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Effects of broodstock conditioning diets on the early life stages of Atlantic cod *(Gadus morhua)*

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Abstract

Broodstock nutrition has been shown to influence the performance of eggs and larvae in marine finfish. The present study aimed to test the hypothesis that a broodstock conditioning diet with increased astaxanthin, taurine and water would have an effect on the early life stages in Atlantic cod. Thus, cod broodstock were fed two diets, a standard broodstock conditioning diet, and an experimental diet containing an additional 30ppm astaxanthin, 1% taurine and 10% dietary water. During the experimental period, egg and larvae samples from three families for each diet were collected, and fertilization success, hatching success, larval length, weight and survival were recorded as well as biochemical analysis on fatty acid composition. Sperm samples were collected, and performance parameters were assessed using CASA. No differences were found in the performance of the two diets. This was in part due to large genetic differences within families of the same dietary group.

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1 Introduction

Due to declining catches of Atlantic cod (*Gadus morhua*) in the North Atlantic, aquaculture has been considered an important way to meet demand and reduce pressure on wild cod stocks. However, cod aquaculture has been met with several major challenges along the way to reaching its full potential. As with most other commercial marine species, egg quality and juvenile production have been the major biological hurdles (Puvanendran & Mortensen, 2009). Selective breeding programs and larval rearing protocols have seen vast improvements in larval survival and juvenile growth (Brown, Minkoff, & Puvanendran, 2003) and the industry appears to be making a comeback particularly in Norway. However, there is still a relatively high mortality rate during the early life stages of cultured cod, especially when larvae are still dependent on their yolk sac for survival (Puvanendran & Mortensen, 2009). Broodstock nutrition has been shown to greatly improve the quality of eggs and sperm and ultimately the successful mass production of juveniles (Izquierdo, Fernández-Palacios, & Tacon, 2001; Pérez-Casanova et al., 2015; Watanabe & Vassallo-Agius, 2003). However, little is known about the specific requirements of many marine species in terms of the quality and quantity of essential nutrients in broodstock diets.

Broodstock nutritional studies are often associated with high running costs, which makes them one of the most poorly researched areas of finfish nutrition. Many marine finfish species are relatively large and take several years to reach sexual maturity. Therefore, there is a need for large culture facilities that are suitable for holding large numbers of adult fish for long periods of time. In addition, nutritional research often requires large numbers of replicates in order to provide suitable experimental conditions. These prohibiting factors have helped slow the progress of gaining an understanding of the dietary requirements of finfish broodstock.

To date, most of the studies concerning broodstock nutrition of cod have focused on protein sources and fatty acid composition (Lanes et al., 2012; Olsen et al., 2007; Pérez-Casanova et al., 2015). However, there is mounting evidence that certain antioxidants and amino acids may play a vital role in egg and larval quality. Hansen, Puvanendran, and Bangera (2016) found that the addition of astaxanthin in broodstock diets can improve the condition and fecundity in Atlantic cod. Taurine supplementation studies have shown an increase in the growth of larvae in some species (Pinto, Rønnestad, Dinis, & Aragão, 2013). In addition, dietary water content is thought to improve nutrient adsorption and digestion in marine fish (Chatzifotis, Papadakis, & Divanach, 2005). Using a cod broodstock diet with a combination

of increased levels of a astaxanthin and water content produced higher egg production and egg output, lower egg incubation mortality and higher larval growth and survival when compared to a standard commercial diet (Hansen et al., 2016).

Fish farms require year-round access to high quality eggs and larvae to optimize production and to be sustainable. Yet the cod aquaculture industry still faces issues of mortality, egg quality and larval rearing. If problems with egg and larval quality are related to nutritional factors, then the supplementation of essential nutrients in broodstock diets should improve these issues and requires further research.

Due to the lack of information on Atlantic cod broodstock nutrition, this thesis addresses the following research question: Does a cod broodstock conditioning diet containing increased levels of astaxanthin, added taurine and dietary water contents, have an effect on the quality and performance of eggs and larvae when compared to a standard broodstock conditioning diet?

2 Background on Atlantic cod and broodstock nutrition.

The following chapter briefly describes the history, problems and progress of the Atlantic cod aquaculture industry with particular focus on Norway. In addition, current research on the nutritional supplements and use of performance indicators used in the present study are summarized.

2.1 Status of Atlantic cod aquaculture

Atlantic cod (*G. morhua*) is distributed in several distinct stocks on both sides of the Atlantic Ocean. Regarded globally as one of the most important commercial marine finfish species, cod has been exploited since humans first began to fish. However, due to increases in technology and fishing effort, natural stocks have suffered, which has led to an increased interest in intensive cultivation of the species.
Farming of Atlantic cod has been of interest for over 100 years; however, most attempts were based on ranching of wild caught juveniles and larval production for restocking purposes. The first cultivation attempts date back to the 1880s where Norway, the USA and Canada released hundreds of millions of newly hatched yolk-sac larvae to the sea each year for nearly

90 years. However, the success of these releases has not been documented (Nardi, Prickett, van der Meeren, Boyce, & Moir, 2021)

In the 1980s, small-scale intensive production to support the land-based processing industry of Atlantic cod had already started in Norway due to fluctuations in wild populations. It wasn't until around 1990 when modern hatchery methods were first developed using cultivated rotifers and Artemia (Rosenlund & Halldorsson, 2007). These first attempts produced high numbers of metamorphosed larvae, however deformities and cannibalism, due to sub-optimal feeding and rearing conditions led to high mortality rates (Svåsand et al., 2004). Despite these encouraging results, the interest in cod aquaculture faded as it was not seen as economically feasible at the time.

Following the 1992 collapse of the Northern cod stock in Canada, global landings of Atlantic cod were in steady decline (FISHSTATJ, 2021). Reduced quotas led to lower wild catches and higher market prices of both wild and farmed cod. This sparked new interest into commercial production and a boom in investment followed by rapid increase in biomass during the period between 2000 – 2008 (Fiskeridirektoratet, 2021) (Figure 1).

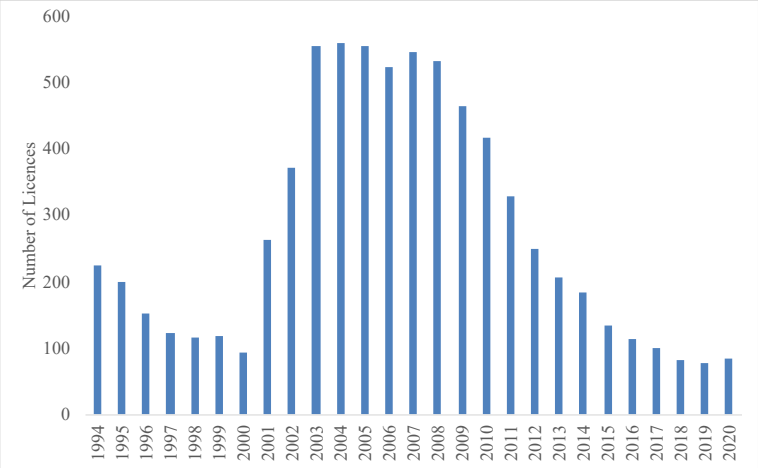

Figure 1 Number of Atlantic cod aquaculture licenses in Norway issued by the Fisheries Directorate.

Source The Norwegian Fisheries Directorate (www.fiskeridir.no/English/Aquaculture/Statistics)

However, this growth was relatively short lived. The financial crisis in 2008, coupled with difficulties across all growth stages, premature onset of sexual maturation and increased quotas of many wild cod stocks, led to the almost complete collapse of the cod aquaculture

industry by 2014. In 2007 there were 547 grow out licenses in Norway that produced around 10,000 tonnes for sale, in 2020 there were only 85 licenses with no production (Fiskeridirektoratet, 2021) (Figure 2).

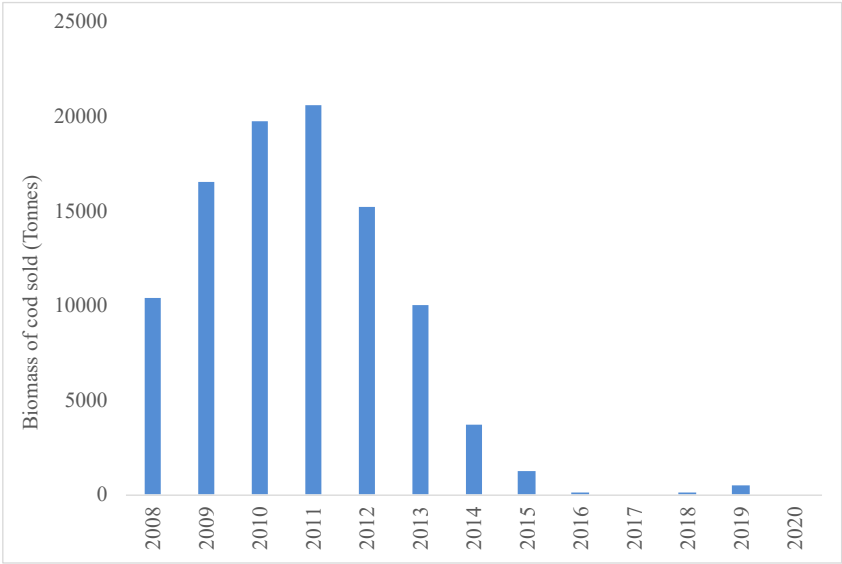

Figure 2. Production of cultured cod sold in Norway from hatchery reared juveniles between 2008 – 2020.

Source The Norwegian Fisheries Directorate (www.fiskeridir.no/English/Aquaculture/Statistics)

Despite the recent lack of commercial interest in Atlantic cod aquaculture, research and breeding programmes have continued in many countries, with most work being undertaken in Canada, Iceland, Norway, UK and USA. In Norway, authorities decided to establish a national breeding program for cod in 2002. The National Cod Breeding Station, based in Kraknes outside Tromsø has been operated by Nofima since 2003 and has the facilities to produce around 300 families per generation (Puvanendran & Mortensen, 2009). The aim of the program was to breed farmed cod with better growth properties and disease resistance than wild cod in the hope that the industry will gain momentum again in the future. This has been achieved to some extend through selective breeding and the development of production protocols and the program is currently on its fifth generation of selectively breed broodstock.

