Effect of different arachidonic acid levels in broodstock diet on egg quality of ballan wrasse *(Labrus bergylta)*

Thesis for the degree
Master of science in Aquamedicine

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Bergen 31.7.2021 Ruben Aldal Sætre

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Abbreviations:

AGD	**Amoebic gill disease**
ARA	**Arachidonic acid**
NL	**Neutral lipid**
PL	**Polar lipid**
IMR	**Institute of Marine research**
FHF	**Fisheries and aquaculture research financing**
NIFES	**National institute for nutrition and seafood research**
NOFIMA	**Norwegian institute of food, fisheries and aquaculture research**
SGR	**Specific growth rate**

Abstract

The production of ballan wrasse (*Labrus bergylta)* is growing and is becoming a focus area for the aquaculture industry. As a result of rising louse numbers and problems with resistance, biological methods such as cleaner fish is being introduced and used in aquaculture.

Our project focused on improving the health of cleaner fish and develop a method for estimating number of eggs spawned. For this captive ballan wrasse broodstock was fed with different levels of Arachidonic acid to investigate the effect on eggs.

When analyzing it seems that an ARA level of total fatty acids closes to the medium ARA diet (approximately 2,3 % of total fatty acids) is the best for an optimum broodstock diet for production of eggs. ARA added in feed also seems to help the fish recover from some diseases and improve their health status.

A subjective method for determining the number of eggs was also developed, and image analysis was used and compared to regular eye-scoring results. Eggs were weighted and counted to verify estimation methods and suggest that the image analysis is more accurate.

Both methods produced rough estimates, but larger sampling size could increase the accuracy. Additionally, eye scoring with a power adaption yielded similar estimates and could be a useful tool for the ballan wrasse farmers to better estimate egg production.

1. Introduction

1.1 Aquaculture of salmon in Norway and challenge with salmon lice

With its long coastline, Norway is the world's largest producer of Atlantic salmon (*Salmo salar)* (Bailey & Eggereide, 2020). The estimated production numbers gathered from Statistics Norway were in 2019 approximately 1 100 000 tons with an export value of 72,5 billion Norwegian kroner. Production of salmon is currently the second-biggest export product from Norway, after oil. Furthermore, the Norwegian government has a strategy that involves a rapid growth toward fivefold production within 2050 (Bailey & Eggereide, 2020).

The production cycle of salmon is divided into a freshwater phase and a seawater phase, were the on-growing phase normally takes place in open sea cages. Salmon lice *Lepeoptheirus salmonis* and *Caligus elongatus* (termed sea lice from here on) are ectoparasitic copepods that can cause serve health issues for the fish during the seawater phase.
Sea lice are a natural parasite to wild salmon and seatrout (*Salmo trutta trutta)* and has been problematic since farmed salmon production started in the 1960s. The parasite feeds off the skin and mucus of the fish and accumulate in numbers that ultimately cause osmoregulatory disturbances and in the most severe cases result in death (L. A. Hamre *et al*., 2009).

Sea lice are the biggest challenge in expanding Atlantic salmon's aquaculture production in Norway, and are responsible for both economic and environmental damages (Barrett *et al*., 2020). With steady numbers of sea lice, there is limited growth in the production of salmon in Norway, and in 2015 the loss related to sea lice was estimated by EWOS/Cargill to be roughly 10 billion Norwegian kroner (Bruarøy, C., 2015; Misund, 2019).

With a decrease in wild salmon and sea trout numbers, the Norwegian government has identified that the number of sea lice is the primary cause for this decline (Forseth *et al*., 2017).

Since 2009 "Lakselusforeskriften" has regulated the production with 0.5 female lice allowed on each salmon (FOR-2012- 12-05-1140). If the farmers are unable to hold the lice numbers

below this, they must start to threat the fish and in worst cases if the lice numbers don't go down over time, they have to reduce the production (Lakselusforeskriften). These regulations have led to a development in different methods for combating the lice effectively.
Historically lice have been combatted by chemicals such as pyrethroids, avermectins, hydrogen peroxide, chitin inhibitors, and organophosphates (Myhre Jensen *et al*., 2020). However, with an increased resistance to these chemicals and an adverse side effect on non-target organisms' other methods were required. Methods such as mechanically flushing and usage of warm water are well developed and frequently used today, as well as other methods like lasers. Additionally, biological methods have been developed such as the use of different cleaner fish like wrasses and lumpsucker.
The challenges with existing methods for delousing is severe with chemicals and mechanical delousing having consequences for both environmental and fish health for the salmon. Cleaner fish is a satisfactory method and environmental more friendly method for removing the lice (Skiftesvik *et al*., 2013).

Cleaner fish were first introduced in 1988, and since the 2010s, it has been actively used for combating sea lice. First wild caught wrasses (both corkwing and ballan wrasse) were used, with corkwing wrasse (*Symphodus melops)* being the most common species. When commercial farming was started ballan wrasse was prioritized over corkwing wrasse. Later farming of lumpsucker (*Cyclopterus lumpus)* was found to be easier to produce than wrasse and is now the second most farmed species in Norway (Helland *et al*., 2014).

1.2 Cleaner fish

Since cleaner fish was first introduced it has risen to become one of the biggest support industries for the production of Atlantic salmon (Bolliger, 2020).
Years of treatments with chemicals and mechanical delousing have left the industry with a massive problem with resistance, and there is a need for other alternatives.
Cleaner fishes were introduced as a more environmentally friendly alternative to chemical treatment. For combating sea lice, the aquaculture industry uses different cleaner fish for continuous biological delousing.
The cleaner fish are transported to the net pens together with the salmon, continuously delousing the fish throughout the production cycle. The cleaner fish prey on the parasite, and research has shown beneficial effects (Skiftesvik *et al*., 2013). The cleaner fish were put in cages with no access to hiding or feed in the beginning, but through increased usage, the aquaculture industry has developed solutions for improving fish health and lifespan of the cleaner fish.

1.2.1 Labridae family

The Labridae family, known as wrasses, is a diverse family of fishes with over 548 different species worldwide (*FAMILY Details for Labridae - Wrasses*, n.d.). They are the second largest family of marine fishes and are found in tropical reefs. The different species have an extensive range of colors, sizes, and shapes, but the species is known for its powerful jaw and protractible mouth and well- developed teeth. Wrasses usually prefer shallow waters, being close to the coast and tend to like rocky cliffs where the food resources are vast. In Norway, there are six native species, with the ballan wrasse being the biggest (Blanco Gonzalez & de Boer, 2017).

1.2.2 Ballan wrasse

Ballan wrasse is the largest of the wrasse species in northern Europe and is found from Morocco in the south to Trondheimsfjorden in the north (Tresurer *et al., 2018*). The fish tend to like temperate temperatures, and during the winter when temperatures drop below five degrees the fish will go deeper and into hibernation. Ballan wrasse can grow until approximately 2 kg, 60 cm in length and live for 25 years. The individual differences in color and pattern can vary a lot. The ballan wrasse is known for anatomical characteristic like having a short intestine and

missing both stomach and pyloric caeca (agastric). Ballan wrasse tends to like rocky grounds and rigid structures, the fish do not have specific habitat and can live in the shallows down to deep waters as well as close to harbors and docks. Their diet is highly diverse, but studies have shown that the fish feeds of invertebrate, but there is also much that is unknown (MW & ME, 2005). The fish usually stay near the coast and are feeding during the fall and further down during the winter. During the spring, the fish actively feeds and gains weight and energy before the spawning period where feeding is limited.

Figure 1: Ballan wrasse. *Modified from IMR.com info site about Ballan wrasse. A specimen of ballan wrasse with is large lips and spiny dorsal fin clearly visible.*

When spawning the fish is organized in a harem (B. Grant *et al*., 2016), where each male has many females. The fish is also a batch spawner and will spawn multiple times over a period, typically in the spring. Ballan wrasse is a monandric protogynous hermaphrodite, meaning the fish starts its life as a female and later changes sex and becomes a male. Studies has shown that the fish is group-synchronous multiple spawners, meaning that fish within a tank will synchronize its spawning (Muncaster *et al*., 2010a). The process of sex change has been a field of focus, and studies have done some research on this subject (Muncaster *et al*., 2010b).

1.2.3 Anatomy and ontogeny

L. bergylta is a compact ray finned fish that has a recognizable protractoral jaw and is known by its large lips. Its common name in Norwegian also suggest that its lips has given its name:

“leppefisk”. The fish has also an solid tail fin and spiny dorsal fins and relatively big head (Lukasz *et al*., 2014). Its powerful jaw enables the fish to push forward the mouth, and the solid teeth are suited for eating both solid and attached feed.
The embryonic development of egg is similar to other marine spices such as cod, but there are some notable differences.
After fertilization, the eggs go through 8 stages until the larvae are hatched (D’Arcy *et al*., 2012). From larvae it goes through more stages, first as a larva with yolk sac. After the fish has consumed the yolk, it lives for a while without going to the weaning phase, which is critical and often with high mortalities.

1.3. Farming of ballan wrasse

Initially, all cleaner fish were caught in the wild by nets and ruses, and often transported all over Norway. With a rise in demand for cleaner fish the numbers of fish caught made and impact on local environment and led to industrial farming of cleaner fish in a large scale.

Numbers from the Directory of Fisheries showed that 61 035 000 cleaner fish were deployed in 2019. According to the food authorities there were 51 licenses for commercial farming of cleaner fish, and with even more being implemented and planned.
Today, corkwing wrasse and lumpsucker dominates the industry, with ballan wrasse coming in third but, lumpsucker and ballan wrasse are the only commercially farmed species. Other cleaner fish spices are small mounted wrasse, and rock cook. The two cultivated spices, ballan wrasse and lumpsucker, both have their benefits with the lumpsucker preferring colder temperatures and the ballan wrasse preferring higher temperatures (Haugland *et al*., 2020; Yuen *et al*., 2019).

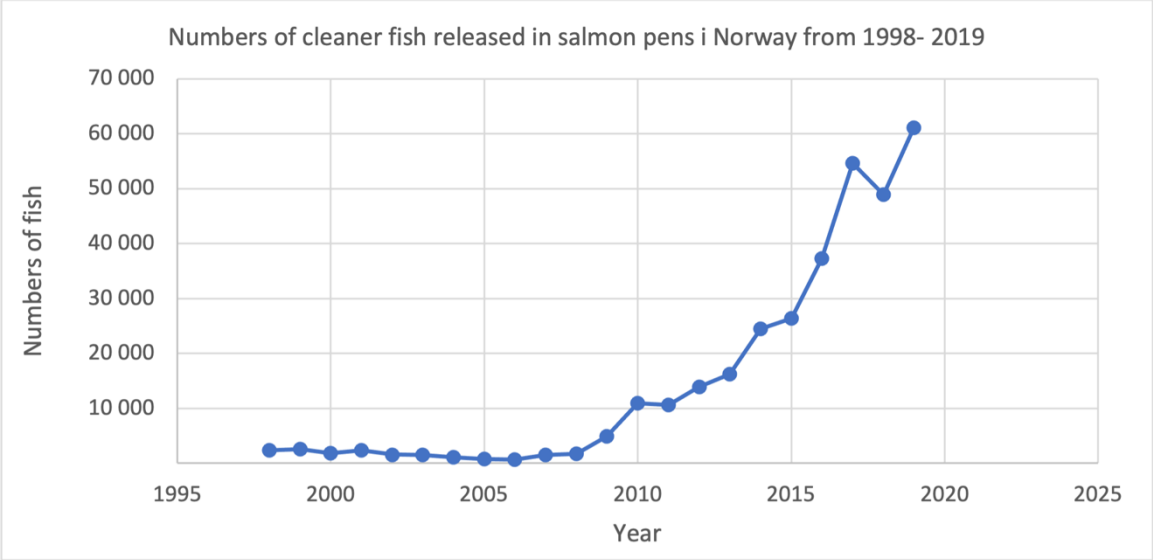


***Figure 2**: **Cleaner fish released in salmon pens in Norway**. Numbers of cleaner fish in Norwegian aquaculture from directory of fisheries with development of fish in salmon pens from 1998 (numbers from directory of fisheries) (numbers in 1000).*

Intensive production of ballan wrasse was started in order to reduce the need for wild caught ballan wrasse in the aquaculture industry. Because of the environmental impact of removing hughe numbers of fish from their local environment the government started to investigate these effects (Skiftesvik *et al*., 2013). In the commercial production of ballan wrasse, there is today no efficient measurement to estimate numbers of eggs produced (Bridie Grant, 2016). The farmers are only giving approximate numbers through observations, and there is no precise estimate to predict the number of eggs produced. When the fish are spawning on a substrate layer it is beneficial that each mat can be incubated, photographed, and scored individually. This study takes note that hopefully each mat has been produced by the same fish, since ballan wrasse is fierce in spawning. The farmers could have a great benefit from understanding better and predicting the actual numbers of egg produced. This way the farmers could be able to plan and predict the production cycle much better.

Marine Harvest (now Mowi) was the first company to start with intensive farming at Øygarden, Vestland. The facility was previously used for cod farming but was reused as a facility for farming ballan wrasse. Most fish that are farmed today has its origin from wild caught fish. Experimental testing using farmed fish as broodstock has been tested without success. Thus, will be a theme for further improvement of the production. A tool for measuring this will be interesting for quantification and better predict the outcome of juvenile fish.

When wild broodstock fish are caught they are hold in big tanks for a year before they are ready to spawn. In a tank there are usually 20-30 females and 3-5 males. The fish are cultivated and manipulated by light and temperature to induce and start the maturation and producing eggs and sperm. When the spawning period is closing mats are placed in the tank to provide spawning substrate for the fish, and mats are collected each morning. When discovering eggs, the mats are placed in incubators in separated smaller tanks. After 4 days the eggs start hatching and embryos with its yolk sac is free swimming in each tank before being transferred to tanks for feeding. When being fed they are in the start given rotifers together with clay. They algae/clay is used to simulate "real conditions" and give the larvae hiding. After a while they are given bigger feed proportional to their size, such as artemia etc. As soon as the larvae is big enough, they are given solid formulated feed. This transition period, weaning is critical and often connected to rising mortality.

1.3.1 Ballan wrasse broodstock feed

Commercially there are few specialized feeds to ballan wrasse, and the fish is known to be very selective in feeding (Kousoulaki *et al*., 2015). When using live feed, specialized tanks are also necessary for growing and enriching with different nutrition to make a more suitable feed.
Studies has showed that a cold extruded diet added extra lipids and phosphorus should be used in the ballan wrasse feed (Kousoulaki *et al*., 2021). Feed fed to ballan wrasse is also often enriched with shrimp meal to be more attractive to the fish.

