Field Test" (OFT) with a shelter was selected. This test was chosen because it has already use in many fish behaviour studies, for example for *Salmo salar* or *Dicentrarchus labrax* (Benhaïm et al, 2016; Ferrari et al, 2014), and it is useful to use a test where the interpretation of result is always standardise. For this experimentation a fish is placed inside a small box in one corner of one tank. This box which is a shelter starts closed, but after five minutes acclimation, it is opened and the fish reaction will be observed. According to the reaction of the fish and the observation of different parameters it's possible to assess an indicator of coping strategy, however to confirm the coping strategy multiple tests across different times and contexts are required. In the overall study a special software called EthoVision is used to quantify behaviour. In this report manual observations of selected variables will be carried out.

In this study four identical rectangular tanks (figure 6) which present the following dimension Length=40 centimeters Width=30 centimeters Height=25 centimeters were used to create observation arenas. Inside the rectangular tank, in one corner, a small white PVC box was fixed. This shelter has an opening whole (door) on one side (4x5centimeters). This hole can be open and close by a sliding trap-door (figure 7). Each arena was filled with 7 litres of water, so the water level was equal of the height of the shelter and identical in each arena. Water was taken from the farm system so was the same composition and temperature as the tanks, and lux in arena was lux 75$\pm$3. This is necessary because data collected will be treated by software which uses the difference of contrast between the fish which appears black on the movie and the rest of the arena which appears white. This water level was selected to avoid the fish swimming above the shelter, because the software can't differentiate when the fish goes inside or above the shelter.

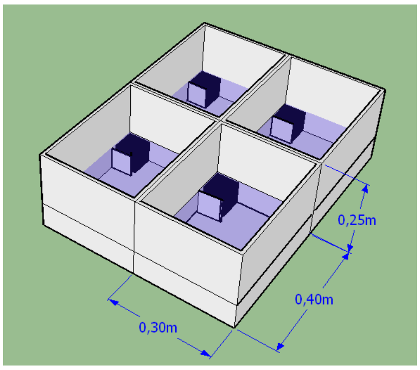

Figure 6 : The arena for assessment of behaviours with dimensions and the shelter open (Personal realisation, on sketchup)


Figure 7 : The arena for OFT with the sliding trap for close the shelter (Personal realisation)

During the experimentation the fish tested were individually, this was possible because each fish has a unique marker (see section 2.2.3). Fish were transferred from the farm tank to the experimentation arena in a small opaque box filled with water. Once at the arena, fish were installed firstly in the small box of PVC, the shelter with the door closed. The fish will stay five minutes in this shelter. During all tests fish need to be handled gently and carefully. All of these precautions are taken to avoid stressing the fish, because if the fish it's already anxious at the beginning of the experimentation this could be distorting the result.

After the period of the five minutes passed, the door was opened and the fish were filmed uninterrupted for twenty minutes. During these twenty minutes no people were present inside the room to reduce stress. For this report three variables were manually collected based on the observed behaviour in the arena.

2.2.2 Acquisition of data

Behaviour data was acquired through filming fish in black and white and to increase the contrast of colour between the fish and the arena will be deposed on a lights box, all the four tanks will be equally light (figure 7). The camera used will also be positioned directly above the arena and it will be record at 30 frames per second with a resolution of 640 × 480 pixel. Moreover, the camera it's connected to a computer with IC-capture software to manage and save the movies.

After the realisation of the film the overall project will use a software that will be use to realise the acquisition of the data. Some detail is provided for interest. This software it's call video-tracking software package Ethovision XT (8.5) (Noldlus Information Technology, Netherlands). It will use the difference of contrast between the salmon which appears in black and the tank which will be white, to analyse different parameter like the total distance travelled, Average Velocity corrected to Body Length etc. To realise this the software detects the centre of gravity of the fish and assigning position (x,y) which will move during the experimentation (figure 8).

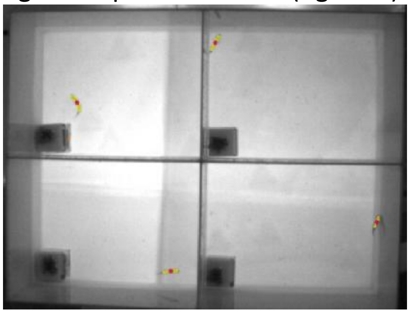

Figure 8 : The OFT during analyse by the software Ethovision XT (Leeper, 2017)

Moreover, the software will analyse the twenty minutes of fish film. It will couple the move of the salmon by is position inside the tank. Indeed, the tank will divide in two virtual zones which are the border and the centre in addition of the shelter zone (figure 9).