Atlantic cod aquaculture is still a relatively new industry and faces many biological and production challenges in order to be economically sustainable and stable. However, with 460,000 juveniles sold in Norway 2019 (Fiskeridirektoratet, 2021) and the cod farming

company, Norcod AS, planning for an annual production of 30,000 by 2025 the outlook for Norway looks promising (Nardi et al., 2021). As the production of cod aquaculture increases, then so will the demand for good quality juveniles. In addition to good husbandry practices and production protocols (Brown et al., 2003; Puvanendran & Mortensen, 2009; Rosenlund & Halldorsson, 2007), hatchery production can be improved by controlling the nutritional quality of broodstock diets.

2.2 Astaxanthin.

Astaxanthin is a lipid-soluble carotenoid that usually exhibits a red/orange coloration. In nature it can commonly be found in microalgae, yeast, salmonids, and numerous crustaceans including krill (Lorenz & Cysewski, 2000). In the marine environment, fish generally obtain astaxanthin via bioaccumulation. At the primary production level, microalgae or phytoplankton which are naturally high in astaxanthin are consumed by zooplankton or crustaceans who are then consumed by fish resulting in biosynthesis up the food chain (Foss, Renstrøm, & Liaaen-Jensen, 1987). As a dietary supplement in aquaculture feeds, astaxanthin is a high cost ingredient, making up 10% -15% of the total feed cost in the salmon farming industry (Butler & Golan, 2020).

Astaxanthins' current use in commercial diets is mostly limited to increasing coloration in the muscle of salmon and exoskeletons of crustaceans (Higuera-Ciapara, Félix-Valenzuela, & Goycoolea, 2006) as well as pigmentation enhancement in ornamental species (Jiang et al., 2019; Paripatananont, Tangtrongpairoj, Sailasuta, & Chansue, 1999). However, it has also been found that dietary carotenoid supplementation can improve growth and performance, reduce mortality rates, and is considered an essential nutritional component for reproduction in many fish species (Torrissen & Christiansen, 1995). Although the effects of dietary astaxanthin on fertilization and egg survival was found to be non-essential for reproduction in Atlantic salmon, *Salmo salar* (Christiansen & Torrissen, 1997).

An early study by Grung, Svendsen, and Liaaen-Jensen (1993) found that supplementing astaxanthin in the diet of farmed cod and salmonids resulted in an increased concentration of astaxanthin in the eggs. Astaxanthin levels in the eggs of wild cod were also found to be higher than that of farmed cod due to their respective diets (Grung et al., 1993). This suggests that because captive animals do not have access to natural sources of carotenoids, there is a need for supplementation into broodstock diets. Sawanboonchun, Roy, Robertson, and Bell

(2008) found that astaxanthin supplementation produced an increase in fecundity and fertilization rate in farmed cod, which demonstrates the benefits to egg quality and larval production. Hansen et al. (2016) reported that increased water content and astaxanthin levels in Atlantic cod broodstock diet could improve body condition and fecundity.

Besides being an essential component for reproduction, astaxanthin is also an important antioxidant. It is also suggested that astaxanthin plays a role in the respiration processes in eggs (Pavlov, Kjørsvik, Refsti, & Andersen, 2004).

2.3 Taurine.

Taurine is an amino acid that is recognized as an essential nutrient that exists naturally in most animals (Salze & Davis, 2015). Fishmeal and animal by-products generally have high concentrations of taurine (0.5% - 1%), however it is not usually found in plant proteins (Kotzamanis et al., 2020). It is important for the sustainable expansion of the aquaculture industry that it reduces its reliance on marine fishmeal ingredients. However, feeds designed for carnivorous marine fish species that contain high levels of plant proteins such as soy have shown reduced growth performance (Francis, Makkar, & Becker, 2001). Taurine supplementation could be one method to support protein substitution in fish feed. Gaylord, Teague, and Barrows (2006) found that rainbow trout (*Oncorhynchus mykiss*) fed a completely soy-based protein diet supplemented with taurine had improved production performance. When common dentex (*Dentex dentex*) were fed a plant-based diet with low levels of taurine they displayed reduced growth and performance. By supplementing the diets with taurine, growth and performance was restored (Chatzifotis, Polemitou, Divanach, & Antonopoulou, 2008).

Studies have indicated that taurine plays a key role in the nutrition of cultured marine fish (El-Sayed, 2014) but differs widely between fish species (Pinto et al., 2013). It is also believed that dietary taurine is an extremely important amino acid for fish larvae development. The natural prey of marine finfish larvae tends to be high in taurine when compared with that of commercial diets, and supplementation studies have shown an increase in the growth of larvae in some species (Pinto et al., 2013).

A dietary taurine supplementation concentration of 1.1% was found to have a positive effect on fertilization rate, fecundity, egg diameter, egg protein content, larger yolk sac volume and larval quality in greater amberjack *(Seriola dumerili)* (Sarih et al., 2019). The addition of

dietary taurine in yellowtail *(Seriola quinqueradiata)* broodstock feed, improved spawning performance. Resulting in increased oocyte growth, spawning success and reduced egg abnormalities (Matsunari, Hamada, Mushiake, & Takeuchi, 2006). This would suggest that supplementing taurine in broodstock diets could have a positive impact on egg quality and larval development in Atlantic cod. Lipid metabolism in fish is also shown to be enhanced by dietary taurine by increasing lipid digestion, absorption, emulsification and body lipid deposition as well as aiding in the formation of bile salts (Salze & Davis, 2015). A study undertaken by Sampath, Rathnayake, Yang, Zhang, and Mai (2020) analyzed all published research data regarding the role of dietary taurine in fish nutrition after the year 2000. Based on the positive effects of dietary taurine supplementation, they suggest that the optimal dietary taurine content is around 1%.

Although taurine has been studied for a number of important cultured fish species and it´s requirement has been identified, it´s specific roles are still poorly understood. Furthermore, its function as an essential nutrient in Atlantic cod broodstock conditioning has not been studied.

2.4 Water.

The effects of dietary water content has been studied in relation to fish growth (Grove et al., 2001) and digestion (Chatzifotis et al., 2005) but has not been studied in relation to reproduction on its own. Dry aquaculture diets tend to contain very little water due to storage and handling issues yet dietary water could play a major role in the digestion and absorption of nutrients in marine fish (Chatzifotis et al., 2005). This is especially important when conditioning broodstock, as consumed nutrients are passed on to eggs and larvae (Izquierdo et al., 2001). Increased dietary water could assist broodstock with osmoregulation and increase nutrient absorption and digestion.

Adding dietary water to European sea bass (*Dicentrarchus labrax*) feed did not affect fish growth parameters but did increase resistance to a *Vibrio anguillarum* infection (Przybyla, Fievet, Callier, & Blancheton, 2014). Bromley (1980) found that dietary water content did not significantly influence the growth, composition, condition factor or food conversion efficiency of turbot (*Scophthalmus maximus L.*). Conversely however, Grove et al. (2001) reported that there was a relationship between dietary water contents and nutrient retention as well as an improvement in growth for the same species.

2.5 Fatty acids.

Fish require three important essential fatty acids (EFA) for successful reproduction, normal growth and development of offspring, docosahexaenoic acid (DHA, 22:6n−3), eicosapentaenoic acid (EPA, 20:5n−3) and arachidonic acid (AA, 20:4n−6) (Izquierdo et al., 2001). All vertebrates, including fish, require a dietary source of EFA as they cannot be synthesized (Tocher, 2003). By altering the fatty acid composition in broodstock diets, it is possible to alter the composition in the eggs in relatively small yet influential ways (Salze, Tocher, Roy, & Robertson, 2005). These EFA have been shown to be crucial determinants of fecundity, egg quality and hatching success in Atlantic cod (Lanes et al., 2012; Røjbek et al., 2014; Salze et al., 2005). Yet the specific requirements of these individual fatty acids and their ratios has yet to be quantified in Atlantic cod regarding reproduction and early life stages (Tocher, 2010).

2.6 Sperm quality.

Control of sperm quality is a major issue for the aquaculture industry, yet more focus is often placed on the quality of eggs and larvae than that of sperm despite the fact that the performance of male fish can influence the quality of eggs and larvae (Rurangwa, Kime, Ollevier, & Nash, 2004). In general, environmental and husbandry conditions are more related to egg quality than sperm quality (Pavlov et al., 2004). However, sperm production and quality are key determinants for successful hatchery management, and it has been shown that broodstock nutrition can play an important role in male spawning performance. Fatty acid profiles followed a pattern from broodstock diet to sperm in rainbow trout (Labbé, Loir, Kaushik, & Maisse, 1991; Watanabe, Takeuchi, Saito, & Nishimura, 1984) which could possibly play a role in sperm performance and ultimately fertilization. The addition of antioxidants to conditioning diets have been shown to have a positive effect on sperm quality (Cabrita et al., 2014). In Senegalese sole (*Solea senegalensis*), the supplementation of polyunsaturated fatty acids (PUFA) into broodstock diets increased sperm velocity and the percentage of progressive sperm, especially when complemented by the addition of antioxidants (Beirão, 2011). These findings suggest that the addition of taurine and astaxanthin to Atlantic cod conditioning diets, both of which have powerful antioxidant capacity, could have the potential of increasing sperm quality in this species too.

Sperm quality can generally be considered a measure of the ability of sperm to successfully fertilize an egg. Therefore, any quantifiable characteristic that directly correlates with the fertilization capacity of sperm could be potentially used as a measure of sperm quality. In the past, these performance indicators have mainly included motility, sperm concentration and ejaculate volume. All of which has a decisive influence on successful artificial reproduction in fish and can be assessed subjectively using a microscope (Pavlov et al., 2004). However, several tools have been developed to analyze and assess the quality of fish sperm in more detail in order to assist in the improvement of artificial fertilization methods. On such method of measuring the components of sperm motility is computer-assisted sperm analysis (CASA). CASA is a rapid method of quantifying different sperm motility parameters by tracking sperm movement which is videotaped via a microscope and then analyzed by computer software (Rurangwa et al., 2004) .