1.3.2 Problems with cleaner fish

In 2020 the food authorities launched a campaign for improving welfare and survival of cleaner fish. In the aquaculture industry cleaner fish have been seen as a sunk cost, and mortality is very high due to different challenges (Erkinharju et al., 2021). The campaign gathered information from the farmers showing almost 42% mortality rate of cleaner fish transported to the net pens. Data observations from 2019 showed that 61 035 000 cleaner fish were released and 42 % died, which result in an all-cause mortality of 25 634 700 fish.
Towards solving and decreasing the high all-cause mortality rate in the industry more focus has been used on fish welfare. Since the cleaner fish is, by definition, under the law of a production animal, they should be free of unwanted and adverse issues compromising fish health and welfare. Diseases, currency, problems with eating, and occasionally being eaten by the salmon are problems the cleaner fish faces on a daily basis while inhabiting the pens.

Studies have shown that when released in a more exposed environment with, for example currents, the fish are struggling and dying in a high rate (Yuen *et al*., 2019). In fact, there are limited studies on cleaner fish compared to salmon, and much is still unknown about cleaner fish welfare.

One of the main problems with intensive farming of cleaner fish, and especially ballan wrasse, is high mortalities in the early stages (Kousoulaki *et al*., 2015; Piccinetti *et al*., 2017). Ballan wrasse has a very vulnerable larvae stage and is difficult to farm commercially, and until the weening stage the mortality rates are particularly high (Kousoulaki *et al*., 2015).

There are also physiological differences between different species of cleaner fish, where the lumpsucker is adjusted to colder temperatures than ballan wrasse. However, both species will, with oscillations and higher temperatures, have mortalities (Yuen *et al*., 2019).

The health of cleaner fish is in a constant debate, and there is a need for radical changes for improving the general health and welfare of this fish. A potential problem that will affect the industry is disease transmission to salmon and vice versa (Erkinharju *et a*l., 2021).

Some bacterial and viral diseases, such as atypical furunculosis, can be transferred. And some parasites are also known to do the same. Another problem is that cleaner fish is not used to open water. Most salmon cages in Norway are out in the open sea, and cleaner fish are not suited for these conditions. Both lumpsucker and ballan wrasse is inferior swimmers compared to salmon, and with currents and more significant movement they tend to be stressed, and their de-lice efficiency is reduced (Hvas *et al*., 2021).

1.4 Arachidonic acid

This study looked at both farmed and wild cough ballan wrasse to investigate the different nutritional requirements for commercial farming. They concluded that adjustments should be made in ARA and iodine for broodstock, and iron for broodstock and juveniles. With ARA being 3-5-fold higher in the wild-caught than in the farmed fish the assumption was made that added ARA was needed (Hamre *et al*., 2013). Arachidonic acid (20:4n-6)/ARA is a fatty acid containing 20 carbon atoms, four double bindings with the first one at nr six from the fatty acids methyl end, that can affect the eicosanoid metabolism. ARA is a major fatty acid in different physiological processes and its importance has been in focus lately (Bell & Sargent, 2003; K. Hamre *et al*., 2013). Previous studies have not highlighted ARA role as an essential fatty acid (EFA) but more studies has showed its importance (Bell & Sargent, 2003) ARA is essential for normal development and survival of juvenile marine fish and have also confirmed

that elevated levels can improve this (Bell & Sargent, 2003). However, as growth rate, the ARA role is not clear where more studies are required.

Physiological ARA affects the juveniles ability to handle stress and regulating of cortisol by up regulating through the hypothalamus – pituitary – interrenal axis (Bell & Sargent, 2003).
In Atlantic halibut studies improves have been observed when adding extra ARA in broodstock feed. The eggs and larvae quality has been increased. Also from other marine species adding ARA is suggested (J. Castell *et al*., 2003). If the larvae and juvenile have higher ARA levels it should affect the quality in production. The effect of ARA in feed is being studied, and the effect and importance for new dietary formulas is being evaluated. Studies has shown that ARA added to pellets had an improved effect on its broodstock offspring (Bell *et al*., 1997; Bruce *et al*., 1999). This study aims to use this information in order to see if different levels of ARA have an impact on the quality and survival of ballan wrasse, and will use the study of (K. Hamre *et al*., 2013) for a better understanding. Part of this study will look at the effect on both quantity and quality of eggs, while checking if ARA has an effect on broodstock growth and health as well.

1.5 Aim of the study

Project Clean life cycle focuses on quality criteria for cleaner fish and the effect of broodstock nutrition on improving health and survival for cleaner fish. In order to improve production of ballan wrasse there is a need for better knowledge about feed for ballan wrasse broodstock. Issues with survival and health have been an important focus in the cleaner fish industry and the biology and requirement for ballan wrasse needs to be in focus.
This study will focus on how different ARA levels in ballan wrasse broodstock feed will affect the quality of eggs.

I. How different ARA levels in the broodstock feed affect egg quality.
II. How does ARA levels in feed effect the health and growth of broodstock.
III. Develop an objective alternate to traditional eye scoring to better estimate the number of eggs.

2. Materials and Methods

2.1 Experimental design

The study was done at the Institute of marine research (IMR from here) with funding from FHF, as part of the project Clean life cycle. The fish farming and sampling were done at IMRs Austevoll research facility, and the lab work is done at IMRs Nordnes facility.
Since there is no commercial broodstock line of ballan wrasse, wild fish were caught outside Austevoll with a net as broodstock two years ahead (2017) of the experiment to acclimate and settle fish into captivity to ensure spawning. The fish was then screened for sex and divided into different tanks with the same sex ratio in each tank.

The temperature was adjusted during the year and increased during the early spring/late winter to induce maturity and for the fish to follow its natural cycle.
The fish were held in three large tanks 3 meters in diameter and given regular food (Skretting clean soft with added shrimp 25%) until august 2019 when the project started. Tanks C8, C9, and C10 also represent the different diets (table 2).

The ARA enriched feed (diet) is a unique feed produced as cooperation between IMR and NOFIMA and had a calculated ARA difference (table 1) based on studies from Hamre *et al.*, (2013). The feed was produced by NOFIMA and made with cold extrusion for binding and materials is listed in figure.

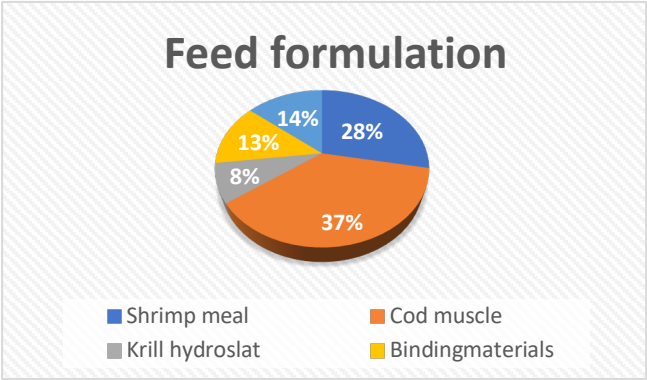


Figure 3: Feed formulation given to Ballan wrasse broodstock. *Feed formulation with different ingrediencies from K. Kousoulaki. The different ingredienses in the feed listed with percentage of total feed*

Table 1: Differences in percentage of ARA of total fatty acids added in feed. *Intentional difference in percentage of ARA added in the different treatments/ ARA diets of total fatty acids as planed when the project started.*

	Low ARA(C8)	**Medium ARA(C9)**	**High ARA(C10)**
Percentage of ARA of total fatty acids	1 %	1.5 %	3.5 %

Before the study started, all fish underwent treatment for diseases, and "weak" and injured fish were taken out of the experiment.

Each fish was tagged with a PIT-tag for individual follow-up. By doing this the fish could later be individually weighted, and signs on the outside as well as possible sex-changes could be observed, and their health status could be followed over time.

Ballan wrasse is a batch spawner and is organized as a harem (Bridie Grant, 2016). In this study, approximately 30 females and 3-4 males were released in each tank.

Table 2: Ballan wrasse broodstock overview of numbers of fish, sex ratio and average weight in different tanks. *Ballan wrasse initial broodstock with sex ratio and different average weight at the start of the study. Average weight with STD.DEV.*

Treatment*:*	**Low ARA**	**Medium ARA**	**High ARA**
Fish in total(n)	32	33	34
Female(n)	28	31	30
Male(n)	5	2	4
Average weight females(g)	813±234	830±185	839±171
Average weight males(g)	1215±226	1187±62	1182±169

During this period, the broodstock was anesthetized with sedation (Tricaine Methanesulfonate (MS222)) and weighed and lengthened five times a year, including two times looking for general health status and possible visible diseases (AGD, parasites).

Before the fish spawned, mats (bought from Lone tepper AS) were cut from a carpet roll and squared in correct dimensions (50X50 cm) for comparing the hatch in each treatment. A tag was placed in the middle of the mat with a hook made from plastic band.

When spawning was first detected by competent personnel using a brush to look for eggs, mats were placed out in the broodstock tanks (10 each time).

The following day the mats were put in incubators (figure 4) and treated with formalin for disinfection

Special incubator tanks were used, with the mats hanging from suspensions (figure 4). Oxygen was added through a tube and waterflow/exchanged of 2.5L/min. The water in the incubator held a temperature of 12 degrees.

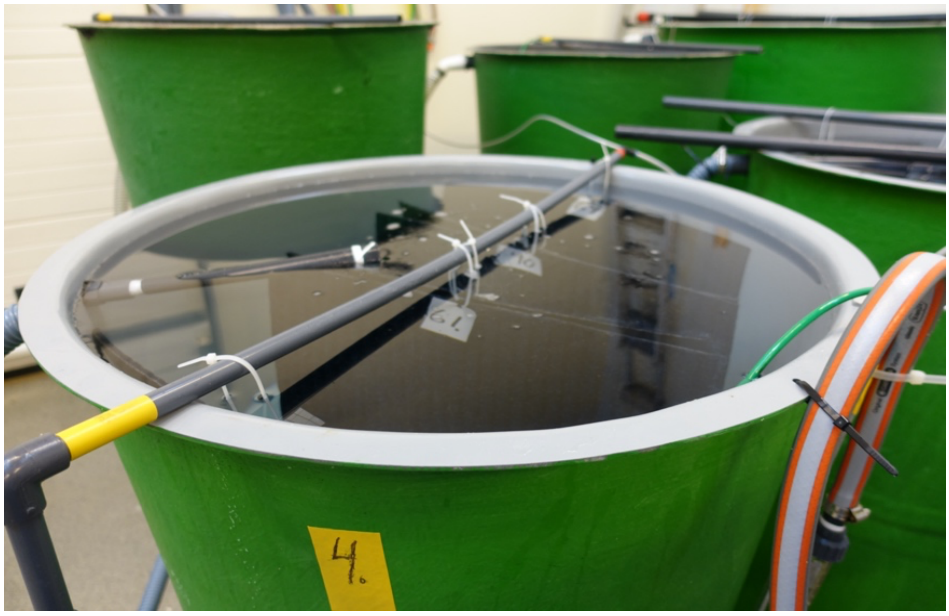


Figure 4: Incubation tanks with mats suspended from a plastic rod. *Incubation of mats suspended from a rod with its back against each other so that the surface faced water.*

Eggs were kept there for three days before being scored and photographed. The mats were then carefully returned to the incubators for hatching.

When taking the photo twenty mats were also scraped entirely for eggs for sampling and weight analysis. This was done with a plastic spoon scarping the entire mat, and eggs were placed in a 45mL plastic tube and frozen.

Some mats were also scarped for eggs and kept in 96% ethanol for dry-weighting analysis, and some kept at ice in minus 80 degrees Celsius for fatty acid profiling.

Eggs were collected during the spawning period from March to June, and the sampling was done as regularly as possible. However, due to the Covid-19 pandemic there was a problem with accessibility to the facility, but the sampling was still done as well as possible.

2.2 Quantification of egg amount:

Part of this project was to develop an alternate method to traditional eye scoring for quantifying the numbers of eggs produced. A new protocol was developed to standardize all possible parameter. A camera rig was made from steel and plastic for taking the photos used in the image analyses. During the study eggs was also frozen and later counted to estimate how mange eggs/pr gram. Also, for quantifying samples were taken and should be placed in alcohol to later dry weighing them for a known reference value for comparing to the analyses.
By doing this an exact number is possible to use to test the different methods.
The quantification of eggs was standardized to one gram mass for all samplings. By doing this a conversion factor is made to later use in calculation of numbers of eggs when weight of sample is measured (g). The following equation was used for this calculation:

$$\frac{Number\ of\ counted\ eggs\ in\ control\ sample}{weight\ of\ control\ sample(g)} = conversion\ factor$$

$$Coversion\ factor * weight\ of\ sample(g) = numbers\ of\ eggs$$

2.2.1 Mat analysis

For this experiment mats were specially produced ahead of the spawning. The mats were as earlier described made from carpet material, 50x50 cm, in a dark blue color and used as a substrate for the fish (figure 5), after recommendation from MOWI Labrus that had success with the very same color and size. The analysis of the number of eggs on the mats were done using two different methods—one with scoring by personnel (eyesore) and one based on image analysis. The image analysis was done using a program named ImageJ. This is an image-edit program that can analyze and estimate the percentage of color differences in pixels. By doing this the eggs will be seen as a different color than the rest of the mat, and we are then able to differentiate and estimate the number of eggs. For verification of the accuracy manual counting of one gram is done, and weighing of the same mats were also done, to give an exact number of eggs as possible and then compared with the Image analysis and the eye score calculated numbers.
Eggs were dry-weighted and counted on a plate giving a baseline for the quantification of the number of eggs. To get a calculated number the samples that were scraped off were also

evaporated of the ethanol, and dry weighted. The evaporation was done by water bath since the ethanol evaporates at 67 degrees Celsius, leaving just the eggs and some water.
After this is done, the eggs are freeze-dried, and the leftover water removed by sublimation. This process works by lowering the temperature, and with low pressure the water will go over to an ice face, and the ice will be removed when depressurize and adding heat, leaving just the eggs in a dry state.

2.2.1 Eye scoring of mats

The first method is done with eye score done by personnel at IMR. This is individually done but all personal that did this is competent and also went through a small visual training with the senior technical. Table 3 is a description of how the scoring system is designed. Eye score was done "one site" by competent personal at IMR Austevoll, and each sheet was filled in immediately after looking at the mats (appendix A6). The mat is divided in 4 zones and each zone gets is individual score since it is often the fish don't spawn over the whole mat.
After filling in the sheets, the mats were photographed and stored for analysis. This was done 787 times in total.