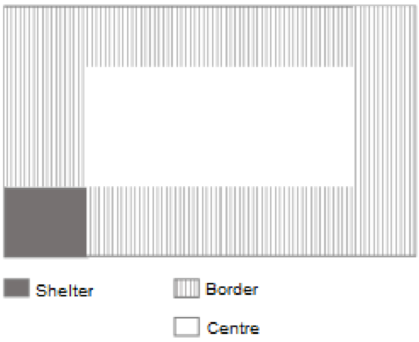

Figure 9 : Showing the virtual zones for the OFT arena created in Ethovision XT (8.5) to assess proactive-reactive behaviour of fish (Ferrari et al, 2014).

Finally, with this experimentation the software can collect all of these variables:
- Total Distance travelled (cm),
- Latency of individual to emerge from the shelter (s),
- Number of returns to the shelter of the fish (Frequency of return)
- Mean distance from the shelter (cm)
- Average Velocity (Vel. in BL s-1),
- Average absolute Angular Velocity (Vang º s-1), (which correspond of the head move of the fish)
- Time spent in virtual centre zone or border zone of the tank (s) (figure 9)

The analysis of this variable will permit to obtain information of the coping style of the fish when combined with multiple other tests at different times and contexts. For example, a fish will considered shyer if it take lots of time to go out of and back frequently in the shelter during this twenty minutes. While, a proactive fish will passed more time out of the shelter and passed more time in the rest of the tank.

2.2.3 Selecting and tagging fish

The objective of the OFT with shelter it's to determine the behaviour of the fish, and the data will be collected by a special software. However, this experimentation need to be repeat because like it has see in the first part to know exactly the coping strategies of the fish it's necessary to test the fish several times. Indeed, with just one test it will just obtain an indicator of coping strategy styles. For this reason, in the overall study the OFT with shelter will be realised two times and a further swimming and stress test will also be performed. Only the first OFT with shelter will be reported here, but details of tagging will be given since it was a part of the overall methodology for this report.

For each feed treatment, height fish were selected for each replicate tank, also the number of fish tested so for each diet a total of thirty two individuals will be assessed. Fish of similar size were selected for this study.

Selected fish were tagged to identify them and be sure to follow always the same fish in the overall study. Tagging was carried out with a low impact method called Visible Implant Elastomer (VIE). This product consists of coloured elastomer and a curing agent. These two products were combined before being injected into the fish, like this the mixing solution will solidify and stay visible for several months. This injection technique and VIE tags are fish safe (Northwest Marine Technology). Fish were anaesthetized during this procedure to reduce stress. Three colours were used to create height unique tags per tank, with two points the first on the dorsal fin and the second on the tail fin. Different combination are realised to differences all the height fish selected by tank for the experimentation. After the tagging, fish were left to recover for one week before the starting of the behaviour experimentation so tagging stress does not affect measurement of behaviour.

Figure 10 : Example of one juvenile salmon tag with two points on the dorsal and tail fin with pink colours (Personal realisation)

2.2.4 Advancement of the study

This internship will finish at the end of July and at the dead line for this report just a few part of the experimentation have been completed. In this context just the first OFT with shelter was completed, the second will be do the 10 July. Moreover, the software is not available for the moment. It will only usable at the end of the month when all the experimentation will be finish.

In this context, the present study will present only the first result available, which correspond of the first OFT realise. Just three data will be collected, not by the software, but by an observation of all the record at half speed by a person. The data collected will be only for three diet which are the commercial diet (COM), the soy protein concentrate (SPC) and the black soldier fly with chitin (BSFC+).This data correspond on :

- The latency for the fish to kit the shelter at the beginning of the experimentation
- The number of return of the fish inside the shelter during the 20 minutes
- Time pass by the fish inside the shelter during the 20 minutes

To finish, counting this dataset, the fish will consider inside the shelter when all of his body it’s complete inside, with is head and caudal fin. This first result will be use to see the first trend of the fish coping style. It will be use in a perspective of the final result of this experience.

2.3 Statistical Analysis of the behaviours experimentation

Concerning the variable collected by the OFT, the first step for this quantitative variables it’s to see if it’s parametric or non parametric data. For this reason a levene’s test or test for equality of variance will be realise. This first test it’s necessary to check all the conditions are realises to achieve an Anova test. Indeed, in a second time, an Anova test will be realising. However, to do this test it’s necessary to have a sample superior of thirty individual, and to have to have quantitative and parametric data. For this reason the acquisition of the data had respect this two rules.

After an Anova test of one factor will use to test the three data collected one par one. The objective of this test will be to see if there are difference of reaction between the fish feed by the three different diet or not.

To conclude a two way Anova will be use. The objective in this case will be to see if there are behaviours difference between all the tank of the different diet and the data collected.

It will present here both the one-way and the two-way ANOVA as was part of the analytical process carried out during the internship. These statistics test will be realised on the software Minitab.