A few studies have looked at the parameters that are assessed using CASA for Atlantic cod. Skjæraasen et al. (2009) analyzed the sperm characteristics of wild and farmed cod in order to assess reproductive success in relation to sperm competition. They showed that sperm velocity (VCL) was the best determinant of male fertilization success in Atlantic cod and males with the highest motility (MOT) and percent progressive sperm (PROG) had the highest reproductive success. In another study, Rudolfsen, Figenschou, Folstad, and Kleven (2008) found that Atlantic cod males displaying progressive sperm were positively associated with fertilization success, suggesting that the faster sperm outcompete the average sperm in fertilizing the egg.

Due to the lack of information on Atlantic cod broodstock nutrition, the objectives of this thesis are therefor to investigate the effects of broodstock conditioning diets on:

- Fertilization success
- Sperm quality
- Hatching success
- Larval development
- Larval survival

This work aims to test the hypothesis that Atlantic cod broodstock fed a conditioning diet with increased astaxanthin, taurine and dietary water would have an effect on quality and performance of eggs and larvae.

3 Materials and Methods

In order to explore the role of taurine, astaxanthin and dietary water contents on the reproductive performance in Atlantic cod, the present study followed a quantitative research design, using a controlled experiment of two broodstock conditioning diets. The effects of the diets were measured using performance indicators for sperm, eggs and newly hatched larvae. Biochemical analysis was used to compare the fatty acid compositions of the diets, eggs and newly hatched larvae.

3.1 Location and experimental period.

The experiment was conducted at the Tromsø Aquaculture Research Station (Havbruksstasjon AS), Center for Marine Aquaculture (CMA) in Kraknes (Tromsø). The feeding trail commenced in January 2019 and ended in July 2020. Biochemical analysis was performed at the University of Tromsø (UiT) the Arctic University of Norway.

3.2 Ethical considerations.

All studies at UiTs´ research stations are carried out in accordance with the Norwegian regulations for use of animals in experiments. The experiment was part of activities carried out by the cod breeding program within an already approved facility. For eggs and larvae no application or approval was needed and terminal measurements on fish juveniles were performed on fish euthanized with an overdose of anaesthetic (Benzocaine, 120 mg $^{L-1}$) by trained and licensed personnel. All efforts were made to minimize fish suffering.

3.3 Experimental fish and design.

The experimental set up employed in this study is graphically outlined in Figure 3.

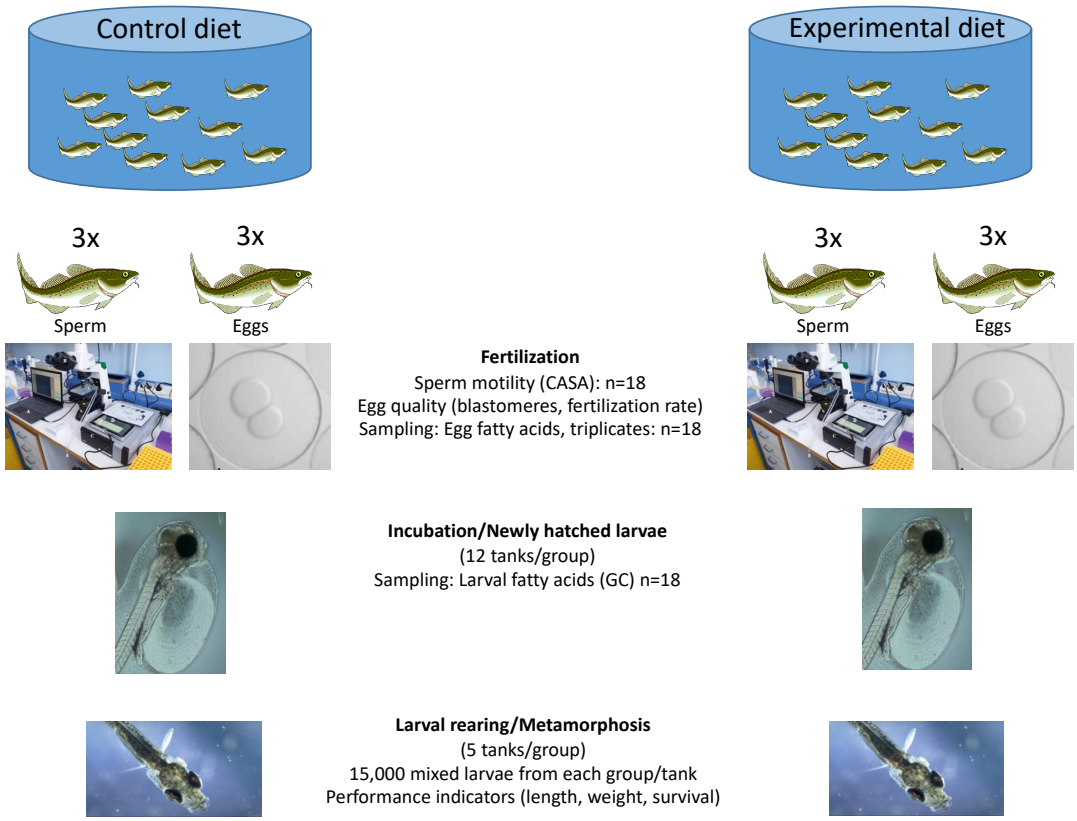

Figure 3. Experimental set up indicating sampling protocol throughout the experimental period.

Fish used for this experiment were from the 2016-year class (F5) from the National Cod Breeding Centre (NCBC), in Tromsø, Norway. Broodstock were kept in two 25 m^3 tanks containing 100 passive integrated transponder (PIT) tagged individuals each and fed the diets for 6 weeks prior to spawning (Figure 4). Fish were fed to satiation every second day. Light manipulation followed a standard photoperiod protocol. Temperature during spawning ranged from 3.5 to 3.8°C. Sampling was undertaken throughout the period from March to August 2020.

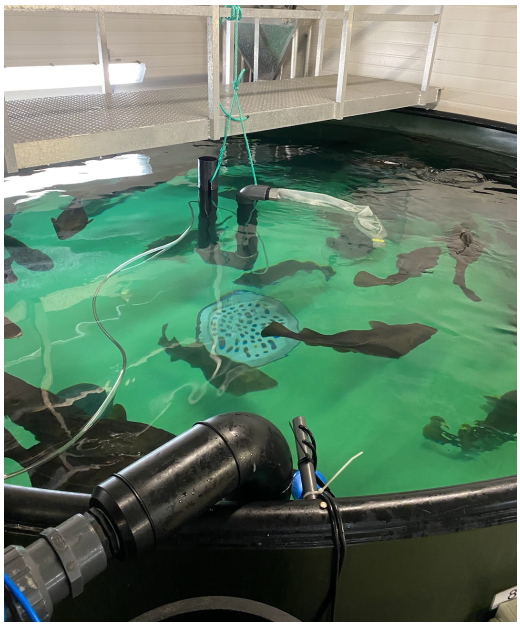

Figure 4. Broodstock holding tank where PIT tagged individuals were fed one of the conditioning diets.

3.4 Diets

The control diet was a standard broodstock conditioning diet (Vitalis Cal, Skretting, Norway, protein 58%, lipid 18.02%, moisture 10%, astaxanthin 50 ppm). The treatment diet (Trofi AS, Norway, protein 57%, lipid 20.80%, moisture 12%, astaxanthin 80ppm, taurine 1%) was made into pellets after the addition of 10% extra water then frozen at -20°C. Pellets were thawed for approximately 24 hours prior to feeding. Samples from each diet were analyzed for their total lipid contents and relative fatty acid compositions (see § 3.9).

3.5 Spawning and egg incubation

Figure 5. Gametes from one family undergoing fertilization divided into four jugs to be placed into incubation tanks.

During the peak spawning period (31st March – 18th April 2020) broodstock were selected based on their quality of eggs and sperm. Gametes from three males and three females were stripped from each tank and eggs were fertilized using a standard dry fertilization method with no exposure to sea water prior to activation, as described in (Hansen & Puvanendran, 2010) to produce three families per diet. Stripping was achieved by applying slight abdominal pressure towards the genital duct to release the eggs. Gametes from each family were hydrated and left to fertilize for 20mins then washed and divided into 4 incubation tanks per family (Figure 5). Incubation tanks received a water flow rate of 1.5L/min and constant aeration to evenly mix the eggs in the water column. At approximately 6hrs post fertilization, three random replicate samples of eggs from each incubator (n = 3 for each family) were taken and placed in a single layer on a petri dish (Figure 6). 100 eggs from each incubator were assessed for fertilization success from images recorded using a dissecting microscope equipped with a digital camera. All eggs considered fertilized had embryos at least at the 2-cell stage.