Table 3: Eye score scoring system. *Eye score system developed to score mats together with technicians at IMR Austevoll station. Different eye score listed, and their description was made to better understand the number of eggs produced in one mat.*

Eyescore	Description
0	Zero to no eggs on the mat
1	Some eggs, spread scarlessly
2	Some eggs, maybe lumps and in layers
3	Eggs in layers and in lumps
4	Eggs almost covering the whole mat and in layers and lumps

2.2.2 Image analysis

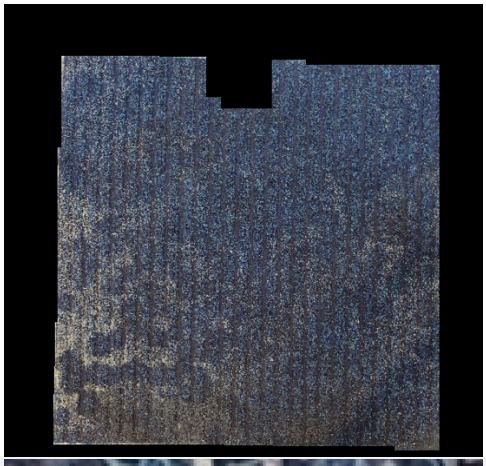
For image analysis quantification of the numbers of eggs produced, ImageJ was selected. ImageJ is a java based open-source image-processing program that is developed by University of Wisconsin and released in 1997.This program is able to differentiate in color and measure amount/percentages covered by different colors in the picture.

For standardizing the photos, a rig was made from metal and a plastic tray and used to make each photo as similar as possible. A Sony DSC-RX100M4 camera was used. A rig was developed to ensure that the pictures of mats were taken at the same distance from the mats (figure 5). The rig was standardized with a place for the camera and a mark where the mats should be placed for simplification.

The image analysis was done by image analysis program (ImageJ). The method produced scores that when comparing to actual amount on the mats were good. The data treatment was done during the summer after the process had been developed (figure 6). However, as with eye score, the method underestimated the numbers off eggs when the score is high.

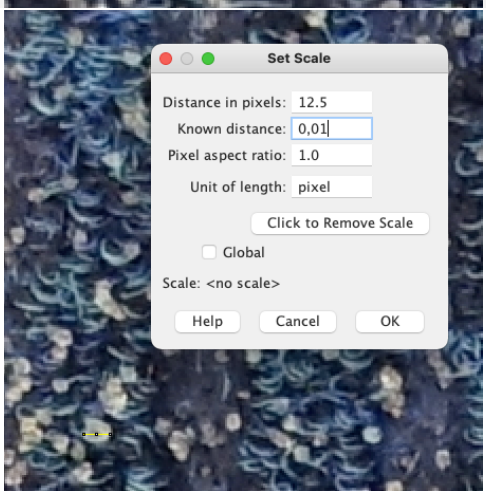
Analyzing the photos of each mat for the number of eggs to give a percentage of mat covered with eggs was done in summer 2020. The standardized method for analyzing the mats in ImageJ was done as followed:

Figure 5: Image of a mat covered with egg. *An image taken with the camera rig of a mat with its tag and eggs in different layers. Note that the tag for identify each mat is visible in the middle and suspending from a strip. This is the same strip used for suspending the mats when incubating (figure 4). This is a before picture of the mat.*

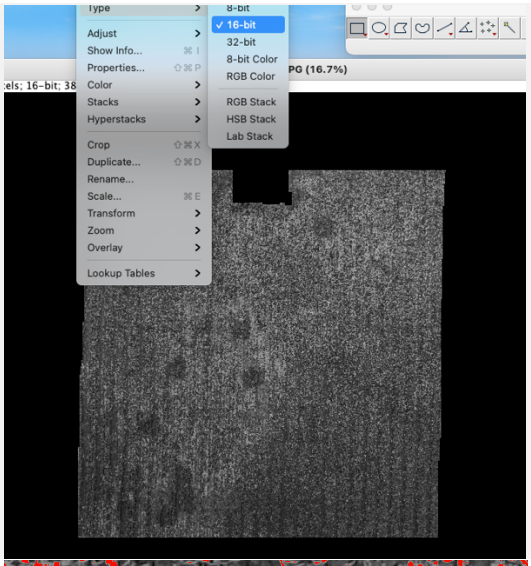


1. The outside of mat was first cropped so that as little as possible of the outside was visible. The mat is placed in the rig with the tag clearly visible in the picture. The cropping is manually done with a snipping tool
2. The tag is then also cropped out of the picture as best as possible.

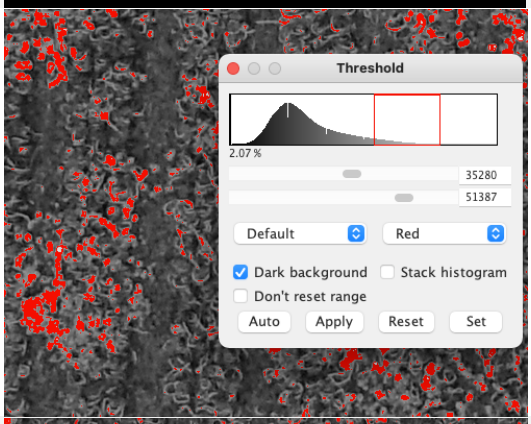
3. A scale was set for measurement of the eggs. A line of the egg was drawn to mark the egg



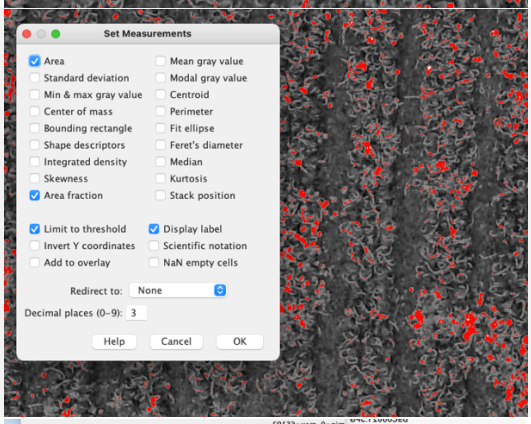
4. The distance was set to measure each egg



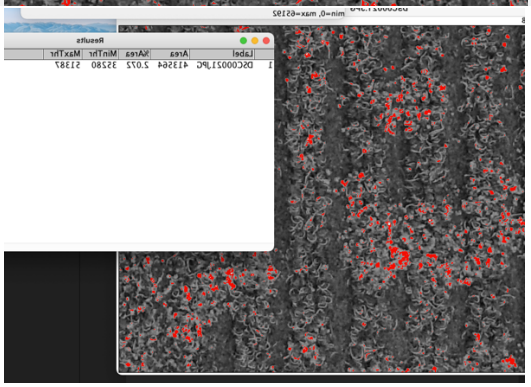
5. Then the photo was changed from a 32-bit photo. The photo was changed to a binary photo 16-bit.



6. A threshold was selected to differentiate between eggs and the mat, A threshold was manually selected. A close-up section of the mat to highlight that the eggs is marked red was done as good as possible, leaving as little as possible of the mat being red.



7. The measurements were selected to analyze each mat. The area fraction is selected to look at the percentage of the picture covered with eggs.
8. The mat is then measured



9. The results are shown in percentage with the threshold levels as well.

Figure 6: Image analysis data process for estimation of numbers of eggs. *Progress of mat analysis in ImageJ. Each stage is part of a protocol for an estimation of numbers of eggs on a mat.*

2.3 Fatty acid analysis in neutral and polar lipid.

A standardized method for analyzing different fatty acids was used to analyze to find the deposition of ARA and total fatty acids in eggs. Since ballan wrasse eggs are small (<1mm) and sticky, getting them homogenized was done by using a crusher as Hamre et., al (2013) did. Both the fatty acid composition in neutral and polar lipids was researched, as well as the percentages of total fatty acids.

We are doing this for separation with gas chromatography with hydrogen and comparing levels of fatty acids in their diet to see if there is any retention of the fatty acids given in the broodstock diet. For this method, an internal standard (19:0) is used as a known value to measure and identify fatty acids. In this experiment an internal standard was made together with competent personnel at IMR Nordnes. For control a salmon liver with known fatty acid composition and a control card was used, to verify the method being done correctly.

Since chemicals are used and quickly evaporated when in contact with air, safety measures were necessary. A covenant closet was used during the whole process. Many of the chemicals used are dangerous and cancer-inducing substances, so extra thick latex gloves were also used. Before the method started, the internal standard was made with the supervision of lab technicians.

This was done by weighing 0.0005 mg metylnonadekonat (19:0) together with chloroform: methanol, and the dilution needed to be fully dissolved. After this was done the measuring flask was put aside and marked for later use.

The samples were then first defrosted 30 min before being weighed. Approximately 1 gram of eggs was taken from each sample and placed in a glass tube.

The eggs were homogenized with a blender/crusher as best as possible for 45 seconds in 2mL of chloroform: methanol solution in a glass tube. This left the mass of the eggs.

The blender was then rinsed in a new tube of 2 mL chloroform/methanol to extract as much as possible of the sample. Both glass tubes were then placed in the freezer (-20 Celcius) to extract the lipids overnight for the separation of fatty acids.

After the blender was used, it was also rinsed thoroughly in the same mix (chloroform/methanol) in another glass tube and dried as well as possible to get rid of excess material on the blender before the process was repeated.

The next day the samples were defrosted and shaken thoroughly on a whirling mixer for 30 seconds for each tube. The samples were then filtrated to extract the lipids with a vacuum block with 4mL of the same methanol/chloroform into a sovirel tube.

This process works so that only the lipids will go through the filter and down to the sovirel tube, leaving the rest of the sample outside the filter.

The lipid extract was then evaporated in a RapidVap system at speed 50%, heat 40 %, and pressure 300 Mbar for approximately 20 minutes with close surveillance and regulation of the RapidVap. After the evaporation, the 200 ul of 2%metanol/chloroform was added in the sovirel tube and thoroughly mixed again.

The materials were then filtrated through a new column into a new sovirel tube with first neutral lipids with 2% methanol: chloride and later on polar lipids. For getting the polar lipids, 5 mL of methanol was used for separating the lipids.

After the sample was divided into two parts, an internal standard was added to both sovirel tubes. Then the sample was evaporated again and added NaOH. The samples were then shaken and placed on a block heater for 15 min at 100 degrees.

After being heated, the samples were cooled down with spring water and added BF3. Then they were shaken and heated for five more minutes.

The cooling process was then repeated, but 2mL hexane and 2 mL distilled water were added. After this process has been done, the samples can either be extracted or frozen for later analysis in the finale stage.

When extracting the lipid phase, a table centrifuge was used up to 3000 round per second. When centrifuging the sample will separate, and the hexane phase can be extracted. For securing that the whole lipid phase is collected, the process can be repeated by adding another 2 mL of hexane and the fatty acid phase is extracted.

After this, the hexane sample with lipids extract can be stored or analyzed right away.

For final analyzing the extract is first diluted with more hexane (1 mL) before being placed in the machine.

The machine works with hydrogen and allows the different fatty acids to travel down a colon. The further the fatty acids travel, the longer it is. By using the internal standard, it is also possible to quantify the amount of different fatty acids.

For doing this, a software called Chromeleon (Thermo Scientific Chromeleon Chromatography Data System (CDS) software) was used to quantify and identify the different fatty acids. For securing that the quantification is done the right way, manual integration will be done as well

guided by technicians. This is possible due to the known value of internal standards and the placement along the fatty acid's axes.

2.4 Health status

During the sampling when registering growth, the health status of the broodstock fish was checked and registered for follow up during the period. Both parasite and other visible diseases were notified and registered for follow up of fish health status.

Amoebic gill disease (AGD)

A visual evaluation of the gills was performed to check for Amoebic gill disease caused by *Paramoeba perurans* on all broodstock individuals. This was done visually by opening a looking at the gills and separating the arches. A patchy grey area is associated with AGD pathology and classified as this. No scoring of AGD was done, just the actual presence of AGD was notified.

2.5 Statistical analysis and data treatment

Data were analyzed in Microsoft Excel and in R-studio. Growth data were handwritten under the sampling. For the eye-score the score was written in schemes and transferred to excel for analysis. An Kruskal-Wallis test was exercised for the lipid analysis. This test was used because of its usage with data that has a significant difference between the groups tested.

An ANOVA test with a TUKEY HSD post doc test was used for looking at the differences between the diets for significance. The same test was used for egg diameter vs lipids for analysis. For graphical showing, the data was presented in both Excel and R, using both scatterplot and column. For adaption of curve, both linear and power adaption was tried to fit the data when plotted.

Specific growth rate (SGR)

SGR was used as a measurement of growth for the broodstock during the period using the following formula:

$$SGR = \frac{lnWeightM_2 - lnWeightM_1}{Days} * 100$$

The weight for each sampling point was used for measuring the SGR and done for all diets.

M = each month, Days = days from one sampling to another. For analysis a two-way ANOVA were used to look at the effect. For analyzing the effect of growth against the different diets and time a TUKEY HSD was exercised.

3. Results

3.1 Analysis of feed

Analyzed percentage of ARA of total fatty acids in the broodstock feed was higher than the initial planned values (table 4). The low ARA diet was analyzed to 0.70 % more ARA than formulated. Medium ARA diet was 0.80% higher, and the high ARA diet was 2.10 % higher than the initial values presented in table 4.

Table 4: ARA percentages in feed fed to the broodstock. *ARA percentage of total fatty acids in the three different diets fed to the broodstock. The percentage difference is calculated and compared to the initial levels of ARA.*

Diet	Low ARA	Medium ARA	High ARA
Initial planned percentage of ARA (%)	1 %	1.5 %	3.5 %
ARA levels analysed in feed (%)	1.7 %	2.3 %	5.6 %
Percentage's difference (%)	0.70%	0.80%	2.10%

3.2 Quantification of egg amount:

Size samples of eggs was weighted and counted. Six samples were taken during the sampling period and eggs was then counted. All samples (1-6) were standardized to form the original weight to numbers of eggs per one gram. Results from all six samples showed an average egg number of 923 eggs per gram (table 5).

Table 5: Egg estimation for broodstock fish. *Eggs were weighted and counted before being standardized to one gram. Total number of six samplings were conducted having a mean egg count of 923 pr gram. Total egg numbers counted was 4465 (n=78).*

Sampling	Gram	Counted eggs	Eggs pr 1 gram
1	0.52	637	943
2	1.10	1241	1117
3	0.53	592	876
4	1.31	1411	974
5	0.49	582	879
6	1.51	1535	752
	Total		4463
	Average	923	

3.2.1 Rig used to standardize photographing of mats

A standardize rig was developed as part of the project to take consistent photos for image analyses. Results from the image analyses showed consistent photographs that enabled sufficient quantification of egg number on mats from ballan wrasse broodstock fish (figure 7).