3. Results

3.1 Levene's test

Firstly, it's necessary to test the data obtain to check there are parametric and they follow a normal distribution. The levene's test it's realise in this objective with a significance level p value=0,05 and: H0: The variable follow a normal distribution
H1: The variable don't follow a normal distribution

The three dataset were be test individually, to remind there correspond on:
- The latency for the fish to kit the shelter at the beginning of the experimentation
- The number of return of the fish inside the shelter during the twenty minutes
- Time pass by the fish inside the shelter during the twenty minutes

The result obtain show for the latency a p value of 0,335, a p value of 0,203 for the number of return inside the shelter, and a p value of 0,492 for the time pass inside the shelter. The hypothesis h0 it's validated. The three dataset follow a normal distribution, there are parametric, it's possible to achieve the Anova test. Moreover, all of the data uses for these tests are available on annex 2.

3.2 Anova of one factor

3.2.1 Latency to leave the shelter

The first obsevation of the average and standard deviation show an important variation inside the individual of one same diet (figure 11). Moreover the average are quite similar exept fot he BSFC+. The Anova test will be use to see if this first impression it's thrue or not.

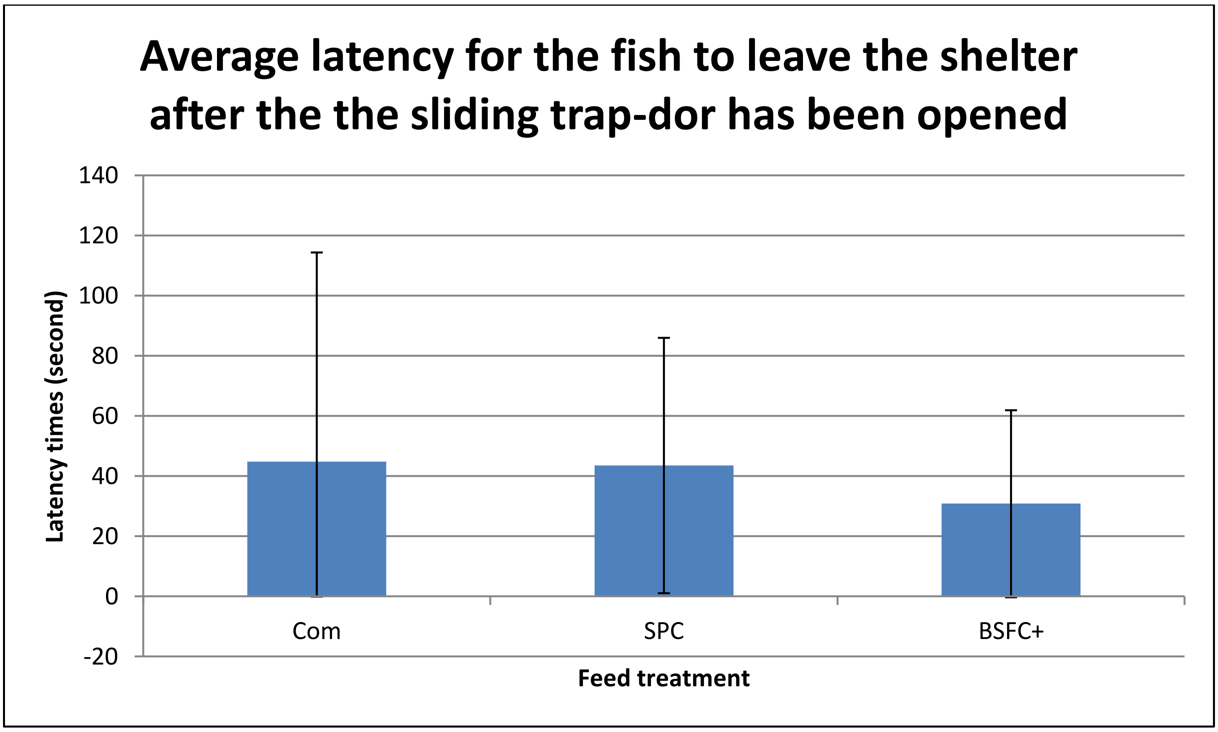

Figure 11: Graph presenting the average latency for the fish to leave the shelter during the OFT

Indeed, this first observation can be check by using an Anova of one factor. The two hypothesis are : H0: There are not differences between the latency time and the feed treatment
H1: There are differences between the latency time and the feed treatment

The p value It's equal of 0,081 and it's superior of 0,05 (Table 1). In this context, the hypothesis H0 it's accepted. For this first data there are not difference of reaction between the different population of fish.

Table 1 : Presentation of the different result obtain with Minitab for the Anova test on the latency for the fish to leave the shelter

Source	DL	SomCar ajust	CM ajust	F Value	P Value
Feed Treatment	2	3813	1907	0,75	0,475
Error	93	236010	2538		
Total	95	239823			

3.2.2 Number of return inside the shelter

The same approach it's realise for the second data collect which is the number of return inside the shelter by the fish during the experimentation. In this case also the average of number of return inside the shelter is quite equal, and the standard deviation is also important (figure 13).