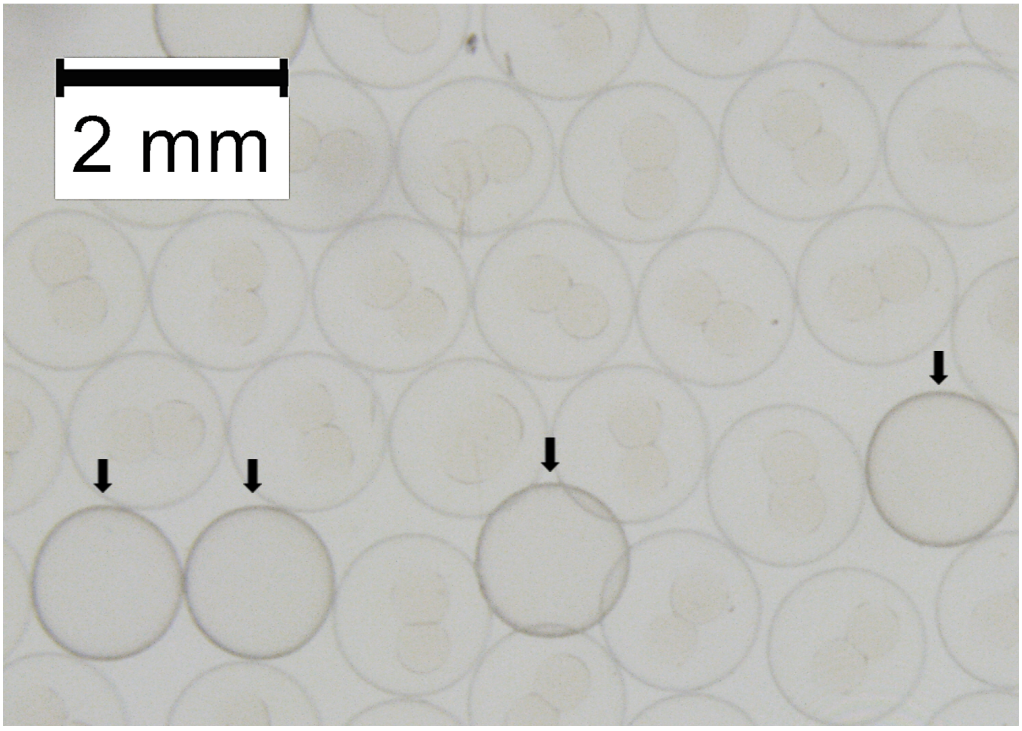

Figure 6. Sample image of gametes during assessment of fertilization success. Image shows unfertilized eggs (black arrows) and eggs at two-cell blastomere stage.

Dead embryos were removed and recorded daily for the first 5 days post fertilization then every second day of incubation until 100% hatched. The total number of larvae in each incubator was estimated at 100% hatched through volumetric sub-sampling. Three fertilized egg samples from each family were placed on dry ice then stored at -80°C for biochemical analysis at later date.

It was not possible to estimate egg survival at hatching or hatch rate as either the initial volume of eggs placed into each incubator or embryo mortality was over estimated. This resulted in a higher number of estimated hatched larvae than the initial number of eggs and in most families exceeding 100% hatched. The same was true for egg mortality where in one family egg mortality was greater than 100% even though there where large numbers of larvae visible in the incubators. To compensate for this, the method described in Hansen and Puvanendran (2010), was used to estimate viable embryos at hatch. Hatching success was calculated from the number of larvae at hatch and the egg diameter using the following formula:

$$\text{Expected volume of eggs at hatch} = \frac{Number\ of\ larvae\ at\ hatch}{1222\ \times\ D^{-2.71}}$$

$$\text{Hatching success (\%)} \frac{Expected\ volume\ of\ eggs\ at\ hatch}{Initial\ volume\ of\ eggs} \times 100$$

The calibration scale (1222 x $D^{-2.71}$) calculated by Kjesbu (1989) was used to estimate the number of eggs in 1mL of eggs in relation to initial egg diameter in millimetres (D).

3.6 Sperm Quality

Sperm samples were collected from one male from each family per treatment diet (n = 3), and control diet (n = 3). Approximately 1mL of sperm was collected per sample by applying abdominal pressure and using a 2mL syringe then placed on ice until further use. To avoid seawater, urine, and feces contamination, the area was wiped dry with paper towel before expressing the milt.

Analysis of sperm-quality parameters was performed immediately following collection of samples. Collected sperm samples preserved on ice were diluted and activated by mixing 5µl of pure sperm in 1500µl solution of cold seawater in an Eppendorf tube at a ratio of 1:300. To ensure uniformity, proper dilution and activation of the mixture in the Eppendorf tube, 5µl clean sperm sample was first pipetted into 750µl of sea water then further diluted and mixed by adding 750µl of seawater and gently pipetting up and down 2-3 times. Approximately 5µl of activated sperm solution was quickly and carefully pipetted into one 20-micron slide chamber (Leica, Nieuw-Vennep, The Netherlands) already mounted on a cooled down mounting stage of a microscope (Nikon E-200, Tokyo-Japan) with 10x negative phase contrast. Samples were immediately analyzed with the software (Sperm class analyzer® CASA system, Barcelona-Spain) over 4 fields under the microscope. All processes from sperm activation and sperm analysis per sample were completed in a space of 1 minute and were replicated in triplicate for each male.

The motility variables assessed in the present study were the mean values of percentage motile sperm cell (MOT), sperm cell linearity (LIN), curvilinear velocity (VCL, μms^{-1}) and percentage of progressive sperm (PROG) (sperm with straightness >80%)

3.7 Larval rearing

Two days after eggs were determined to have reached 100% hatching, equal proportions of larvae from each incubator were collected and transferred into five, 190 L circular fiberglass larval rearing tanks for each treatment at a stocking density of 15,000 larvae per tank. Only larvae from 2 families from each diet were moved to rearing tanks for start feeding. Three newly hatched larval samples from each family were collected, placed on dry ice and then stored at -80°C for biochemical analysis at a later date.

From 2 to 5 days post hatch (dph), water temperature for larvae rearing was approximately 5°C. The temperature was then gradually increased by one degree per day from day 6–10 dph to 10°C. Larval feeding and weening followed standard rearing protocols described in Hansen et al. (2016) . Larvae were fed algae (*Nannochloropsis sp*) from 2-12 DPH, rotifers enriched with *Pavlova sp, Chlorella sp*, Multigain® and Phosphonorse® (Trofi AS, Norway) from 2-29 DPH. Then Artemia enriched with Multigain®, Phosphonorse® and Micronorse® (Trofi AS, Norway) from day 25 until day 56. Weaning onto dry feed (AlgoNorse®, Trofi AS, Norway) started at 38 dph, increasing the size of the dry feed as they grow.

3.8 Length, weight and survival

Ten larvae from each tank (n=50 per group) were randomly sampled for standard length measurements every 7-10 days from 3 to 83 dph. Larvae were euthanized with an overdose of anaesthetic (Benzocaine, 120 mg L^{-1}) and measured under a dissection microscope (Figures 7 & 8). Average weight was taken every 14 days from 92 to 176 dph. An estimation of larval survival was performed at 90 dph following the volumetric sub-sampling methods described in Hansen et al. (2016).

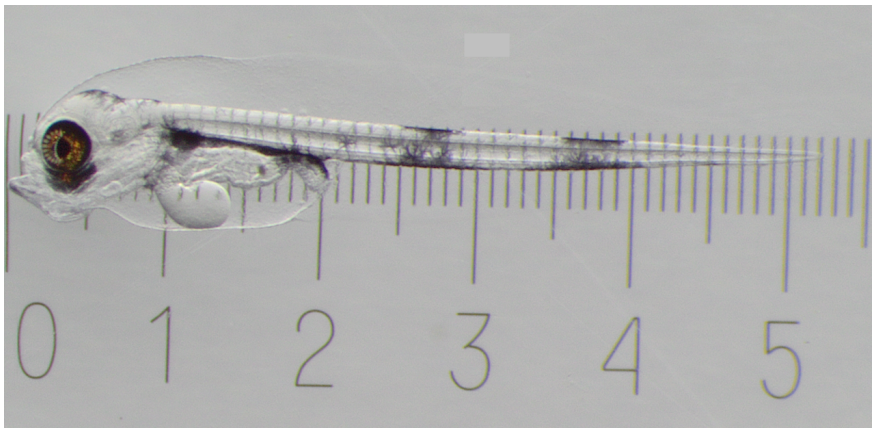

Figure 7. Sample image of a cod larva at 3 days post hatch undergoing length measurement.

Figure 8. Sample image of a cod larva at 63 days post hatch undergoing length measurement.

3.9 Biochemical analysis.

Three 4ml cryotubes where filled with eggs and three with newly hatched larvae from each family and placed into dry ice then kept in a -80 °C freezer until analysis. Before analyzing, samples were freeze dried for approximately 24hrs.

Total lipid contents for the diets was determined using lipid extraction (based on (Folch, Lees, & Stanley, 1957) with some modifications). Approximately 0.5g of sample material was weighed directly into a 15ml centrifuge tube. 9.5ml of dichloromethane/methanol (2:1, v/v) was added as well as 0.5ml of internal standard (IS), heptadecanoic acid (C17:0) in dichloromethane/methanol (2:1, v/v) at a concentration of 10mg/ml and mixed for 30 minutes using a shaker (Heidolph Multireax). Samples were then centrifuged at 4000 x g for 15 min and the supernatant transferred to new centrifuge tubes. 2 mL of 0.9% sodium chloride (NaCl)

was added to each tube then mixed using a vortex. The solution was then centrifuged at 2000 x g for 10min. The water/methanol phase at the top of the tube was removed by using a Pasteur pipette. The dichloromethane / lipid phase was then transferred into a pre-weighed, 8 ml glass tube and evaporated to dryness using nitrogen gas (N_2). The lipid content of the sample was calculated by the following formula:

*% lipid = ((Glass tube with content - Glass tube Empty) / Weight of sample) * 100%*

Total lipid content for eggs and larvae was omitted from the analysis due to small sample quantities, only compositional analysis was performed using the methods described below.

Methylation of the diet lipid samples into FAMEs was based on (Stoffel, Chu, & Ahrens, 1959). Sulfuric acid, (H_2SO_4) was used as a catalyst, combined with heating to increase the reaction rate. Diet lipid samples were dissolved in dichloromethane: methanol (2: 1) to a concentration of 10 mg/ml. 100 µl of sample was added to Duran tubes, then 0.9 ml of dichloromethane and 2 ml of 2% H_2SO_4 in methanol was added to each tube. The samples were placed in a heating block at approximately 100 ºC for one hour. 3.5 ml of heptane and 3.5 ml of 5% NaCl was added and mixed well. Two phases were formed and the upper phase consisting of heptane and lipids was pipetted into new glass tubes (4 ml) and evaporated to dryness using N_2 gas. The samples were then dissolved in 100 µl of heptane and transferred to gas chromatography (GC) tubes.