Figure 7: Development of image analyses method. *Camera rig used for photographing of mats. The camera is placed in the white ring (arrow 1) and the mats is placed in at the plastic tray (arrow 2). The red mark in front marks where the tag end of the mat is placed (arrow 3).*

3.2.2 Image analysis model

Data from the image analysis in ImageJ was put in a scatter plot and compared to weight of the sample. 78 samples were both weighted (converted to numbers of eggs) and analyzed for comparison of the image analysis and the actual numbers produced.
Results showed with a linear adaption had an R^2 value at 0,9422. The line representative for image analysis was estimated til y = 5995,8x (figure 8).

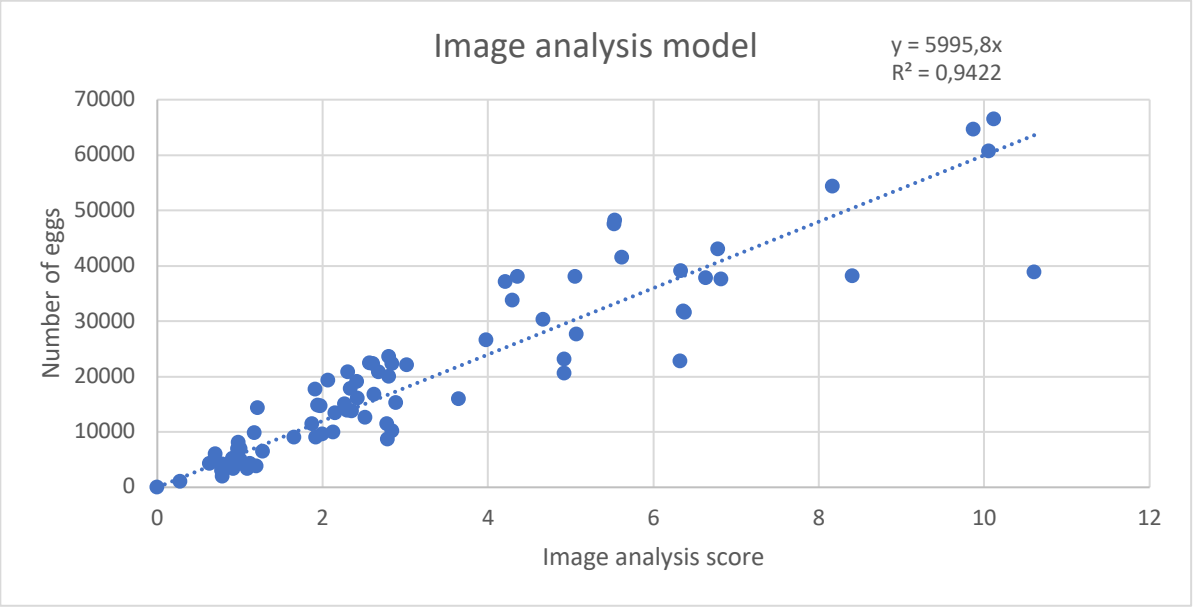


Figure 8: Image analysis model and numbers of eggs produced. *Image analysis model vs numbers of eggs with a linear adaption as marked (y = 5995,8x). The data sets R^2 = 0,9422 and is representative for its adaption to the data set. On the x-axis is the image analysis score while on the y-axis represents the numbers of eggs produced is represented, respectively (n=78).*

3.2.3 Eye score model

The results from the eye scoring of mats was analyzed and put in a plot for comparison of numbers of eggs and the eye score number. As with image analysis 78 samples was compared and both a linear and power adaption was used to estimate the model's accuracy. The linear adaption had an $R^2 = 0,8865$ and the power adaption $R^2 = 0,7938$. For both models an equation was estimated for later use and for the linear modulation was $y = 10721x$. For the power adaption it was $y = 4376,9x^{1,8402}$ (figure 9).

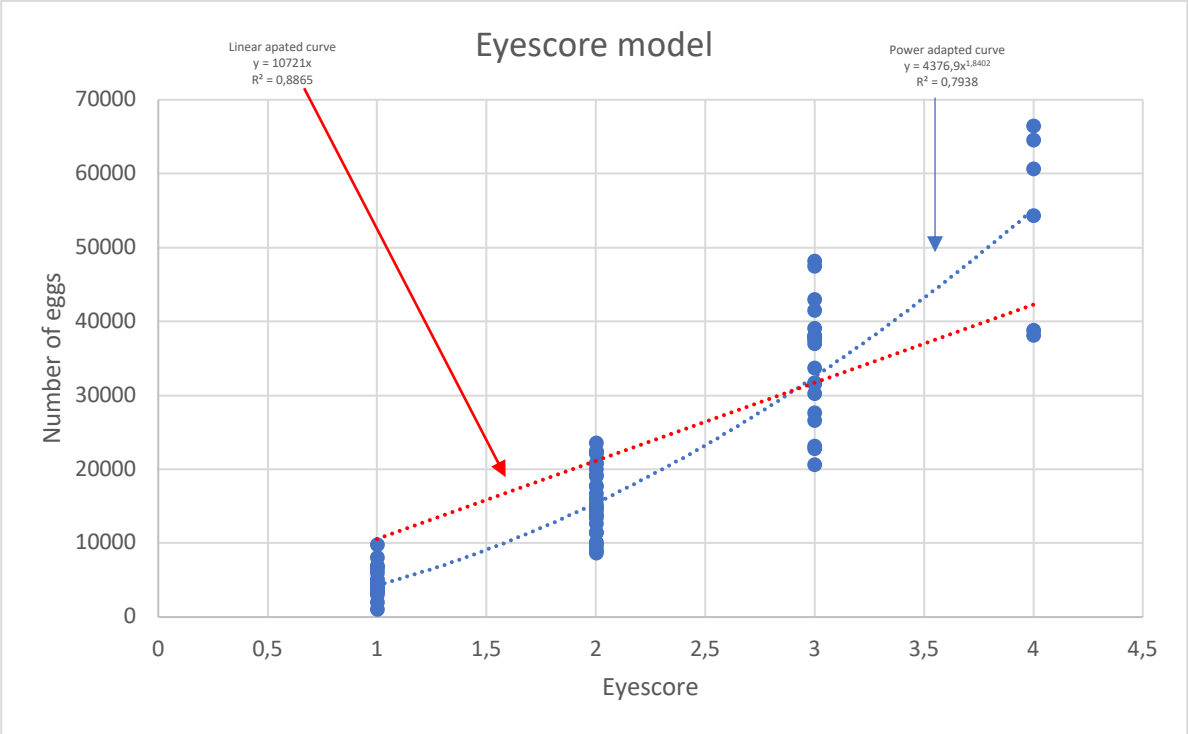


Figure 9: Eye score models and numbers of eggs produced. *Eye score model vs numbers of eggs with both a linear adaption and a power adaption. Red arrow marks the linear adaption while the blue represents the power adaption. Linear adaption ($y = 10721x$) with an $R^2 = 0,8865$. The power adaption ($y = 4376,9x^{1,8402}$) with an $R^2 = 0,7938$. X-value is the image analysis score while the y-axis represents the numbers of eggs produced (n=78).*

3.2.4 Numbers of eggs pr cm^2 of mats for both methods.

The coverage of eggs in one cm^2 was estimated from image analysis and compared to the actual numbers of eggs produced. When adding a linear adaption, the estimated $R^2 = 0,9422$ and the line: $y = 0,0039x$ (figure 10). When comparing the different diets coverage of eggs in cm^2 the medium ARA diets had the highest production of eggs. The same is also observed for females where cm^2 of coverage for each kg female fish the medium ARA diet was superior. The low ARA diet produced lowest numbers in all categories while the high ARA diet was in the middle (table 6).

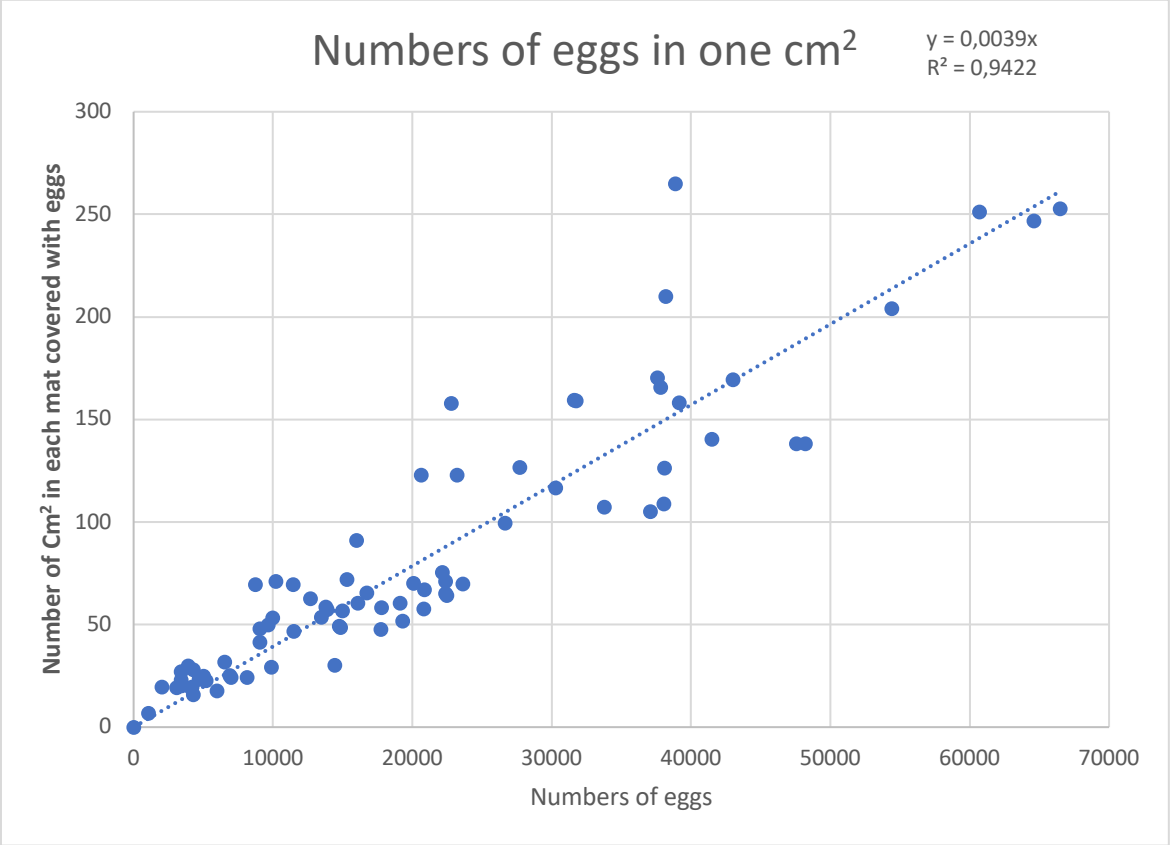


Figure 10: Mat coverage of eggs in one cm^2. *Correlation analysis using numbers of eggs per cm^2 in each mat vs actual numbers of eggs. X-axis represents numbers of eggs and y-axis is coverage. The linear adaption ($y = 0,0039x$) and with an $R^2 = 0,9422$ ($n=78$)*

Table 6: Egg coverage (Cm^2) and numbers for each female with different ARA enriched diets. *Egg coverage (Cm^2) and number of eggs are shown in total for each female in the low ARA diet, the medium ARA diet and the high ARA diet. Also presented are total numbers of eggs produced pr cm^2 per female.*

	Cm^2 in total	Cm^2 pr female fish	Cm^2 pr kg female	Number of eggs pr cm^2 per female
Low ARA (n=24)	6738.68	280.78	0.30	263
Med ARA (n=27)	9013.77	333.84	0.34	374
High ARA (n=27)	8339.83	308.88	0.31	363

3.2.5 Egg production estimation during the spawning period for image analyses and eye score.

Results from both analyses showed the numbers of eggs produced over the spawning period. For both models 561 mats were analyzed and tended to produce larger batches in the start of the spawning period (figure 11, 12). Analysis showed that the medium ARA diet produced the most eggs with the high ARA coming in second and low ARA lowest in both methods (table 7). Numbers of eggs produced by each female was also calculated with both methods showing the medium ARA diet producing best. In the eye score model the low ARA produced second best and high ARA worst while with Image analysis the high ARA produced second best and low ARA worst (table 8).

Analysis was also done to look at numbers of eggs produced by each female pr kg showed the medium ARA diets producing superior with high ARA in second and low ARA works with Image analysis. For eye score medium ARA produced best with high ARA in second and low ARA lowest (table 9).