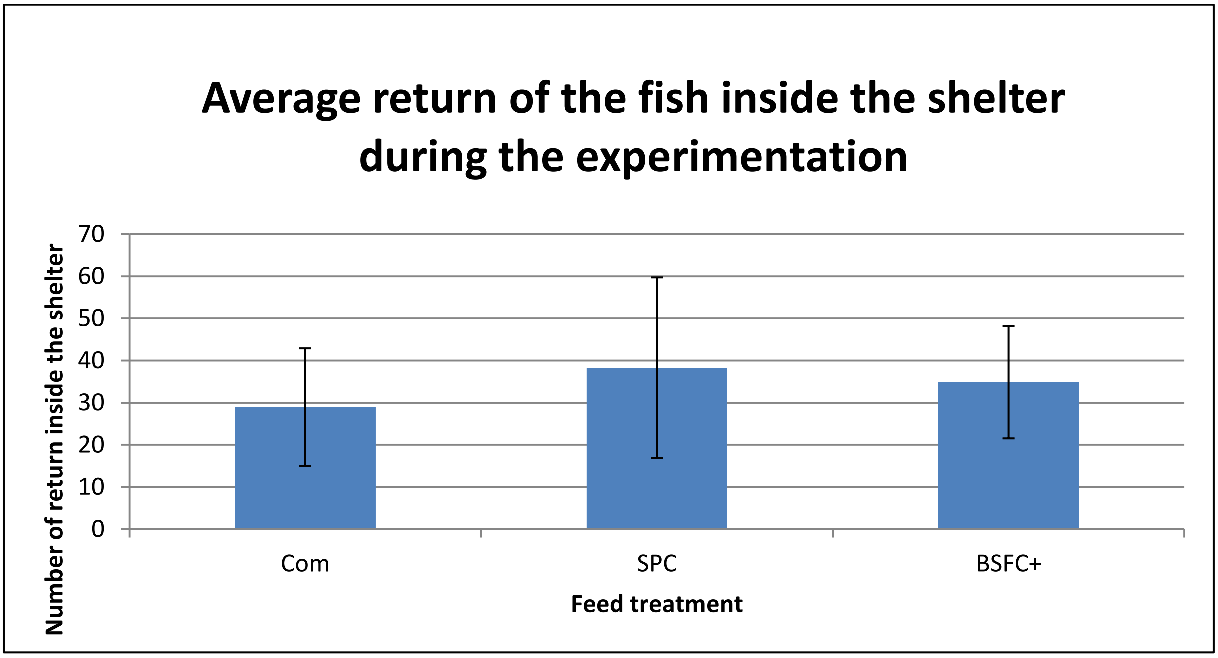

Figure 12 : Graph presenting average return of the fish inside the shelter during the OFT

The Anova tests confirm this impression in this case also. Indeed, the two hypothesis are also:

H0: There are not differences between the number of return inside the shelter and the feed treatment

H1: There are differences between the number of return inside the shelter and the feed treatment

The p value it's equal on 0,081 that is superior on 0,05 (table 2). It's possible to say that the reactions of this fish are all the same for this data collected.

Table 2 : Presentation of the different result obtain with Minitab for the Anova test on the number of return inside the shelter

Source	DL	SomCar ajust	CM ajust	F Value	P Value
Feed Treatment	2	1431	715,5	2,58	0,081
Error	93	25814	277,6		
Total	95	27245			

3.2.3 Time pass inside the shelter

To finish for the last data collected, the observations are the same than for the both previous because the time pass inside the shelter during the experimentation is in average quite equal inside three different diet test. In this case also the standard deviation it's important (figure 14).