Direct methylation was used for fatty acids composition analysis for eggs and larvae based on (Dulavik, Sørensen, Barstad, Horvli, & Olsen, 1998). Fatty acids were saponified and methylated to methyl esters according to (Dulavik et al., 1998). 20mg of homogenized sample was weighed directly into Kimax tubes in triplicate. 2.4 ml of 2M HCl in methanol (with 0.05% butylated hydroxytoluene BHT) was added, then tubes were placed in a heating block at 100 °C for two hours. 2.4 ml of H_2O and 12 ml of heptane was then added and mixed well. The upper phase consisting of heptane and lipids was pipetted into new tubes and evaporated to dryness with N_2 gas. Fatty acid methyl esters (FAME) were recovered with 50 µl of heptane and transferred to GC tubes.

The fatty acid composition for all samples was determined using an Agilent 6890N gas chromatograph equipped with a 7683B autoinjector and flame ionization detector (FID). The carrier gas used was helium and the various fatty acids were separated through a Varian

CP7419 capillary column (50 m x 250 µm x 0.25 µm nominal). The temperature of the injector was 240 ° C and the temperature of the detector was 250 °C.

The individual fatty acids were identified on the basis of the retention time and comparison with known standards. The area percentage was calculated as the area of the individual fatty acid peak in the chromatogram divided by the total area of all the fatty acid peaks. The area percentage of the various fatty acids then gives a good measure of the fatty acid composition in the samples. Values for fatty acid composition are expressed as the amount of individual fatty acid detected as a proportion of the total fatty acids in the sample (%).

3.10 Statistical analysis

All proportional data with non-normal distribution were arcsine transformed. In the cases where the requirements for normal distribution could not be met, non-parametric statistical tests were performed. Fertilization rate, length and sperm performance indicators were analyzed using an independent samples Kruskal-Wallis rank sum test. T-tests were performed to check for significant differences for weight, survival and fatty acids composition. For the sperm-motility data, the mean from the three replicates for PROG, LIN, MOT and VCL were calculated and Mann-Whitney U-tests were used to test for significance due to the small sample size.

Data are presented as mean ± STD or SE and differences considered significant at $p<0.05$. Statistical package used for analysis was R version 4.0.2

4 Results.

Prior to exploring the effect of the diets on eggs and larvae, the family effect on fertilization rate, sperm quality indicators, and fatty acid composition data was tested using one-way ANOVAs or Kruskal-Wallis rank sum tests. Genetic (family) differences were found for nearly all variables, meaning that the data could not be pooled into the two diet treatment groups.

4.1 Sperm quality

Kruskal-Wallis ranks sum tests revealed that there were significant family differences for the sperm activity parameters VCL (control *P*=.039, treatment *P*=.027) and PROG (control *P*=.027, treatment *P*=.027) in both dietary groups and LIN in the control group (*P*=.03). There were no significant differences within families in both groups for MOT and the treatment group for LIN (Figures 9 & 10). However, after pooling the data, independent T-tests showed there were no differences between the 2 dietary groups for any of the analyzed sperm performance parameters (Figures 11 & 12).

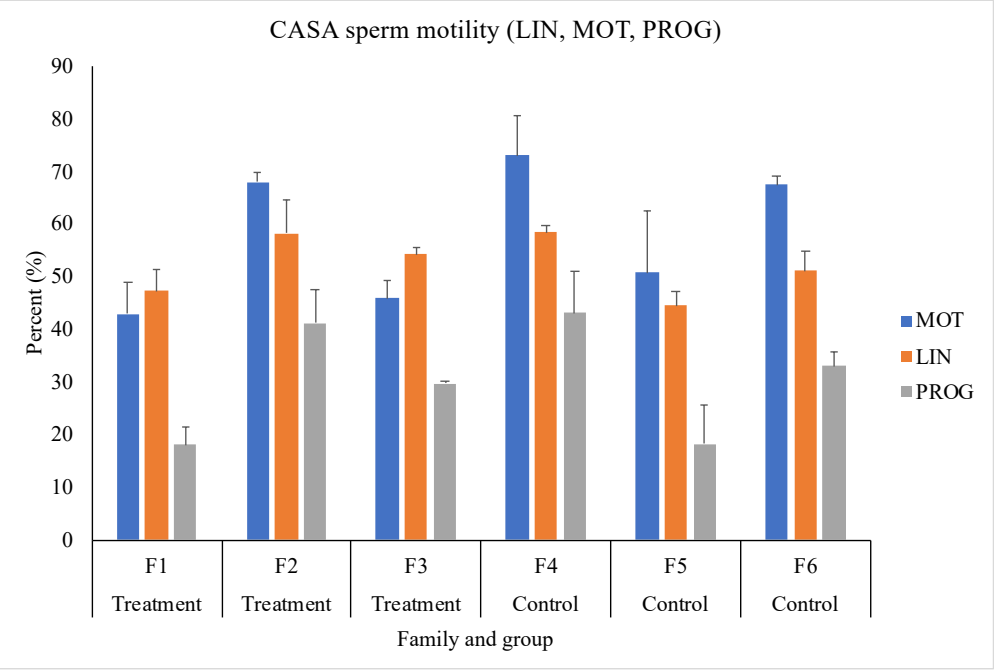

Figure 9. Sperm performance variables MOT, percentage motile sperm; LIN, linearity; PROG, percentage progressive sperm for each family in treatment and control diets.

Figures represent the means of n = 3 fish for each family

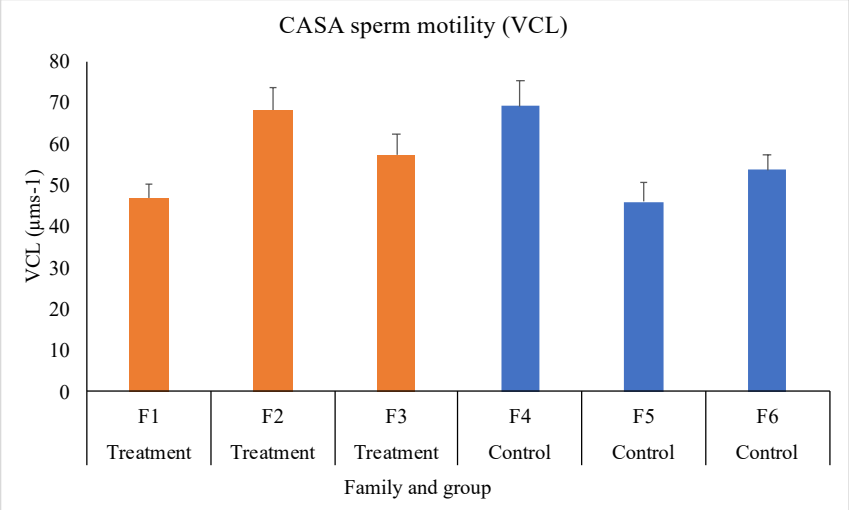

Figure 10. Mean measurements (n=3) of track velocity (VCL) for each family in treatment and control diets

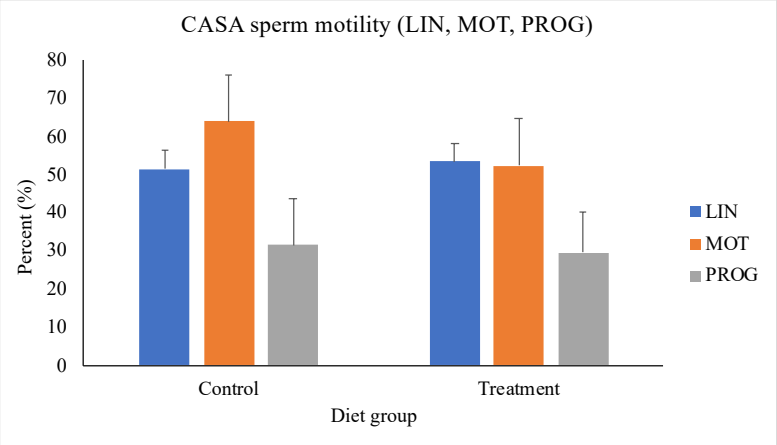

Figure 11. MOT, percentage motile sperm; LIN, linearity; PROG, percentage progressive sperm for each dietary group.

Values represent the means of n = 9 for each dietary group ± s.d

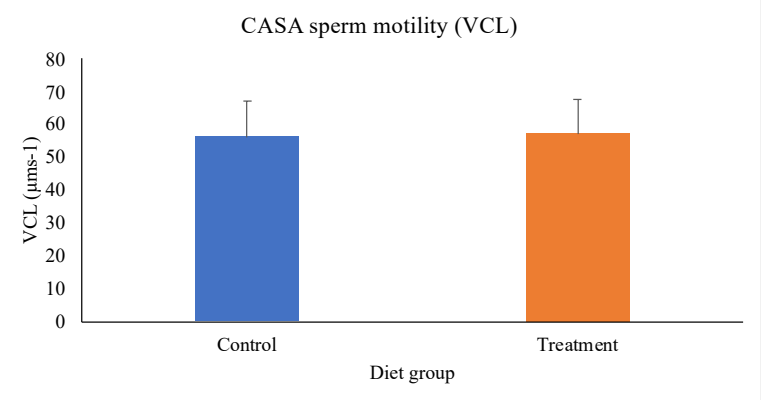

Figure 12. Sperm track velocity (VCL) for each dietary group.

Values represent the means of n = 9 for each dietary group ± s.d

4.2 Fertilization, egg mortality and hatching success

Average fertilization success varied between 41% and 81% for families fed the treatment diet and 71% to 84% for the control. However, a Kruskal-Wallis rank sum test showed that there was no significant difference in fertilization rate between the two diets (Figure 13).