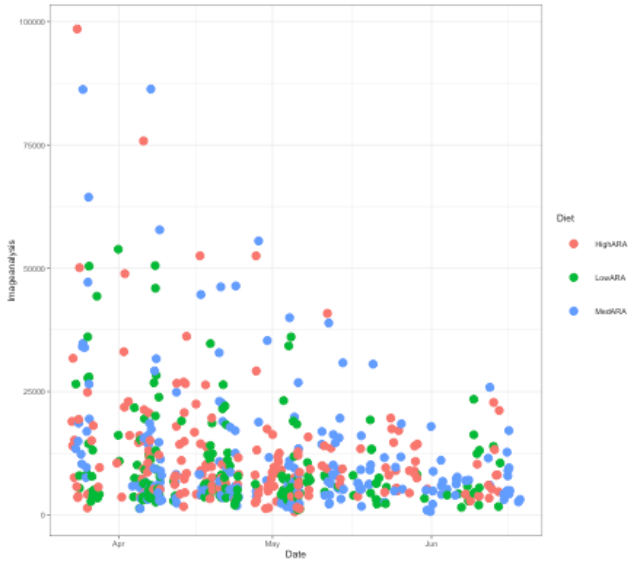


Figure 11: Numbers of eggs produced when using image analysis. *Numbers of eggs produced when analyzing with image analysis over the spawning period. Data is X-axis with numbers of eggs y-axis (in 1000). Color marks the different diets (green=low ARA diet, blue=medium ARA diet, red=high ARA diet. (n=561)*

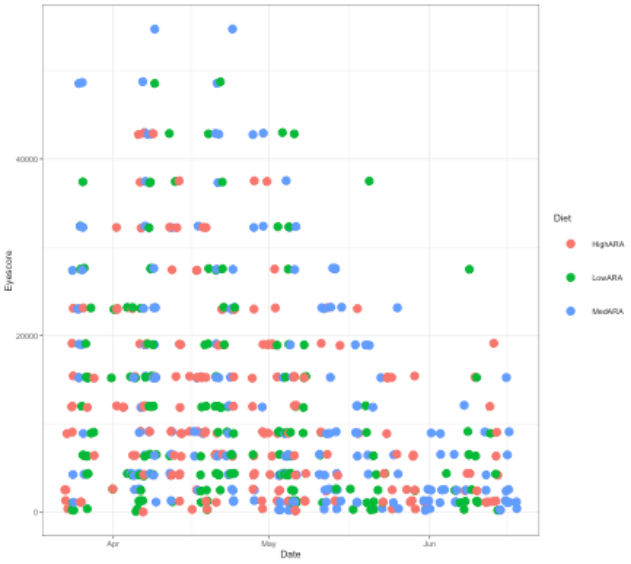


Figure 12: Numbers of eggs produced when using eye score analysis. *numbers of eggs produced when analyzing with eye score over the spawning period. Data is X-axis with numbers of eggs y-axis (in 1000). Color marks the different diets (green=low ARA diet, blue=medium ARA diet, red=high ARA diet. (n=56)*

Table 7: Overview of numbers of eggs produced by each diet by both methods. *Numbers of eggs calculated for the different diets with eye score and image analysis. For both analysis the medium ARA diets produced most eggs with high ARA in second and low ARA lowest. n= numbers of female fish fed each diet.*

Diet	Numbers of eggs calculated with eye score model	Numbers of eggs calculated with image analysis model
Low ARA (n=24)	2 021 809	1 773 337
Med ARA (n=27)	2 556 552	2 372 046
High ARA (n=27)	2 102 871	2 194 691
Total	6 681 233	6 340 073

***Table 8: Numbers of eggs for each female produced in different diets for both methods**. Numbers of eggs produced by each diet for both image analysis and eye score. For eye score the medium ARA diets produced best with low ARA in second and high ARA lowest. For image analysis the medium ARA diets produced best with high ARA in second and low ARA lowest. n= numbers of female fish fed each diet.*

Diet	Numbers of eggs for each female eye score model	Numbers of eggs for each female image analysis
Low ARA (n=24)	84 242	73 889
Med ARA (n=27)	94 687	87 854
High ARA (n=27)	77 884	81 285

***Table 9: Numbers of eggs pr kg female produced by different diets with both methods.** Numbers of eggs produced pr kg female by the different diets and estimated with both methods. For eye score the medium ARA diets produced best with low ARA in second and high ARA lowest. For image analysis the medium ARA diets produced best with high ARA in second and low ARA lowest. n= numbers of female fish fed each diet*

Diet	Numbers of eggs pr kg female eye score	Numbers of eggs pr kg female image analysis
Low ARA (n=24)	90.19	79.11
Med ARA (n=27	95.93	89.01
High ARA (n=27)	78.91	82.36

3.3 Fertilization rate

Fertilization rate was calculated from eye scoring sheets (appendix 6). The fertilization rate for the low ARA diets produced the best score 78.37 %. The medium ARA diet produced second with 74.45 %. The high ARA diet scored lowest with 63.84 %.

***Table 10. Comparison of fertilization rate for each diet.** Fertilization rate in percentage for each diet with low ARA diet producing the highest fertilization rate, medium ARA diet in second and high ARA lowest. n= numbers of sheets the fertilization rate is calculated from)*

Diet	Fertilization (in %)
Low ARA (n=178)	78.37%
Med ARA (n=198)	74.45%

High ARA (n=185)	63.84%

3.4 Hatching rate

Hatching rate was calculated after looking at numbers of larvae produced, and numbers of eggs produced.

Table 11: Overview of hatching rate. *Hatching rate and number of larvae produced. Estimated numbers of eggs was calculated using the image analysis (n=10).*

Diet (n=10)	Number of larvae	Number of eggs	Fertilization percentage (%)	Hatching percentage
High ARA	14000	34 843	95,55 %	59.82%
Low ARA	64000	96 489	57,41 %	33.67%
High ARA	45400	85 768	60,95 %	47.07%
High ARA	53000	85 768	95,47 %	38.21%
Med ARA	27250	45 564	52,77 %	40.19%
Low ARA	73000	101 850	77,02 %	28.33%
Med ARA	36933	42 884	98,65 %	13.88%
Med ARA	30500	37 524	90,72 %	18.72%
Low ARA	21200	31 418	77,37 %	67,48%
Med ARA	70000	183 460	80,88 %	38,16%

3.5 Broodstock water temperature

The temperature in the broodstock tanks was registered during the whole period from august 2019 to august 2020. During this period, the temperature in the three tanks was almost identical, but there were some spikes. In the low ARA tanks, a spike towards the end at 16 ºC was registered. In both the medium ARA diet and high ARA diet, there was an early spike at 16 ºC (figure 13,14,15).

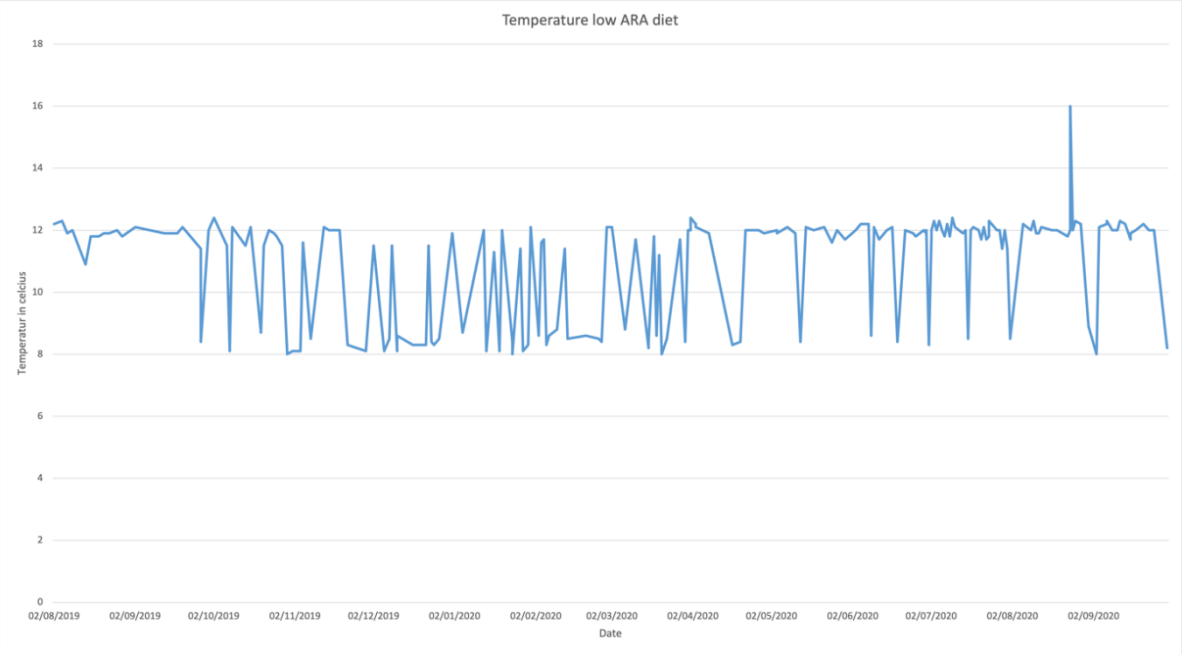


Figure 13: Temperature in the tank fed the low ARA diet. *Temperature during period for low ARA diet fed broodstock. X-axis=date, y-axis= temperature in* ^{o}C.

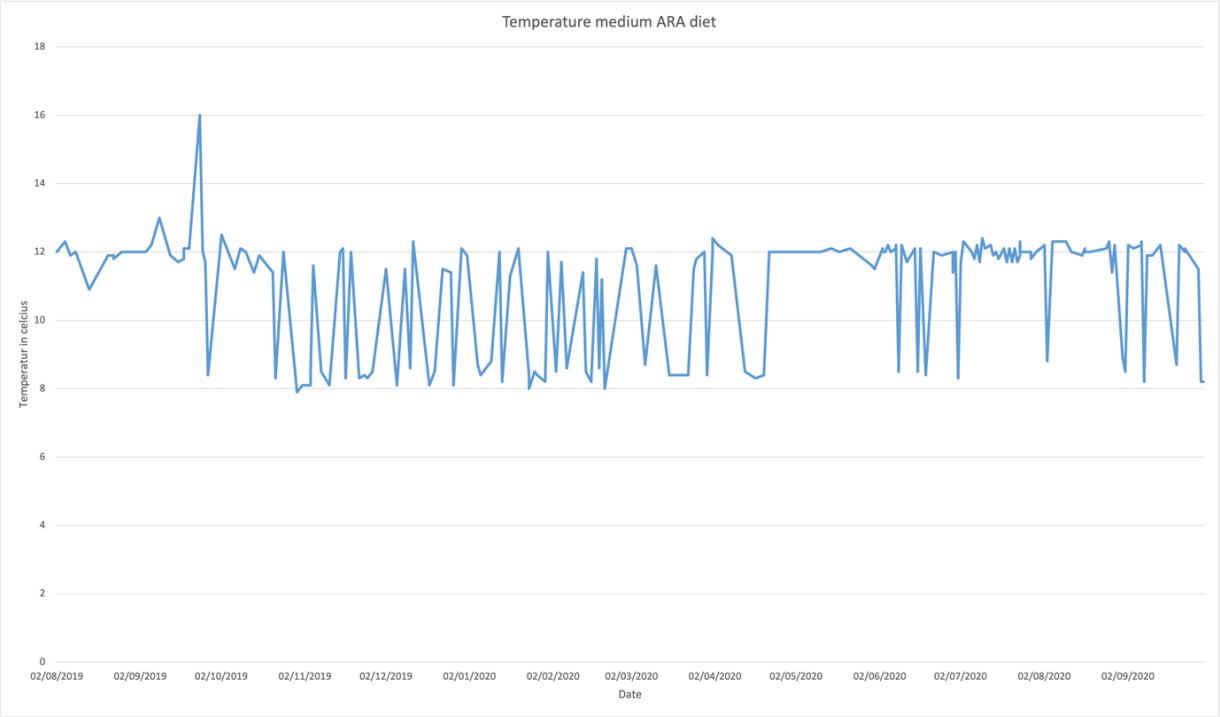


Figure 14: Temperature in the tank fed the medium ARA diet. *Temperature during period for medium ARA diet fed broodstock. X-axis=date, y-axis= temperature in* ^{o}C

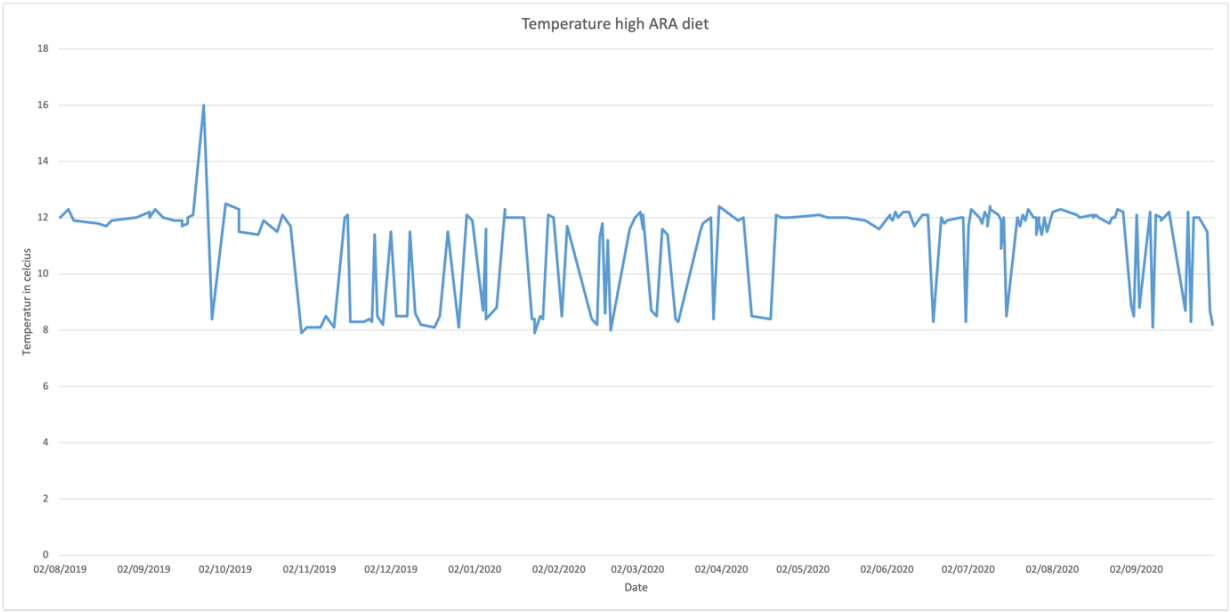


Figure 15: Temperature in the tank fed the high ARA diet. *Temperature during period for high ARA diet fed broodstock. X-axis=date, y-axis= temperature in* ^{o}C

3.6 Broodstock growth

Broodstock fish was weighted five times during the period. The specific growth rate was calculated for each sampling point with the august weighting as a starting point

When making a model for growth in R-studio, ggplot showed the difference in SGR over time (figure 16). The dataset showed that the fish lost weight during the winter and gained weight towards the summer (figure 16).

Time had a significant effect on SGR (P=e-16), while diet had no effect (P= 0.8315). However, when looking if both affected, there is no significance (P = 0.2486).

Since time had a significant effect, a TUKEY HSD was done, February and November (P= 0.00), March and November (P= 0.00), September and November (P=0.00), March and February (P=0.00), September and February (P=0.00). The only not significant effect was between September and March (P=0.9639).