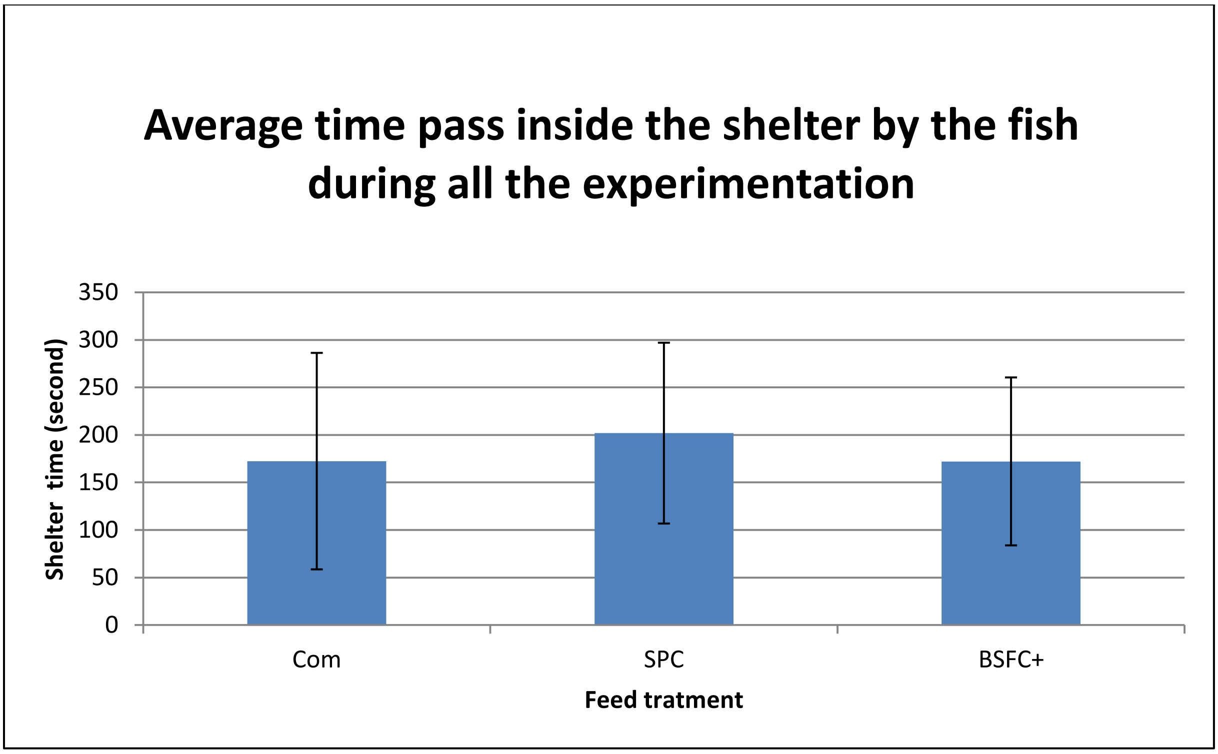

Figure 13 : Graph presenting the average time pass inside the shelter by the fish during the OFT

The Anova tests confirm this impression in this case also. Indeed, the two hypothesis are also: H0: There are not differences between the time pass inside the shelter and the feed treatment

H1: There are differences between time pass inside the shelter and the feed treatment

The p value it's equal on 0,394 that is superior on 0,05 (Table 3). There are not significant reaction between the fish feed by different treatment for this data.

Table 3 : Presentation of the different result obtain with Minitab for the Anova test on time pass inside the shelter during all the experimentation

Source	DL	SomCar ajust	CM ajust	F Value	P Value
Feed Treatment	2	18705	9352	0,94	0,394
Error	93	924255	9938		
Total	95	942959			

3.3 Two way Anova

For this final test the objective it's to see if there are difference between the tank of the different feed treatment and the different feed treatment for the three previous dataset collected. The same approach like previouly it's realise. The three dataset are tested by a two way Anova with two hypothesis: H0: All the data areequal

H1:There are difference between at leats one data

For the data time to kit the sheleter and time passe inside the shelter durng the experimentation the hypothesis H0 it's accepted because p value it's superior at 0,05. For this case it's possible to says that there are not difference for this two data between the different feed treatment but also between the different tank.

However for the second data which correspond of the number go back and go inside the shelter the p value it's lower than 0,05. This mean there are difference for the fish reaction with this data between the different tanks of the feed treatment. This can explain by the very important difference of reaction between the fish, like it's possible to see with the very important standard deviation for this data.

4. Discussion

4.1 Discussion about the OFT with shelter result

The objective of this experiment was to see if there are differences in behaviour of farmed juvenile Atlantic salmon fed insect protein and the juvenile salmon fed traditional protein. The statistical tests used, show there is no significant difference in the behavioural variables measured during the OFT with shelter between the fish from different feed treatment.

Firstly, with this data alone it is impossible to identify precisely the coping style of the fish, but it is possible to analyse this data as an early indicator of behavioural type, which might suggest the coping strategy it would expect after further tests. These first results suggest that the fish tested are more proactive than reactive. This is suggested because almost every fish leaves the shelter in the first minutes of the experimentation for example and in other experimentation it can happen that the fish stay inside the shelter during all the twenty minutes of experimentation, for example in sea bass experimentation (Benhaïm et al, 2016). In contrast the salmon in this experimental display similar coping style which is more boldness and proactive, as every fish left the shelter. Of course sea bass and salmon are not the same fish and the salmon could be naturally more boldness than the sea bass, because it is a migratory fish. But, this initial result is similar to a comparable study carried out last year which discussed that farmed juvenile salmon are overall quite bold in comparison to wild salmon and to other species (Leeper, 2017). There will be further acquisition of new data which will confirm or not this first observation since this report represents only the first result of a larger experimentation which is currently not finished.