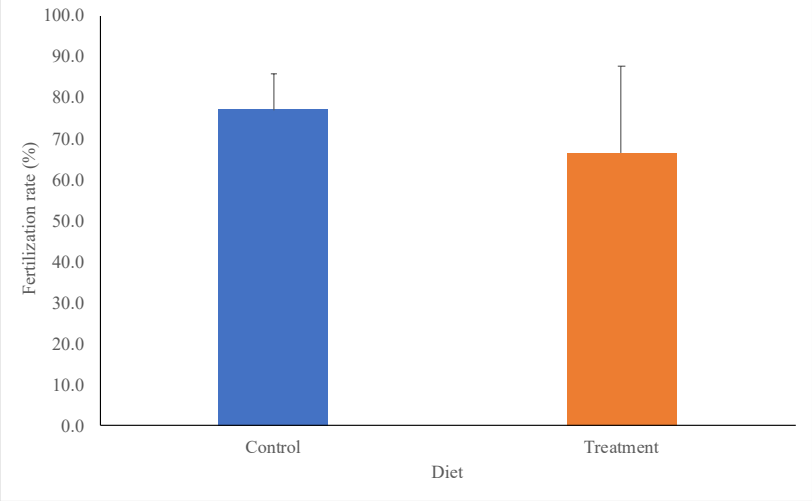

Figure 13. Mean (n=3) fertilization success of Atlantic cod eggs from fish fed different conditioning diets.

Diet did not significantly affect egg mortality between the two groups. Means: Control = 65.61% SD=0.13, Treatment= 64.05%, SD=0.22.

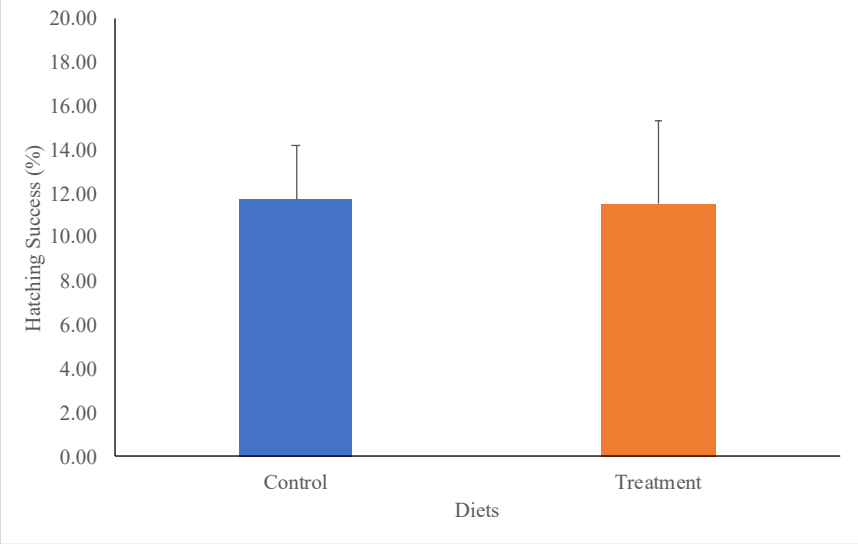

Figure 14. Mean (n=3) hatching success of eggs from broodstock fed the different conditioning diets.

Hatching success ranged from 6% to 16% for fish fed the treatment diet and 7% to 15.6% for the control diet. Diet was not significant in influencing hatching success (Figure 14).

4.3 Length, weight and survival

Larvae fed the control diet had significantly larger length at days 11 (*P*=.00819) and 32 (*P*=.004214) post hatch using a Wilcoxon rank sum test. However, larvae fed the treatment diet had a larger average length at 68 dph (*P*=.04863) (see Figure 15)

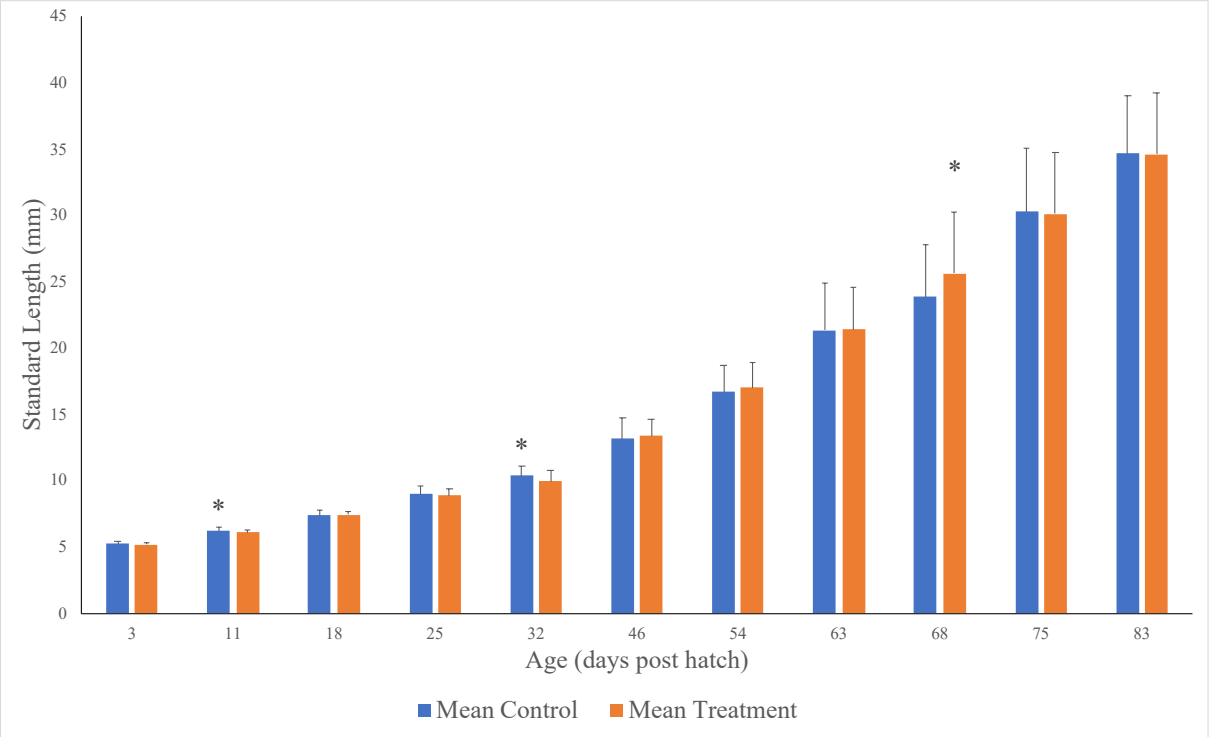

Figure 15. Standard length of Atlantic cod larvae between the dietary treatments from 3 dph to 83 dph (metamorphosis).

* Denotes significant difference at *P*=<.05.

Average survival at 85 dph for larvae fed the control diet was 14%, and 21% for the treatment diet. An independent t-test revealed that diet did not affect larval survival (Figure 16) or average weight from 85-176 dph (Figure 17).

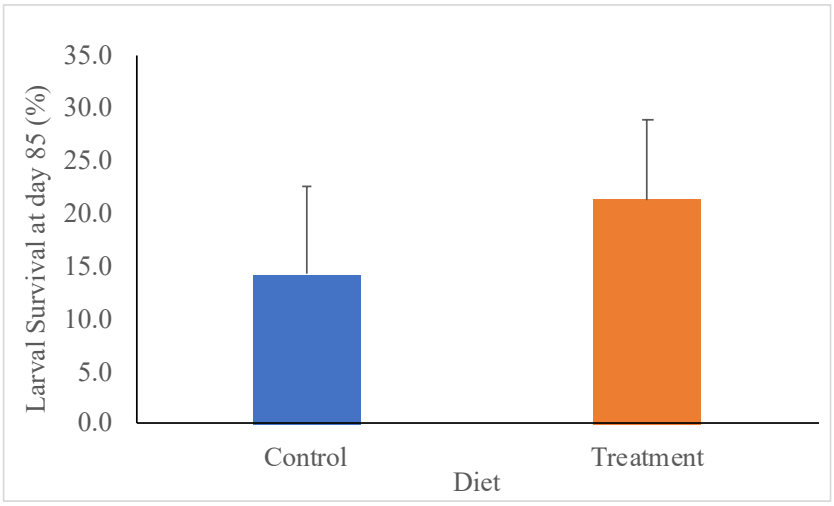

Figure 16. Survival of Atlantic cod larvae from the different diets at 85 days post hatch.

Values show means (n=3) ± s.d

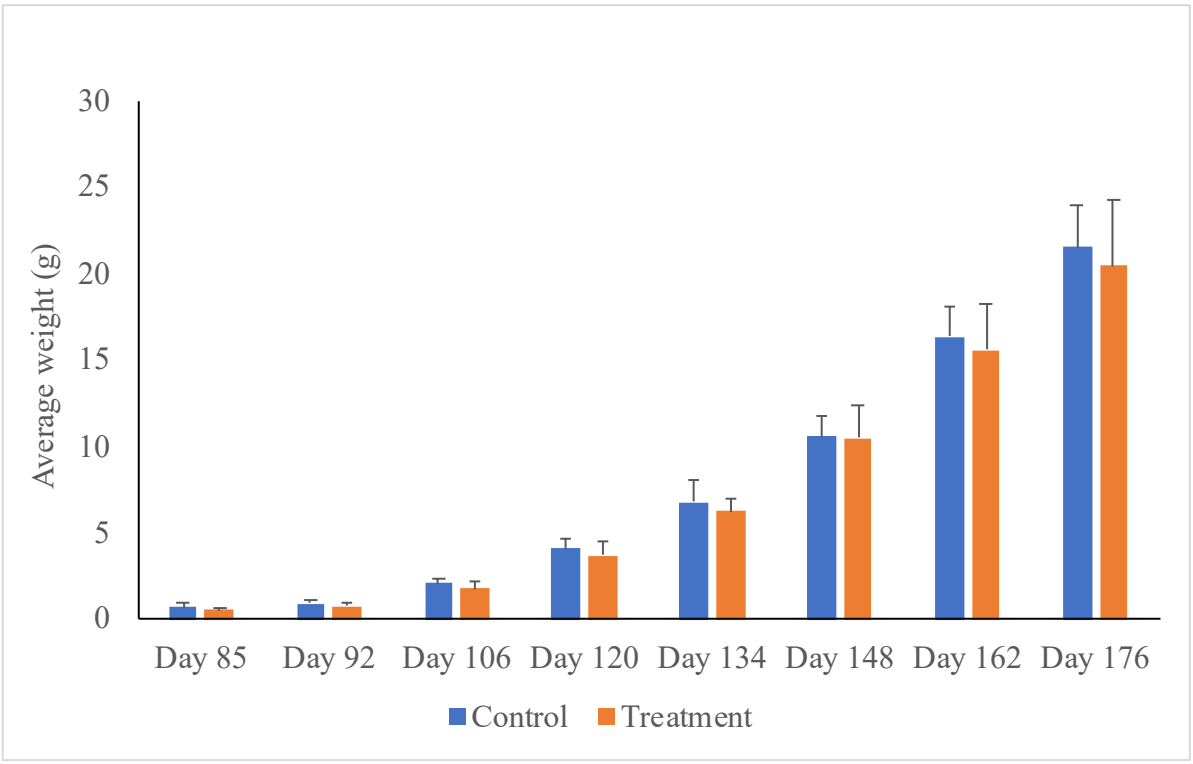

Figure 17. Average weight of Atlantic cod larvae from broodstock fed the different diets from 85 dph to 176 dph.