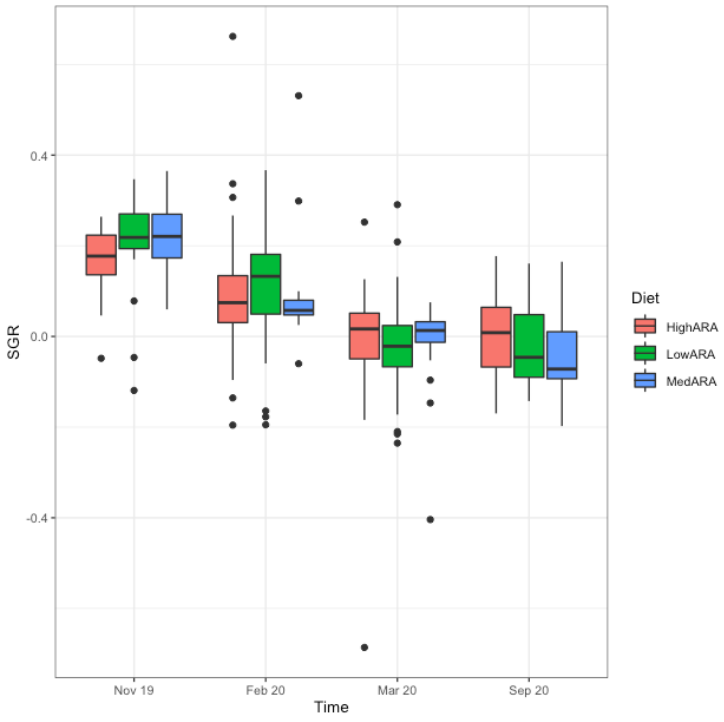


Figure 16: Specific growth rate for the broodstock fish. *SGR over time at four different sampling points.0 Mean of each diet is also marked and the outliers as well (green=low ARA, blue=medium ARA, red= high ARA). Outliners is marked as a black dot over and under the line for extremes. X-axis=Date, y-axis= SGR. n=24 for low ARA, 27 for medium, 27 for high ARA.*

3.7 Broodstock fish health status

Fish health for the broodstock (both sexes) was followed during the period from August 2019 to September 2020. During this period, two fish were euthanized and taken out from the tank. The mortalities were observed in-tank fed low ARA and high ARA.

Some injuries were also observed in the tanks at different times. During the winter, all tanks increased both AGD and different skin injuries (table 12, table 13, table 14).

In the low ARA group, ten fish lost their injuries when observed at the end of September. For fish fed medium ARA diet 12 fish lost their injuries. For a high ARA diet, 18 fish lost their injuries during the time period.

Table 12: Broodstock health status for the fish fed low ARA diet. *Fish health status fish fed low ARA diet (n=33). Different injuries were registered at all sample points. Fish were followed up individually for health status and updates during the period.*

PIT tag	Injuries August 2020	Injuries November 2020	Injuries February 2020	Injuries September 2020
1594		AGD		
4074			Inflated	
4092			AGD	
4130		AGD	AGD	Inflated
4150			AGD	Inflated
4175				
4195			Wound	
4227				
4229			Inflated, AGD	
4261			AGD	
4310		AGD	Parasite	
4316				
4354				split fin, loss of scales
4374				Dorsal fin injury, loss of scales
4444		AGD	Wound, AGD	
4532				
4581		AGD	Inflated with exophthalmia	
4591		AGD, wounds	Inflated, AGD	
4603				Dorsal fin wound, loss of scales
4617			Inflated, AGD	
4736		AGD		
4743				Loss of scales
4765		AGD, wounds	AGD	Loss of scales, humpback
4837				
4868				
4902		exophthalmia	Dorsal fin deformities, AGD	Dorsal fin injury, loss of scales
4929			Inflated	exophthalmia
4961				Inflated
4992			Wound	
9534		Dorsal fin injury		
9568		AGD		
9787		AGD	AGD	
Numbers of injuries		12	17	10

Table 13: Broodstock health status for the fish fed medium ARA diet. *Fish health status fish fed medium ARA diet(n=32). Different injuries were registered at all sample points. Fish were followed up individually for health status and updates during the period.*

PIT tag	Injuries August 2019	Injuries November 2019	Injuries February 2020	Injuries September 2020
2010				
4049			Wounds	Inflated, Loss of scales
4099			AGD	
4124			Inflated	
4160			Wounds	
4198			AGD	
4274		AGD	parasite, Inflated, AGD	Inflated
4286				
4319			AGD	
4336			AGD	Loss of scales
4367		AGD		
4385		Very blue		Loss of scales
4395		AGD	AGD	
4439				
4445				Humpback
4476		AGD		
4511				Inflated, Loss of scales
4565			Wounds, AGD	Loss of scales
4583			AGD	
4590			Inflated, AGD	Loss of scales
4605		Inflated	Inflated	
4607		AGD		
4651		AGD, injury right eye	Inflated	Inflated
4727		AGd	Inflated	
4768		AGD		
4769				
4817			Wounds	
4892				
4921			Inflated, AGD	
4957			AGD	
4964			Wounds	Inflated
4986			AGD	Humpback
5010			AGD	Inflated
Numbers of injuries		10	21	12

Table 14: Broodstock health status for the fish fed high ARA diet. *Fish health status fish fed high ARA diet(n=35). Different injuries were registered at all sample points. Fish were followed up individually for health status and updates during the period.*

PIT tag	Injuries august 2019	Injuries November 2019	Injuries February 2020	Injuries September 2020
4057		Wounds		
4107		Wounds	Wounds	
4140	Eye injury, thin	Eye injury, very pale	Euthanized	
4142		Fin injury	Inflated, AGD	
4178		AGD	AGD	Loss of scales
4212		Wounds	Inflated	
4234				
4275		AGD, fin injury	Wounds	Loss of scales
4279		Exophthalmia	AGD	
4302	Gold	AGD	Inflated	
4379			Inflated	
4401			Inflated, AGD	
4417		Humpback	AGD	Deformed back
4498		Wounds	Inflated, 2 parasites	
4553			Wounds	
4587				
7704				
4609			Inflated, wounds	
4620			Wounds	
4649				
4657			Wounds	Fin injury
4665			AGD	
4718		AGD	AGD	Humpback
4741		Wounds		
4806			AGD	
4822			AGD	
4826			Inflated	Katarakt
4914			AGD	
4931			AGD	
4954				
4959			Very Inflated	
5016			AGD	
5038		AGD, Fin injury	AGD	Fin injury
9770				Loss of scales
Number of injuries		14	26	8

3.8 ARA deposition in eggs in neutral and polar fractions.

When the eggs were sampled as described above, some were frozen at minus 80 degrees Celsius and kept frozen for lipid analysis.
Results from the analysis showed that there was more deposition of ARA in eggs in the high ARA diet for both the polar and the neutral fractions. The deposition of eggs from polar lipids was higher than in the neutral fraction (figure 17, table 15).

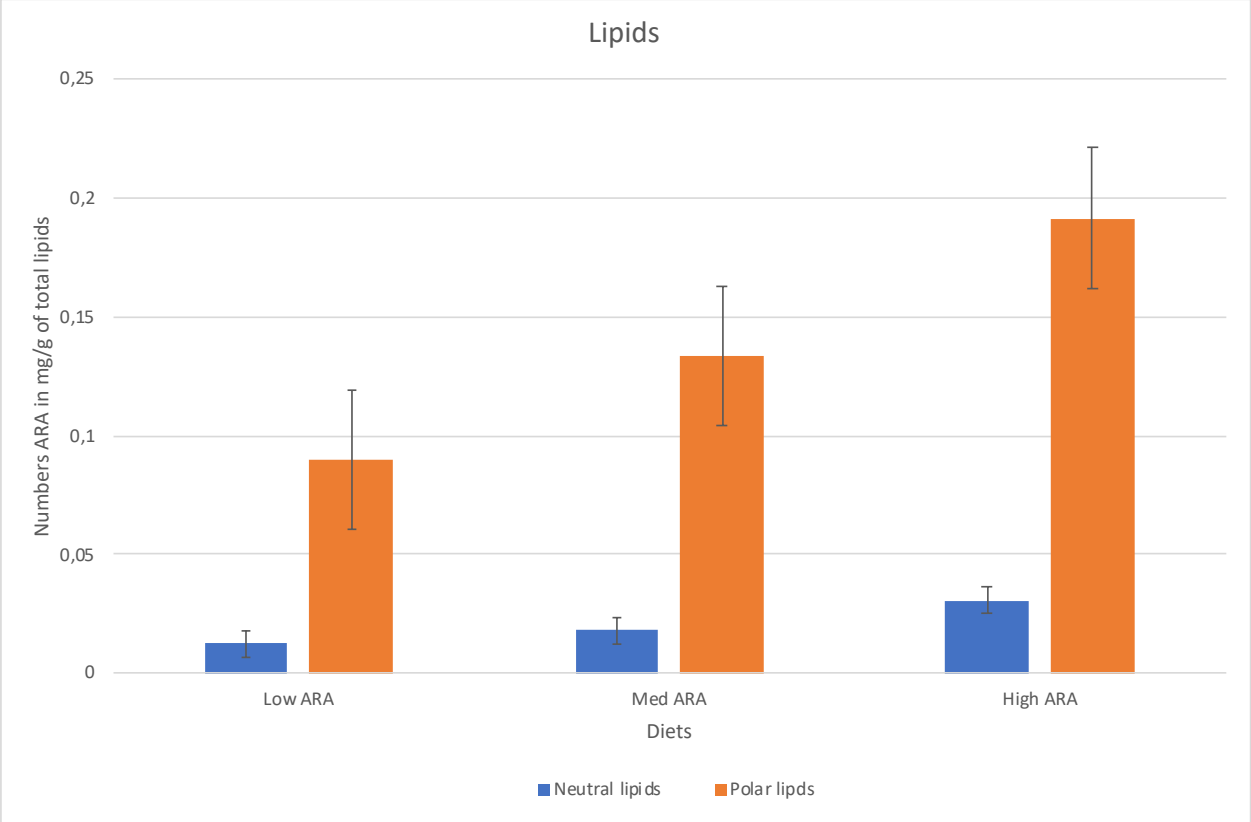


Figure 17: Numbers of ARA in mg/g of total fatty acids in eggs. *Comparison of ARA deposition in eggs in polar and neutral fraction of total fatty acids for the different diets. X- axis represents diets, while y-axis represents percentages of ARA in mg/g of total lipids.*

Table 15: Average percentage of ARA of total fatty acids *Average percentage ARA of total fatty acids when analyzing. divided in neutral and polar fraction. The high ARA diet had most deposition in eggs, while medium ARA in second and low ARA last.*

	Low ARA (n=6)	Med ARA (n=6)	High ARA (n=6)
Neutral lipid	1.57%	1.57%	2.35%
Polar lipid	3.55%	3.72%	5.97%

3.9 Total lipid analyses

A quantification of total number of lipids was done as well. In both fraction polar/neutral the medium high ARA diet tends to have more retention of total lipids. As with the quantification of ARA the polar lipids had more retention in total than the neutral.

For the polar lipids the p-value = 0.4233. For the neutral lipids the p-value = 0.6449

When doing a TUKEYHSD test there is no significance in both tests (appendix 4,5)

In the polar lipid retention, the p-value between the high ARA and Low ARA (P=0.8154). Between the med ARA and high ARA (P= 0.9623). Between the med ARA and low ARA p-value is (P=0.6625).

For the neutral retention the p-value between the low ARA and high ARA is (P=0.700). Between the med ARA and high ARA is (P=0.9604). For the med ARA and low ARA is (P=0.8677).

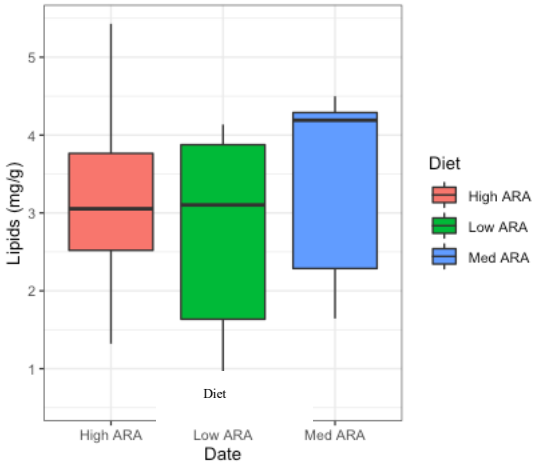


Figure 18. Total number of polar lipids(mg/g). *On the x-axis lipids pr mg/g are shown. Y-axis is different diets. Average is marked with a line in each column (n= 15).*

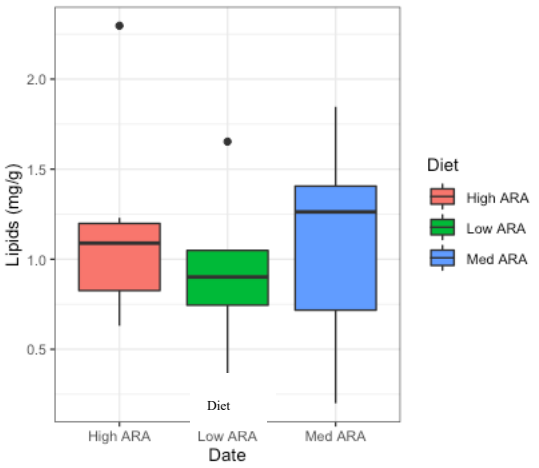


Figure 19. Total number of neutral lipids (mg/g). *On the x-axis lipids pr mg/g are shown. Y-axis is different diets. Average is marked with a line in each column (n= 15.*

3.10 Egg diameter vs lipids

Eggs diameter was measured in order to look for differences in size compared to diet. When analyzed the high ARA diet had the largest eggs, with med ARA having little less and low ARA the smallest.

A Kruskal-Wallis test were performed for looking if there is a significant trend. With P of 0.0068 there is no significance in diets vs diameter. To look if diet had an effect an TUKEYHD test was done with low ARA and high ARA (P= 0.0325). For med ARA and High ARA (P= 0.7517), For med ARA and low ARA (P=0.1914).

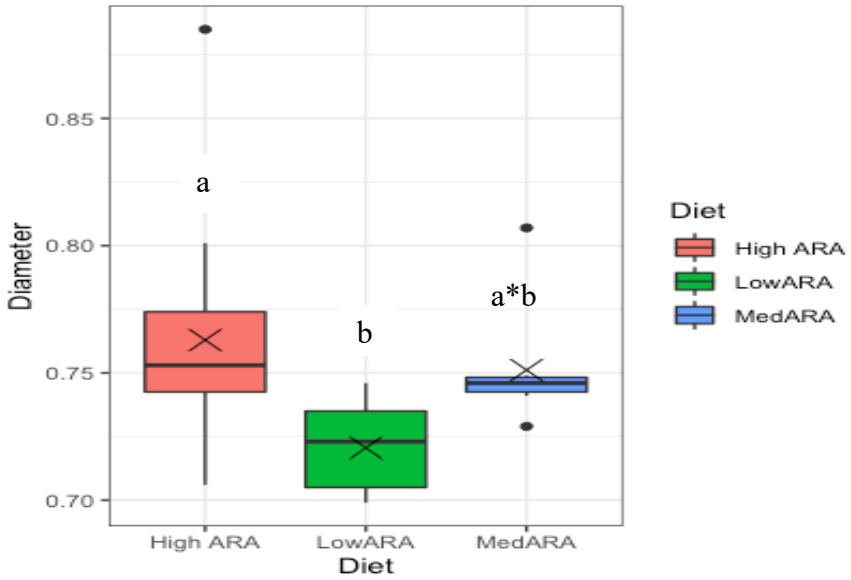


Figure 20: Diameter vs diet*. Diameter vs diet with STD.dev and mean for the different diets (green=low ARA diet, blue=medium ARA diet, red=high ARA diet). Significant data point are marked with different lower letter. (n=28)*

5. Discussion

This study aimed to look at different diets of ARA fed to ballan wrasse broodstock and the effects on its egg development. A novel method for quantifying eggs was also tried and an image analysis was developed to give an alternate to eye scoring.
The goal was to observe the fish from egg to larvae and until the weening phase. However, problems with larvae mortalities obstructed these plans. Covid-19 also interfered with access to the facility for close follow up of the fish, and therefore some of the work could be not done exactly as planned. However, the project was able to proceed with minor modifications.