It is also interesting to see that there is not statistic difference in this first result between the diet BSFC+ and SPC. However in the study of the last years (Leeper, 2017) the salmon feed by Soy protein appears like more reactive, shy. However this trend is not show in this first result. This could be explaining by the limit of the data collected and when the new data will be available this trend will maybe not confirm. Another hypothesis it the concentration of the protein which could be explain this fact, because in the experimentation of the last years soy protein classic had been use and this year soy protein concentrate are use. In all the case the new data will permit to confirm or not this trend.

Also, the standard deviation shows a high variability within the data collected. Indeed, there is big difference in reaction of the individual salmon within the same feed treatment, for example some fish can leave the shelter directly at the beginning of the experimentation and other fish stay inside for some minutes. This high variability is also present within the fish from the same tank, although this was not assessed statistically, only observed for this study. Moreover, all the environmental parameters have been standardising during the experimentation to be the same inside each tank. This suggests that that the behaviour of each fish is also strongly influenced by other factors, like natural variable reactions of the fish . This natural coping strategy may not have been affected by the different diets tested. Moreover, this important variability shows the necessity to do many replicates and analyse more data, element which will be possible by using the software, to see if this trend stays the same or if is it possible to detect an impact of the diet when more data is collected.

Thirdly, this report is registered in a larger project whose objectives are to see if this new diet can be used for the commercial aquaculture of salmon. And these first results are interesting for this utilisation. Indeed, utilisation of insect protein seems to have no negative effects on the fish behaviours which are the same by using insect protein or traditional protein. Moreover, the fish coping style appears more proactive than reactive, and the proactive fish could be more interesting in aquaculture context. This study needs to be comparing with the further results like the fish growth to see if it's really interesting to use this diet in a commercial system based on performance.

4.2 Recommendations for future studies

The observations made during the experimentation suggest that the OFT with shelter had some limitation that may affect study results and it is possible to improve this test. Firstly, despite the low level of water inside the tank during the experimentation, some fish jumped above the shelter. This will present a problem for the future collection of the dataset with the software which can't differentiate when the fish is inside or above the shelter. A system must be developed which avoids this jumping on top of the shelter. The fish may now too big to carry out this experimentation using smaller fish could solve this problem which was not present during the previous experiments with the same arena last year (Leeper, 2017).

The size of fish requires a bigger shelter should be used. Indeed, the fish have been counted as being totally inside the shelter, and for the bigger fish, often the head or the dorsal fin protrude from the shelter even when they are inside. The software will collect the data by using the centre of gravity of the fish. For this reason a salmon partially inside the shelter can be consider inside by the software, so this shelter problem will be solved later in the experiment at the analytical stage. It could be interesting to compare the result obtained by the software and the result collect by manual observation to see if there are high differences. Also, it could be interesting to compare the result of the bigger fish with the other to see if there are differences between this salmon. If it's the case, the size of this shelter has a real influence on the result and need to be bigger to achieve this test with this size of fish.

Secondly, during the beginning of the experimentation the fish lived in group of thirty per tank salmon. However, during this test the fish were alone and there are four fish tested in the four arenas individually. However, if two fish are very close of the wall the arena they may see the other fish and this could alter their swimming path. In this case sometimes two fish have the reflex to stay next to this wall, to "stay together" especially because salmon are schooling species (figure 15). For this reason it wills be important to improve the opacity of the wall of the tank.

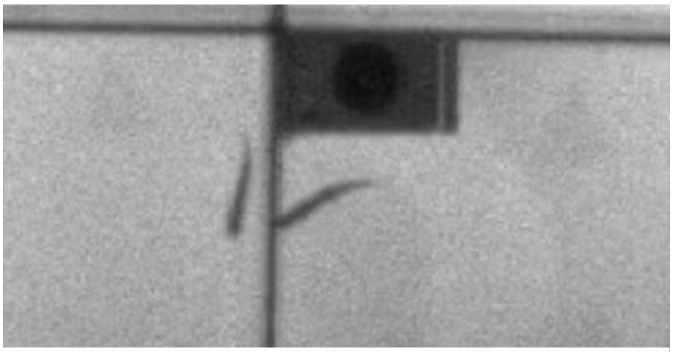

Figure 14 : Two fish staying together during the experimentation

The salmon are farmed with classic aquaculture condition, where light is always on. However during the experimentation there is low luminosity and inside the shelter there is no light. Also, during the experiment for this report, only three variables for behaviour will be collected all dependant of the shelter. However, some fish present all the other characteristic of a reactive fish which show stress, although these were not quantified for this report. Indeed, some fish stay in a corner of the tank, move less, and present a high angular velocity, which are also signs of shyness but they go less inside the shelter (Benhaïm and al, 2016; Ferrari and al, 2014; Leeper, 2017). For this reason they appear like proactive with just this first result, even if they may have other characteristics that overall suggest they are reactive. Maybe this complete change of environment, from the tanks to the arena for the experimentation can add lots of stress to the salmon, and some salmon are afraid by the shelter. This can affect the result of the experimentation. Even if there are five minutes of adjustment inside the shelter and all fish have test with the same condition.