Values show means (n=3) ± s.d

4.4 Biochemical analysis

Biochemical analysis of the diets showed that they were very different in fatty acid composition. The control diet had significantly higher levels of nearly all fatty acids detected (Table 1). However, the treatment diet had significantly higher levels of three fatty acids of particular interest. These were, stearidonic acid (C18:4 n-3, *P*=<.001), cetoleic acid (C22:1 n-11, *P*=.006) and 11-eicosenoic acid (gondoic acid, C20:1 n-11, *P*=<.001)

An independent t-test showed that eggs and larvae from broodstock fed the treatment diet had significantly higher levels of palmitic acid (C16:0, eggs *P*=.0014, larvae *P*=.0015) despite the control diet containing more C16:0 (*P*=.039) (see Table 1). Eggs from the treatment diet also had higher levels of stearidonic acid (C18:4n-3, *P*=.012). However, eggs from fish fed the control diet had significantly higher levels of oleic acid (C18:1 n-9, *P*=.0014) and docosahexaenoic acid (DHA, C22:6 n-3, *P*=.006). Eggs and larvae from the control diet showed higher levels of linolelaidic acid (C18:2 n-6, eggs *P*=.014, larvae *P*=<.001) and arachidonic acid (ARA, C20:4 n-6, eggs *P*=<.001, larvae *P*=<.001). The significantly higher percentage of ARA in the control diet corresponded to higher proportions in the eggs and larvae fed the same diet.

Table 1. Fatty acid composition of diets, eggs and larvae.

	Diets		Eggs		Larvae	
	Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Fatty acid	**N =3**	**N = 3**	**N = 9**	**N = 9**	**N = 9**	**N = 9**
C12:0	0.09	0.17*				
C14:0	5.39	9.40*	1.54 ± 0.17	1.69 ± 0.21	1.15 ± 0.06	1.18 ± 0.11
C14:1	0.08	0.20				
C16:0	19.17*	18.42	19.32 ± 0.34	20.08 ± 0.48*	18.73 ± 0.32	19.56 ± 0.57*
C16:1 n-7	6.64*	5.19	1.68 ± 0.59	1.97 ± 0.02	1.62 ± 0.08	1.64 ± 0.10
C16:2 n-4	0.71*	0.55				
C16:3 n-4	0.67*	0.36				
C18:0	4.21*	2.18	2.68 ± 0.16	2.92 ± 0.43	5.43 ± 0.19	5.72 ± 0.26*
C18:1 n-11	1.40*	1.03				
C18:1 n-9	17.07*	12.63	12.82 ± 0.43	11.97 ± 0.50	11.05 ± 0.21	10.21 ± 0.75
C18:1 n-7	3.34	3.89*	3.07 ± 0.10	3.05 ± 0.14	3.08 ± 0.05	2.99 ± 0.12
C18:2 n-6	8.23	8.34	4.54 ± 0.11	4.24 ± 0.30	3.60 ± 0.04*	3.26 ± 0.16
C18:3 n-6	0.17*	0.14				
C18:3 n-3	1.68	1.70	0.80 ± 0.07	0.79 ± 0.08		
C20:0	0.43*	0.17				
C18:4 n-3	1.90	3.21*	0.41 ± 0.08	0.53 ± 0.10*		
C20:1 n-11	2.25	7.68*	1.38 ± 0.10	1.45 ± 0.17	1.58 ± 0.06	1.53 ± 0.14
C20:4 n-6 (ARA)	1.01*	0.28	1.90 ± 0.06*	1.67 ± 0.05	2.32 ± 0.08*	2.06 ± 0.06
C22:1 n-11	3.66	10.87*	0.74 ± 0.10	0.82 ± 0.05		
C20:5 n-3 (EPA)	10.25*	5.74	17.38 ± 1.24	17.58 ± 0.41	17.40 ± 0.51	18.11 ± 0.86
C22:4 n-6	0.45*	0.27				
C24:1 n-9	0.68*	0.14	0.56 ± 0.14	0.49 ± 0.03		
C22:5 n-3	1.25*	0.40	1.31 ± 0.09	1.31 ± 0.08	1.50 ± 0.06	1.45 ± 0.07
C22:6 n-3 (DHA)	9.26*	7.04	29.87 ± 0.36*	29.43 ± 0.21	32.53 ± 0.15	32.27 ± 0.60
∑						
Saturated	29.29	30.35*	23.54 ± 0.67	24.69 ± 1.12*	25.32 ± 0.57	26.46 ± 0.95*
Mono-unsaturated	31.47	30.76	19.52 ± 1.36	18.94 ± 1.03	17.33 ± 0.39	16.37 ± 1.10
n-3	28	28.96	50.50 ± 11.94	50.47 ± 0.92	51.43 ± 0.72	51.83 ± 11.54
n-6	9.86*	9.02	6.44 ± 0.17	5.90 ± 0.35	5.92 ± 0.13*	5.32 ± 0.22

Results are expressed as percentage of total fatty acids and show means ± s.d.

* Indicates significance at *P*=<.05

5 Discussion

Broodstock nutrition has been shown to influence the performance of fish eggs and larvae. Various studies have looked at the effects of astaxanthin, taurine and dietary water contents on the reproductive success of marine finfish. However, the majority have investigated the substitution of a single component as opposed to a combination of the three. The purpose of

this work was to evaluate the effects of modulating astaxanthin, taurine and dietary water into Atlantic cod broodstock conditioning diets on the performance of eggs and larvae. This was achieved by comparing two diets under experimental conditions and analyzing key performance indicators. The findings of this study suggest that there is no significant difference between the two diets in terms of their effect on spawning and larval performance in cod.

The results of this study point at large variation between families and individual males in regard to spawning and sperm performance indicators. Genetic differences in cod have been well documented for growth (Gjerde, Terjesen, Barr, Lein, & Thorland, 2004), length and body weight (Tosh, Garber, Trippel, & Robinson, 2010), disease resistance (Frenette et al., 2020) and for egg quality (Bachan, Fleming, & Trippel, 2012). The family effect in the present study appears to have had more influence on spawning success than the respective diets. Ideally, future nutritional studies could be improved by using replicates of males produced from the same genetic family crossed with females from another family. Although this is usually not economically possible due to the infrastructure required for such trials.

The present study contradicts the findings of Hansen et al. (2016) in terms of egg or larval performance related to an increase in dietary astaxanthin. This could be due to a number of issues with the experimental design. The most important being that two completely different diets were used in the current experiment which makes it difficult to distinguish what ingredients are influencing the results. For example, the control diet, which is a standard conditioning diet contained significantly more of nearly all of the essential fatty acids. Therefore, it is possible that the positive nutritional effects of taurine and increased astaxanthin were canceled out by the potentially better suited fatty acid profile of the control diet. By using two or more identical diets with different levels of taurine, astaxanthin or H2O, the results would have been more comparable.

Fertilization rate is the most reliable indicator of sperm quality (Pavlov et al., 2004). However, here was no control over sperm volume added to eggs, therefore, any correlations between performance indicators for sperm and spawning success could not be quantified. By adding sperm to eggs in excess and at an unknown volume, as was the case in the present experiment, it is not possible to accurately measure sperm quality. One reason is because high sperm motility is not always essential for successful fertilization. For example, Trippel and Neilson (1992) found that male cod with high levels of immotile sperm achieved similar

fertilization rates to those of males with exclusively motile sperm. This suggests that MOT is not a good indicator of cod sperm quality and by adding milt at a high sperm to egg ratio, there is no opportunity for the higher quality sperm to outcompete the weaker ones. So, in order to accurately use fertilization rate as a good indicator of sperm quality under experimental conditions, a minimum sperm to egg ratio should be used. Under commercial conditions however, where sperm is usually in high supply, the goal is to achieve maximum fertilization while minimizing waste of gametes and a standard sperm to egg ratio of 1×10^5 sperm per egg is recommended (Butts, Trippel, & Litvak, 2009).

Hand stripping of male finfish during artificial spawning can lead to urine contamination of sperm and is often hard to avoid. Urine contamination has been shown to negatively affect the quality of sperm in a number of species (Dreanno et al., 1998; Król et al., 2018; Rurangwa et al., 2004) and it is possible that the males in the present study who's samples displayed lower percentages of motile sperm, had been contained by minute amounts of urine. Further research into the effects of urine contamination on Atlantic cod sperm quality is needed to improve fertilization rates.