To investigate the diet effects, three groups of fish were given an ARA enriched diet approximately eight months prior to spawning to investigate the effect of the broodstock nutrition. During the spawning period, the fish was starved after recommendation from experienced technicians at IMR Austevoll. For looking at ARA effect the deposition in eggs was studied and compared to previous studies (K. Hamre *et al*., 2013).
Results indicates that using both eye score and image analysis could be useful and supply each other for better estimating the numbers of eggs. Analysis also showed that the deposition of ARA in eggs was higher in the broodstock fed the higher levels as well. However, the diets produced significant different numbers of eggs produced with the medium ARA diet producing most indicating that a recommendation for ARA levels should be closer to this level for optimizing egg production

5.1 Growth and health status for broodstock fish fed different diets with arachidonic acid (ARA)

Previous studies have not shown an effect of ARA on growth rate in other marine teleost's (Bell & Sargent, 2003; Masoudi Asil *et al.*, 2017). This is comparable to our results where no significant effect of the diets was found on growth. Only time had a significant effect on growth, and the broodstock ARA enriched diet had no significant effect on numbers of eggs produced by ballan wrasse.
During the spawning period the broodstock was starved in order to avoid debris and waste in the tank. Thus, may affect the growth of the fish and may be one of the reasons for the reduced weight gain during this period. Gill diseases were observed during the autumn and winter but

disappeared towards the spring-summer. AGD was discovered with white patches in the gill filament together with an unknown blister (appendix 7).

A previous study shows that AGD infestation is patchier on ballan wrasse compared to salmon, and that ballan wrasse recovers from the parasite without any significant mortalities (Sture, 2020). Similarly, observations in current study showed that AGD disappeared in later stages towards springtime. Moreover, during the February sampling fish was early inflated and looking mature (table 12,13,14). This is similar to other studies showing an improved reproductive physiology with visible signs in fish near spawning period (Norberg *et al*., 2017). Some external injuries such as wounds and damaged fins were also observed, and some of the fish had visible loss of weight. The external injuries could be caused by the spawning behaviour, which often includes biting. Biting and territorial behavior between males and females or male competition has been observed in previous studies (Bridie Grant, 2016; Potts, 1985).

5.2 Estimation of egg quantity

Estimation of egg quantities were done in two ways, one using visual evaluation (eye score) by personnel at IMR and the other using objective image analysis. In the industry eye scoring is the most widespread, but is not a sufficient method for estimating the number of eggs (Bridie Grant, 2016). This study aimed at evaluating a subjective method (image analysis) of scoring mats compared to an objective method (eye score) individually by farmers.

When evaluating the eye score, a scoring index was used (table 3). The patchy distribution of eggs on the mat substrate could potentially cause imprecise eye score estimates. This means that the score would be uneven and often underscoring the number of eggs. Scoring is also influenced by the individual person that performs the analysis.

Since this is a subjective estimation of egg number, maybe standardizing scoring in the future with clear guideline for estimation should be developed. Today there is no accurate method for estimation of the egg number, besides scraping the eggs of the substrate (Bridie Grant, 2016). A positive effect of eye score is that in-person one can differentiate if the eggs are in layers. Furthermore, the use of both analyses could improve the estimation of eggs produced and should be included in the future research.

However, since the spread of data in both analysis higher when the values are higher it deviates more from the projected line (figure 8,9). This makes estimation of eggs increasingly difficult

when the production of eggs is higher. A possible solution for better estimating this could be more samples towards the top score and weighing.

Both methods use a two-dimensional view to describe three dimensions layers with eggs. The camera and the image analysis are unable to quantify the three-dimensional structure of an egg. However, both models for estimating the numbers have differences compared to the actual numbers that were produced. But by using both methods we are confident that estimation are close to actual numbers of eggs produced.

Results about the improved number of eggs due to ARA effect is similar to studies from other marine teleost's (Furuita *et al*., 2003).

The material used as substrate in the broodstock tanks was the same carpet material that MOWI uses (Lone tepper), and based on their experience, rather than using artificial turf that is used in the industry (B. Grant *et al*., 2016). The effect of the substrate is not known, and maybe in the future it's something to look at. However marine studies of spawning behavior have shown the fish spawning at nests and rocks (Levent Artüz, n.d.). But mimic these condition in intensive farming can be difficult hence one should try to be close to natural condition as possible. There is also limited work done on the substrates effect on spawning, and this may be subject to further studies.

Overall this analysis was developed to give an objective alternative to traditional eye scoring, as alternative methods are limited (Bridie Grant, 2016). However, when making a power curve instead of a linear model, the estimation of eggs is more similar to the eye score, even when the adjusted R value is lower than a linear approach making the models more similar (figure 20). When estimating the number of eggs produced, different methods and results were evaluated to give the industry a tool for better estimation and predictability.

Eggs were photographed and scored on day three after incubation. And when they were scored fertilization rate was also measured as a percentage (table 14). Overall, the eye score produced significantly more eggs than the image analysis. A possible reason for this is the maximum score of four. Results indicates that this is not ideal and maybe in a future research there should be a continuous score. This may be a limitation for mats almost covered with eggs in layer.

Although this method can be limited compared to image analysis with a continuous score where there is no limitation to the estimation of egg numbers.

In the future there should be a better base for further scoring, though this is difficult since each mat is individually different from another. When doing this, and adding the fertilization rate, a possible outcome for prediction of numbers of eggs is possible to estimate. Maybe a system with eye scoring with more than max score 4 in value should be added, as well as a "layer"

score. Where the layers will also be given a value and considered in the estimation of eggs. Since each mat is incubation manually this should be possible to do commercially for farmers. Based on our results, the eye score method with a power adaption is a good proxy to image analysis for estimation of eggs and is a useful tool for estimation of the numbers of eggs. Also using both analyses could for the farmers be a useful tool for better estimating the numbers of eggs produced.

5.3 Deposition of dietary ARA in eggs

Studies have shown that marine fish require ARA as well as HUFA in the diet to improve fish health for broodstock fish (J. D. Castell e*t al*., 1994; Estévez *et al*., 1997).

Brood stock nutrition studies have shown that Omega-3 is vital, but the ARA effect is not apparent and needs further research (Bell & Sargent, 2003; Watanabe & T., 1985). ARA is a significant precursor for the eicosanoid metabolism in marine fish and affect the embryogenesis, development of the immune system, hatching, and larval development in early stages. The fish need extra ARA, but excessive levels will negatively affect the production (Furuita *et al*., 2003).

The fish health of ballan wrasse has been in focus, as a consequence of sky-high mortalities in intensive production. Therefore, it is interesting to evaluate if ARA can enhance fish health during intensive production (Hamre *et al*., 2013). Studies have shown that although reducing stress and improve survival using ARA in the diet it should also be added to the broodstock for more retention in the egg (Bell & Sargent, 2003; Koven *et al*., 2003). This is similar to results from summer flounder where reduced stress was observed in cases where extra ARA was added (Willey *et al*., 2003). In this study an ARA level of 6% of total fatty acids was found to reduce stress. These levels are higher than in the current study but highlights the importance of ARA in the diet.

There is a significant difference in levels of ARA in wild-caught and farmed ballan wrasse (Hamre *et al*., 2013). Later studies have shown that adding phospholipids compared to neutral lipids will increase the fish health and survival rates (Hamre *et al*., 2013).

However the exact effect of ARA on growth is unclear (Bell & Sargent, 2003) which is substantiated by the current results where no significant growth was discovered.

A standard method for analyzing lipids was used, gas chromatography. However, to analyze eggs they need to be homogenized, which was done with a mixing rod. But this was found to be difficult due to the eggs being sticky and not easy to crush. This was contradictory to what Hamre *et al*., (2013) did when they analyzed eggs.

Previously fish were given regular food (Skretting cleansoft mixed with extra mushed shrimp (ca 25 % percent shrimp)) but by using ARA enriched diet there is a difference in the numbers of mats pr female fish laid incubated. The ARA enriched diet also improved the number of days with spawning and increased egg production (figure 11, 12).

The fish fed the high ARA diet spawned eggs with a lot of ARA deposition in eggs, and this may be due to that high ARA diet was overshooting to initial values (Furuita *et al*., 2003). The high ARA diet was made to push the limits of ARA levels and observe possible consequences in egg deposition.

Studies from Japanese flounder (*Paralichthys olivaceus*) have shown that too much ARA will negatively affect both egg and larvae quality, and the fish will not benefit from a diet with these levels (Furuita *et al*., 2003). Both for the polar and neutral fraction where Furuita *et. al*., (2013) found that ARA levels 3.3±0.7 % for neutral and 8.6 ± 0.8 % was too high and had a negative effect on the eggs. The same was observed when adding 0.4% and 0.6% ARA in broodstock diet.

In other studies where different ARA levels where fed to the broodstock, the fed with the highest levels produced significant worse than the other diet. (Emman & Alorend, 2004). Our results, however, did not show any significant differences indicating that the high ARA diet performed notably worse than the other diets (table 15).

However, the different diets had different deposition of ARA levels, showing that the eggs with the high ARA diet had the most deposition in the eggs, which is similar to findings in other marine species (figure 17) (Furuita *et al*., 2003).

The total number of days with spawning was registered where the medium ARA diets tended to produce eggs more evenly and over a longer time period compared to previous years (figure 11, 12). Previous studies on ballan wrasse are conflicting where the ARAs exact effect still needs further clarification (Bridie Grant, 2016).

Previous studies have also shown that the size of the egg is a convenient proxy to use in evaluating the egg quality (D'Arcy *et al*., 2012). In this study, the diet with medium ARA diet produced the biggest eggs in diameter, and not the diet with highest ARA levels. This is

different from similar studies since ARA is known for its role as a structural lipid, and where levels of ARA may be proportional to the egg size (Stuart et al., 2018a).

Our study showed that the medium ARA diet produced the largest eggs in diameter and also produced higher number of eggs. This indicates that higher levels of ARA do not have a positive effect as with other spices. This is similar to studies from cod indicating that ARA added to the broodstock diet will affect the numbers of eggs produced (Roy & Davie, n.d.).
However, other studies from cod also indicates that the size and age of the broodstock will affect the eggs positive (Palakovich Carr & Kaufman, 2009). Our results may indicate that the groups with the highest average female weight also were producing most eggs (table A8-1).
In the future, eggs should be measured over a longer period and over multiple spawning periods for comparing the sizes. Studies from cod also showed that the egg size will decrease later in the spawning period (Kjørsvik, 1994). Previous works from cod also showed that the egg size will decrease when the fish is starved during the spawning period (Kjesbu *et al*., 1991). However, this was not observed in our results despite all groups were starved during the spawning period.
In parallel with the experiment there was also an extra tank that was given regular feed (Skretting cleansoft mixed with extra mushed shrimp (approximate 25 % percent shrimp)). This produced a lot fewer mats with eggs, and the spawning period was also shorter. However, this might not be comparable since the fish had not been in the facility the same number of days as the fish in the experiment but suffice as an adequate comparison in the study.
Throughout the trial period one would assume that the deposition of ARA in eggs would be lower later in the spawning due to the fish using its energy, however in our studies this was not observed.

During the spawning period, technicians observed that the broodstock was actively feeding on the eggs spawned in the broodstock tanks. This is because the fish was starved during the spawning period in order to avoid debris in the tanks, which makes it challenging to gather and collect the eggs.
If the broodstock were to be given feed during the spawning the increased debris could potentially affect the eggs, and also facilitate microbial growth and disease outbreaks. However, starving the fish may affect the levels of ARA since the eggs eaten are high in fatty acids. Previous studies have shown that the long term nutritional effect on starvation is severe when food is confined over a long time period (Sánchez-Muros *et al*., 1998).

There was a notable difference of ARA levels in the analysis of lipid fractions, where polar lipids were measured at higher abundances than the neutral lipids.

Polar lipids are essential as structural lipids that are important in membrane development, and they also have higher retention levels than neutral lipids (Furuita *et al*., 2003).

Though the level of ARA is different in species of fish Furuita *et al.,* (2013) results are similar to results in our studies where the deposition of polar lipids is higher than in the neutral fraction. The fish fed the high ARA diet had the highest deposition of ARA when analyzed and this is similar to Furuira *et al*., (2013) study. In the future eggs should be taken over a long period, when possible, though it may be challenging to synchronize the fish spawning that would be necessary to have a comparable control group

The reason for Hamre *et al*., (2013) recommendation of higher ARA levels was based on the difference in wild caught and commercially farmed ballan wrasse aiming to replicate conditions in the wild. Hamre *et al*., (2013) results had the level of ARA in wild caught ballan wrasse as a reference. Her values were measured to 2.4 ± 0.9 % ARA of total fatty acids. Our results showed similar or higher values for both the medium ARA diet and the high ARA diet (table 15). Both Furuita *et al*., (2003) and Hamre *et al.,* (2013) observed higher levels of total fatty acids. In this study the highest levels of total fatty acid deposition in eggs were found to be the medium ARA diet. This is contradictory from previous studies but may be a consequence of the high ARA diet being in abundance and not absorbed by the fish. Results also showed that the feed had no significant effect on total deposition of lipids in eggs and there is also not any significance between the diets for total deposition in eggs. Previously studies have also shown an improved fertilization and hatch rate for Atlantic halibut *(Hippoglossus hippoglossus*) when adding extra ARA in the broodstock diet values of 2% of total fatty acids effected the fertilization and hatching vs levels of 0.5 or 1 % (Mazorra, 2000; N.R. Bromage, C. Mazorra, A. Davie, E. Alorend, M.P. Bruce, J.G. Bell, 2001). Similar studies from captive yellowtail *(Seriola dorsalis)* also showed that ARA affected hatching rate when ARA was added to the broodstock (Stuart *et al.*, 2018b). Our results showed similar effect with a positive trend that ARA levels will stimulate the hatching rate.