Moreover the opposite example it's observe with salmon which move lots of, present a low angular velocity, and are not afraid to go in the centre of the tank which is consider "less safe". However these fish show high exploration of the arena space and during this exploration they pass many times, but only for one or two seconds inside the shelter. This fish will appear reactive even if there are proactive from the data collected in this report alone. It's like if they are searching food because they do a high exploration of the tank at the beginning, this exploration will decrease and be less important during the last five minutes. For all of these reasons there is the necessity to collect more data to have results more specific, because the variables of this report alone are too few, and depend too much of the fish interaction with the shelter.

Finally, the results obtained in the present study have many limitations. This problem will be in part solved by the realisation of the second test and the investigation of the video by the software which will permits to collect more data. However, with the trend of the first result of the OFT and the potential limit of this test, it could be interesting to achieve in addition a different experimentation. This could be interesting to have new result which will permits to confirm or not these first results.

Conclusion

This study has compared the impact on behaviours juvenile salmon of using insect protein to replace traditional protein use in aquaculture. The objective it's to found new sustainable diet which can use in the salmon aquaculture. This work it's based on using Black soldier fly (*Hermetia illucens*) and the first result of this study show no difference between the behaviours of the salmon feed by this new diet and this feed by traditional aquaculture diet. However, the study is not finish and will continue. For this reason, these first result will be confirm or not with the continuation of the experimentation.

For the moment the data exploit present lots of limit. Indeed, only one test had been realise when at least two are necessary to determine fish behaviours. The data had been collected not by the software but by manual observation so they can present mistake of the observer. Moreover, only three data have been collected (time to leave the shelter at the beginning, number of return inside the shelter, total time pass inside the shelter) all dependent of the shelter when the software can collect seven data. Limit to the test achieve have also been observe during the realisation of the experimentation, like when two fish stay next to a wall, to "stay together". For all of this reason these first result need to take like potential trends of the fish behaviours. Also with the potential limit of the OFT it could be interesting to realise another test.

The observation of these first result, with this in mind their present limit, are similar with previous study where a quite equal experimentation had been realised. Indeed, the salmon test present more proactive indicator than reactive, and the proactive fish are supposing present more interesting performance in an aquaculture context. For all of this reason these first trends are really interesting and the use of insect protein could be interesting for the aquaculture production. However, this is only trends for the moment and they need to be confirm or not by the continuation of the experimentation.

To conclude, this study is enrolled in a bigger project where the objective it's to see if this insect protein could replace the traditional protein and not affect the performance of the salmon farm. To remind the salmon growth and gut microbiome will also be test. When this behaviours experimentation will be finish, the objective will be now to compare the result obtain with the other test realise, to see for all the parameters test if this insect protein are really interesting in a aquaculture context. And if all of this result show is positive the utilisation of the insect protein in salmon aquaculture could be a really interesting new option.

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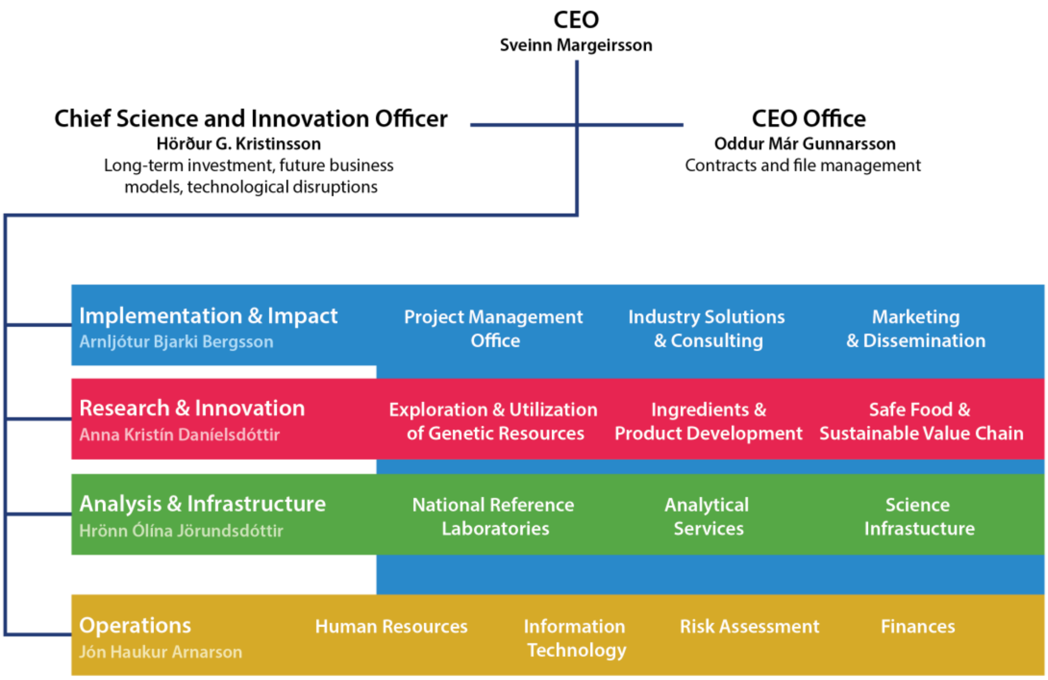
Annex

Annex 1: Organisation of the Matis company



Annex 2 : The different dataset collected with the first OFT

Feed Treatment	Latency time	Number of return	Time pass inside the shelter
COM	7	30	77
COM	28	27	129
COM	57	25	179
COM	13	22	85
COM	16	13	79
COM	5	17	63
COM	9	55	150
COM	87	26	270
COM	58	29	551
COM	92	28	246
COM	31	24	126
COM	99	35	305
COM	138	21	201
COM	24	19	94
COM	56	18	135
COM	82	30	242

Feed Treatment	Latency time	Number of return	Time pass inside the shelter
COM	15	17	95
COM	11	19	71
COM	14	12	58
COM	23	12	73
COM	40	19	123
COM	378	16	484
COM	6	36	154
COM	17	34	129
COM	59	65	304
COM	9	25	154
COM	15	58	243
COM	13	32	144
COM	5	49	160
COM	13	37	131
COM	6	55	156
COM	7	21	107
SPC	64	59	286
SPC	159	38	334
SPC	14	73	303
SPC	23	28	126
SPC	13	81	336
SPC	9	33	130
SPC	8	21	98
SPC	13	12	46
SPC	24	34	152
SPC	30	20	164
SPC	17	26	111
SPC	31	14	62
SPC	27	30	105
SPC	80	27	224
SPC	75	25	269
SPC	4	26	131
SPC	32	41	145
SPC	160	38	302
SPC	24	24	132
SPC	73	18	144
SPC	88	30	205
SPC	69	25	208
SPC	8	58	280
SPC	9	24	272
SPC	37	101	366
SPC	23	35	121
SPC	62	85	348

Feed Treatment	Latency time	Number of return	Time pass inside the shelter
SPC	52	36	213
SPC	12	49	134
SPC	13	50	325
SPC	13	24	87
SPC	128	40	302
BSFC+	35	42	154
BSFC+	12	29	87
BSFC+	15	29	173
BSFC+	11	24	89
BSFC+	10	31	99
BSFC+	14	58	228
BSFC+	12	31	111
BSFC+	10	31	88
BSFC+	47	58	292
BSFC+	15	34	119
BSFC+	172	56	313
BSFC+	31	37	182
BSFC+	26	17	110
BSFC+	25	50	238
BSFC+	15	18	123
BSFC+	33	29	121
BSFC+	63	21	150
BSFC+	37	27	357
BSFC+	65	43	459
BSFC+	7	18	68
BSFC+	70	21	127
BSFC+	31	28	166
BSFC+	49	31	191
BSFC+	25	36	130
BSFC+	15	23	117
BSFC+	14	28	113
BSFC+	45	41	258
BSFC+	20	28	126
BSFC+	6	66	166
BSFC+	24	29	126
BSFC+	11	41	169
BSFC+	22	61	259

35 ALLÉE FERDINAND DE LESSEPS
37200 TOURS

Thomas BONNAFOUX
2017-2018

Impact of treated and untreated insect dietary proteins (*Hermetica illucens*) on the behaviour of farmed juvenile Atlantic Salmon (*Salmo salar*)

Summary: This study will interest on the farming of the Atlantic salmon (*Salmo salar*). The objective will be to see the impact on the utilisation of insect protein of Black soldier fly (*Hermetica illucens*) on the alimentation of juvenile salmon. This new diet will be compared with traditional diet. The final objective it's to see the potential impact on the fish behaviours of this new protein.

Résumé : Cette étude s'intéresse à l'utilisation de nouvelles sources de protéines dans l'élevage du saumon Atlantique (*Salmo salar*). L'objectif est de voir si l'utilisation de nourritures basées sur l'utilisation d'*Hermetica illucens* va avoir un impact sur le comportement des poissons. Ce nouveau procédé sera comparé avec des aliments traditionnellement utilisés dans l'aquaculture du saumon.

Mots Clés : *Salmo salar*, Aquaculture, *Hermetica illucens,* Comportement animal

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