6. Concluding remarks

The image analysis was developed to give a subjective method for estimation of the numbers of eggs. However, eye scoring with a power adaption is similar in numbers and should be a

useful tool for the farmers for estimation of numbers. By using both methods to support each other one could get better estimates of numbers of eggs.

ARAs effect on quality of eggs is evident both in deposition in eggs and in numbers of eggs produced. However, ARA does not have a significant effect on growth, but ARA seems to help the fish to recover faster, at least for some diseases, and generally appears to improve their health. The broodstock diet matched the intentional levels of ARA and the increased deposition in eggs is notable. Compared to the standard diet all experimental diets seem to work well and affect the production positively. The low ARA diet did not seem to affect the fish negatively, neither did the high ARA diet. However, it seems that an ARA level of total fatty acids closest to the medium ARA diet (2,3% percentage of total fatty acids) is the best for an optimum broodstock diet for production.

7. Future perspectives

In future research more tanks and bigger tanks should be used to remove possible individually tank effect in the different tanks. Due to the COVID-19 pandemic it was not possible to follow fish from egg to juvenile until the weaning phase, which was planned. For further research this should be done to look at ARA effect until this stage. Further research could also look at the estimation/fecundity of ballan wrasse. The image analysis model should include sampling from more than one year to collect more data. By doing this the estimation should be more accurate and the prediction better for the estimation of eggs.

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9. Appendix

Appendix 1 Process of gas chromatography, different chemicals used

Table A1: Chemicals used in gas chromatography. *chemicals used to separate fatty acids from ballan wrasse eggs*

Methanol (CH_3OH)
Chlorfom ($CHCl_3$)
Natriumhydroxid (NaOH)
Bortrifluorid (BF_3)
Cleansed water (H_2O)
Hexan (C_6H_{14})

Appendix 2 Statistical analysis specific growth rate

Analysis of variance (2-way ANOVA)

Response: Specific growth rate

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Diet	2	0.0042	0.00210	0.1846	0.8315
Time:Diet	6	0.0900	0.01501	1.3170	0.2486
Residuals	359	4.090	0.01139		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

TUKEY HSD

TukeyHSD(aov(SGR ~ Diet, data = Sgrxtime))
Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = SGR ~ Diet, data = Sgrxtime)

Response: Diet

	diff	lwr	upr	p adj
Low ARA-High ARA	0.011317039	- 0.03149970	0.05413378	0.8081585
Med ARA-High ARA	0.001641304	- 0.03966178	0.04294439	0.9951906
Med ARA-Low ARA	0.009675735	- 0.05233777	0.03298630	0.8548029

TUKEY HSD

TukeyHSD(aov(SGR ~ Time, data = SgrXtime))
Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = SGR ~ Time, data = SgrXtime)

Response: Time

	diff	lwr	upr	p adj
Feb-Nov	-0.110084658	-0.15039010	- 0.06977922	0.0000000
Mar-Nov	-0.217393025	-0.25769847	- 0.17708758	0.0000000
Sep-Nov	-0.224912791	-0.26544026	- 0.18438532	0.0000000
Mar-Feb	0.107308367	-0.14772143	- 0.06689530	0.0000000
Sep-Feb	-0.114828132	-0.15546264	- 0.07419362	0.0000000
Sep-Mar	-0.007519765	-0.04815427	0.03311474	0.9639997

Appendix 3 Egg diameter vs ARA diet

Kruskal-Wallis rank sum test

data: Diameter by Diet

Kruskal-Wallis chi-squared = 9.9742, df = 2, p-value = 0.006826

TukeyHSD(aov(Diameter ~ Diet, data = DiameterXdiet))

Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = Diameter ~ Diet, data = DiameterXdiet)

Response: Diet

	diff	lwr	upr	p adj
LowARA-High ARA	-0.04235354	-0.08157150	-0.003135569	0.0324706

MedARA-High ARA	-0.01178409	-0.05232777	0.028759586	0.7517530
MedARA-LowARA	0.03056944	-0.01182861	0.072967495	0.1914858

Appendix 4 PL lipids test

Kruskal-Wallis chi-squared = 1.7193, df = 2, p-value = 0.4233
TukeyHSD(aov(mg ~ Diet, data = dfL))
Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = mg ~ Diet, data = PL)

Response: Diet

	diff	**lwr**	**upr**	**p adj**
Low ARA-High ARA	-0.5014072	-2.626948	1.624134	0.8154525
Med ARA-High ARA	0.2161902	-1.909351	2.341731	0.9623509
Med ARA-Low ARA	0.7175975	-1.407943	2.843138	0.6625045

Appendix 5 NL lipids test

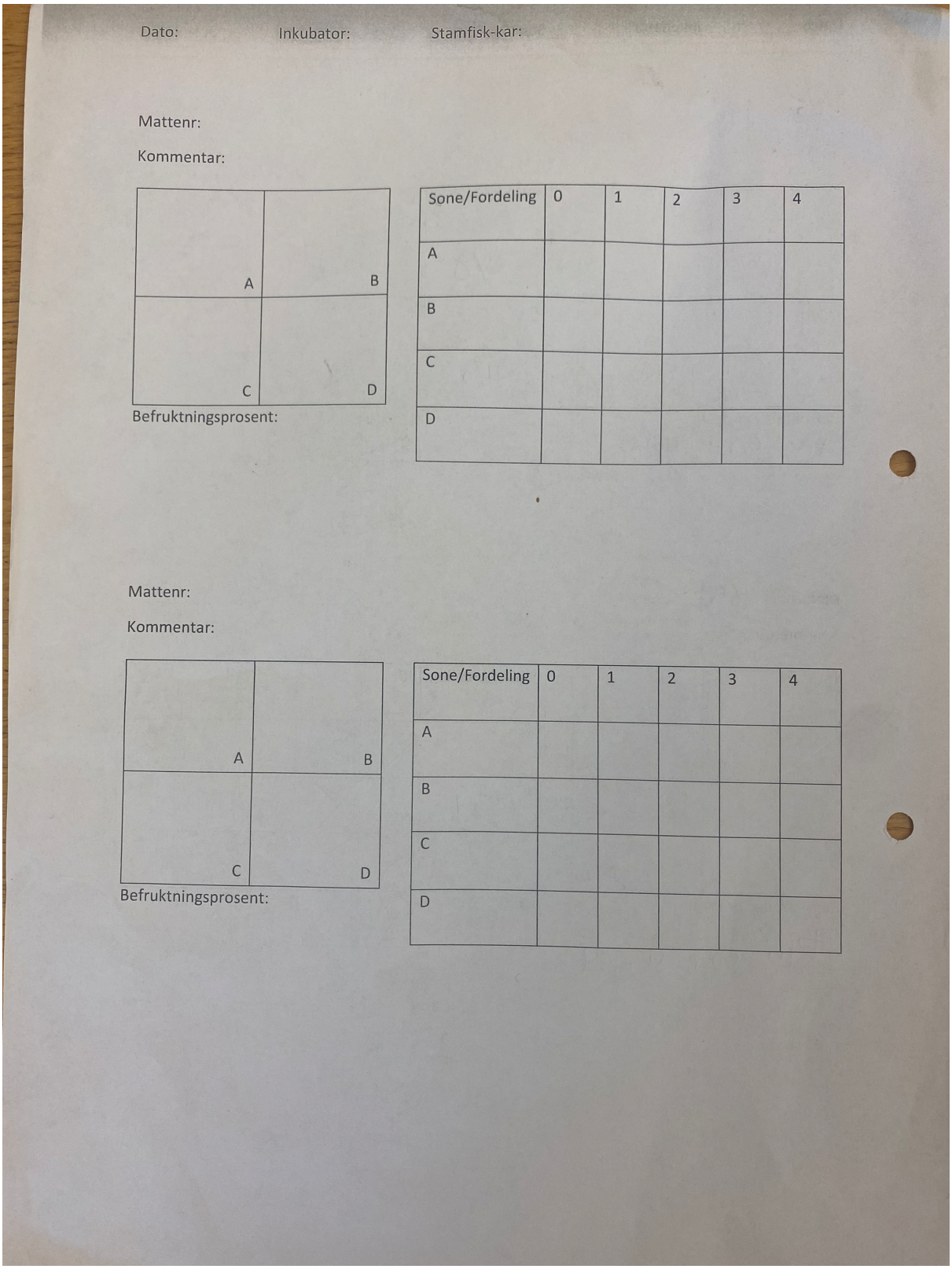
Kruskal-Wallis rank sum test
data: Mg by Diet
Kruskal-Wallis chi-squared = 0.87719, df = 2, p-value = 0.6449
TukeyHSD(aov(Mg ~ Diet, data = dfLL))
Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = Mg ~ Diet, data = dfLL)
Respone: Diet

	diff	**lwr**	**upr**	**p adj**
Low ARA-High ARA	-0.2484347	-1.0748617	0.5779922	0.7200857
Med ARA-High ARA	-0.086230	-0.9126573	0.7401965	0.9604234
Med ARA-Low ARA	0.1622043	-0.6642226	0.9886313	0.8677456

Appendix 6 Scoring system for eye score analysis.

Figure A6 Scoring sheet of eye scoring. *Date, incubator, and tank up in the left. Each zone marked as A, B, C, D. And eye score as "sone/fordeling. Feralization percentages is under overview of mat was also noted in each zone.*



Dato: Inkubator: Stamfisk-kar:

Mattenr:

Kommentar:

A B C D

Befruktningsprosent:

Sone/Fordeling	0	1	2	3	4
A					
B					
C					
D					

Mattenr:

Kommentar:

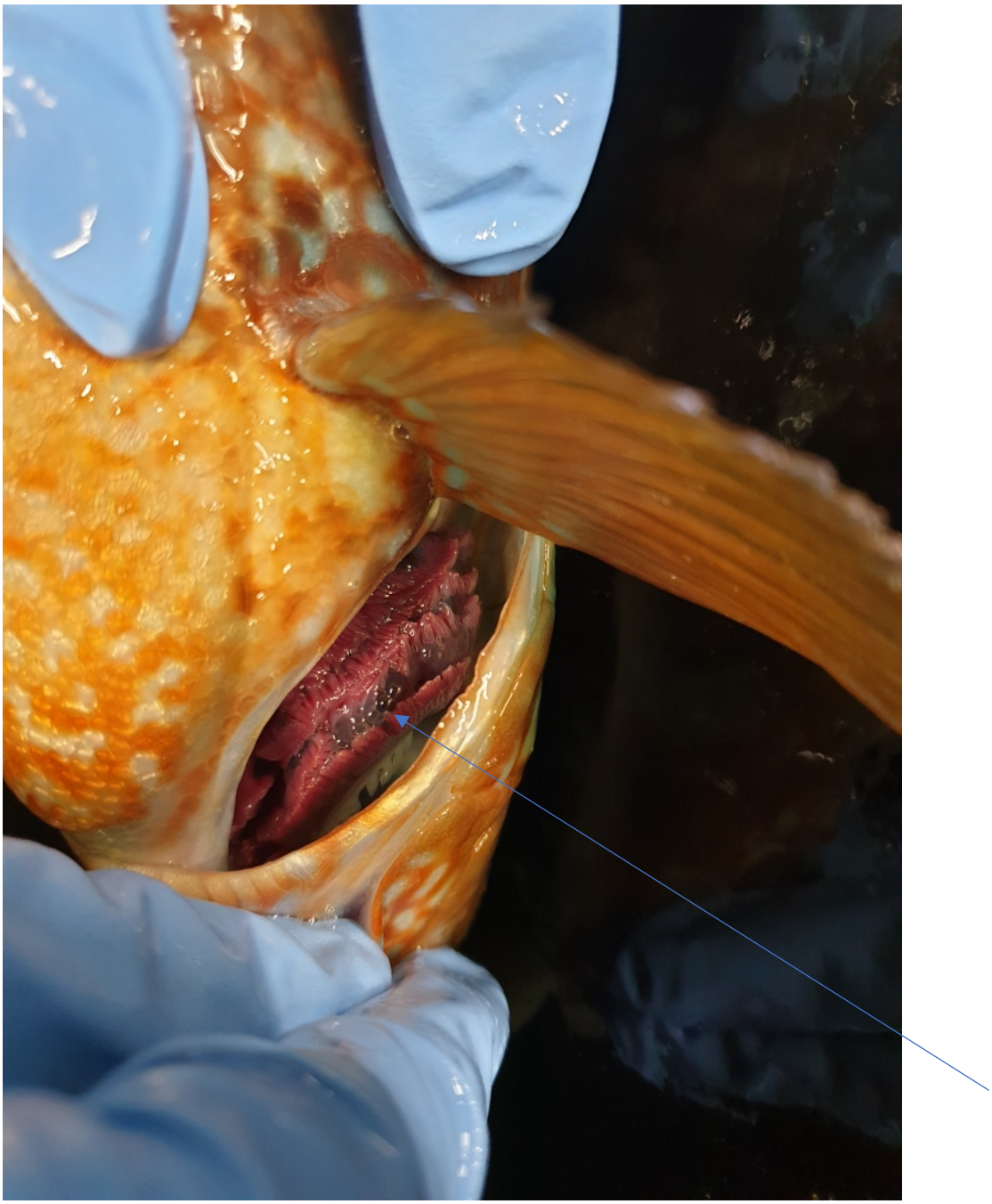
A B C D

Befruktningsprosent:

Sone/Fordeling	0	1	2	3	4
A					
B					
C					
D					

Appendix 7 Unknown blister discovered on broodstock.

Figure A7: Unknown blister. Unknown blister discovered under November sampling. blister clearly visible in the gills but not named. Arrow pointing to the blister.



Appendix 8: Tables used in measurement of weight, number, biomass, gram produced by each fish, and numbers of mats produced by each individual fish.

Weight of the eggs was also calculated and the medARA produced most gram for each emale fish. The lowARA diet produced more than the high ARA (table A8-2)

During the spawning period number of mats was registered. The medium high ARA diet produced most mats for each female with the low ARA diet coming in second. The high ARA diet produced the lowest numbers of mats (table A8-3).

Table A8-1 Female stat for number, and total biomass

Diet	Average weight	Number of fish	Total biomass in each tank (kg)
Low ARA	934	24	22 416
Med ARA	987	27	26 649
High ARA	987	27	26 649

Table A8-2 Numbers of gram produced by each female fish

Diet	Numbers of gram produced by each female fish (g)
Low ARA	128.62
Med ARA	135.19
High ARA	121.32

Table A8-3 Numbers of mats produced by each female. Comparison of different diets and number of mats.

Diet	Numbers of mats for each female
Low ARA	7.25
Med ARA	7.37
High ARA	6.81