Broodstock nutrition is crucial for producing high-quality eggs and larvae, and the fatty acid composition of diets is known to be important for the success of their development (Izquierdo et al., 2001) Salze et al. (2005) found a strong relationship between the levels of ARA in eggs and fertilization, hatching success rates and cell symmetry. ARA levels in the control diet (1.01%) were significantly higher than in the treatment diet (0.28%). ARA was also significantly higher in the eggs and larvae of broodstock fed the control diet, which is in agreement with Røjbek et al. (2014), who suggested that the ARA in cod eggs is related to its dietary content and its accumulation in oocytes during development and is of high importance for egg quality. However, this did not affect spawning or larval performance and are in contrast to the findings of Røjbek et al. (2014), who reported that eggs from cod fed a diet with high ARA content had significantly higher fertilization and hatching success than those fed low levels of ARA. Because the eggs and larvae from broodstock fed the standard commercial diet had significantly higher levels of ARA, they could be regarded as having higher quality although this was not evident in the differences in any of the larval and egg performance indicators.

Two additional fatty acids of particular interest were cetoleic acid (CA, C22:1 n-11) and alpha-linolenic acid (ALA, C18:3n-3), both of which the treatment diet had significantly

higher levels (Figure 18). Cetoleic acid is a good indication that fish oil from North Atlantic origin was used in the treatment diet. ALA is found in plant oils and is a precursor to EPA and DHA (Tocher, 2003). Research has shown that CA helps with the synthesis of ALA to EPA and DHA in salmon and humans liver cells (Østbye et al., 2019).

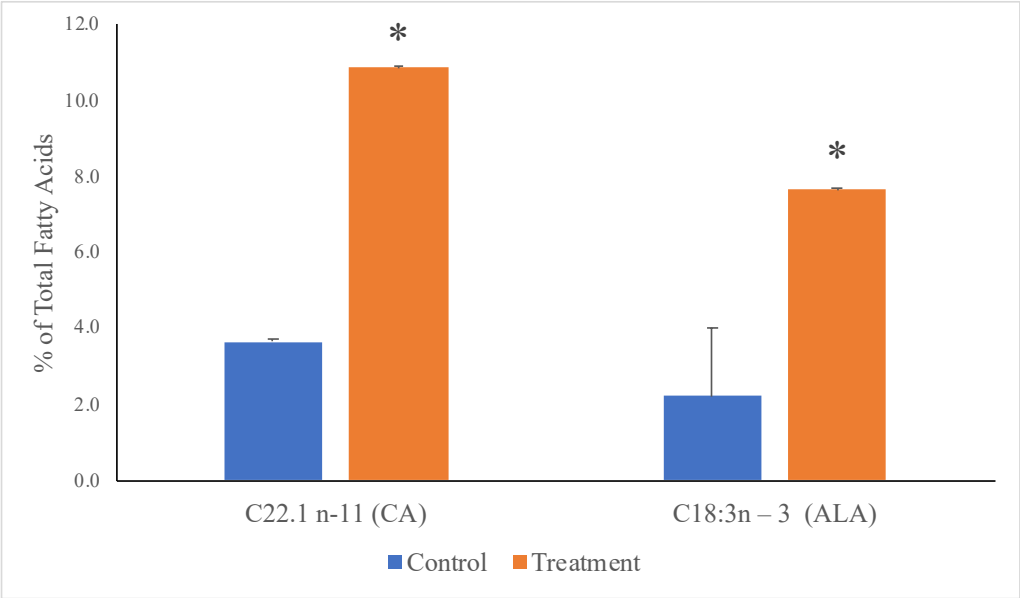

Figure 18 Cetoleic acid (CA) and alpha-linolenic acid (ALA) levels in the diets.

Values represent the mean percentages ± s.d of the total fatty acids. * Indicates significant at P = <0.05.

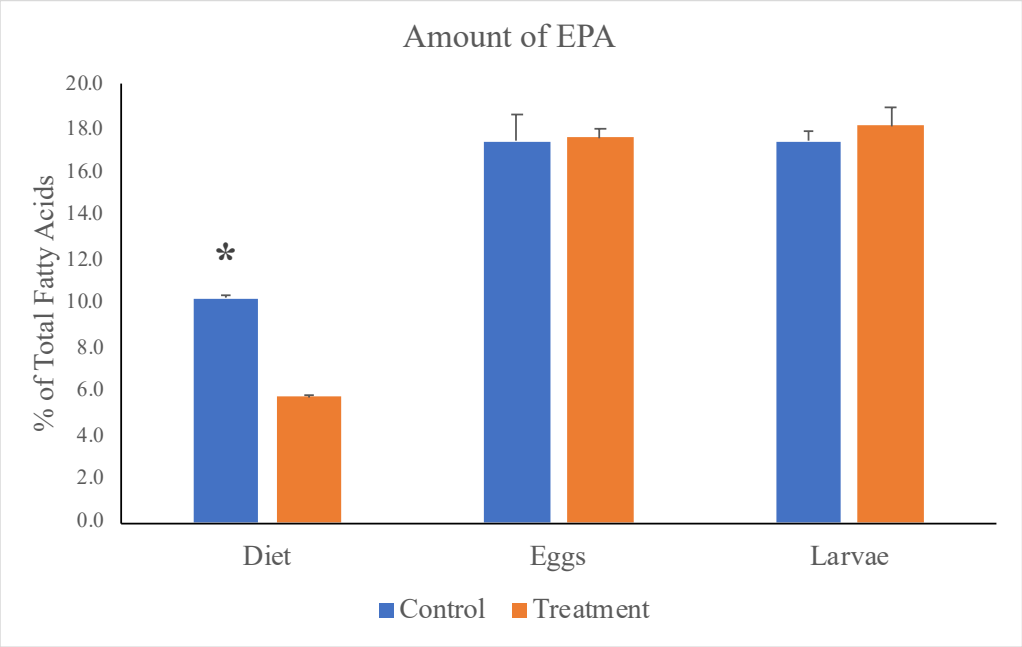

Figure 19 Mean percentage ± s.d of eicosapentaenoic acid (EPA) present in diets, eggs and larvae samples

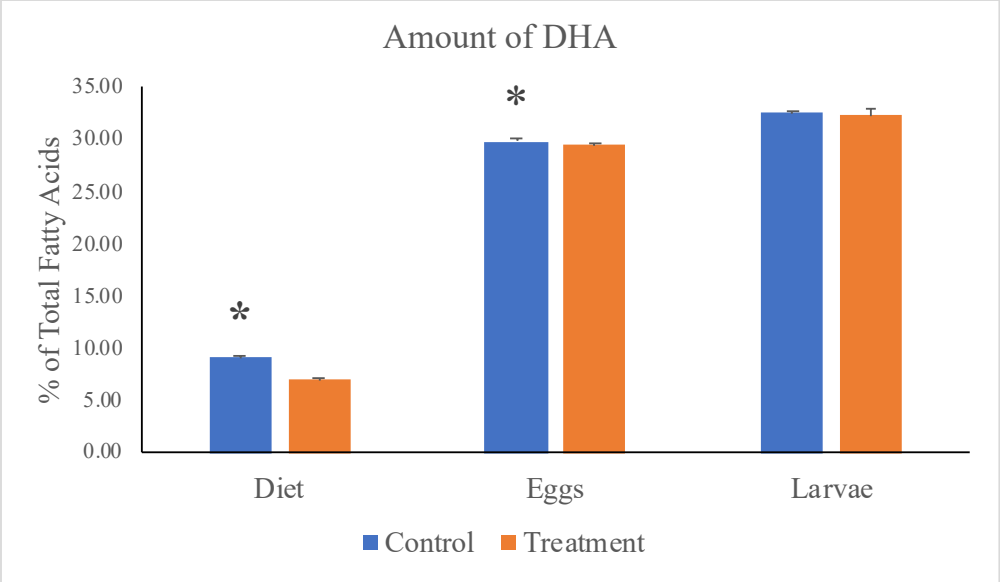

Figure 20 Mean percentages ± s.d of docosahexaenoic acid (DHA) present in diets, eggs and larvae samples

In the present study, even though the amounts of EPA and DHA in the control diet were significantly higher than the treatment, the levels in the eggs and larvae were equal for both groups (Figures 19 and 20). This could result from the conversion of short chain fatty acids such as ALA and SDA into EPA/DHA through synthesis from cetoleic acid.

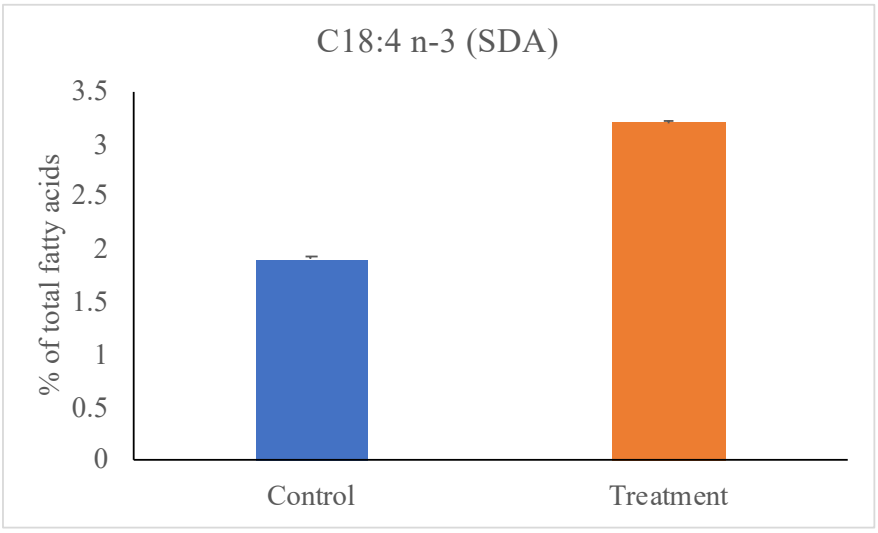

Figure 21 Mean percentage ± s.d of stearidonic acid (SDA) in the diets.

A second possible explanation for the equal amounts of EPA and DHA in both dietary group's eggs and larvae despite the treatment diet containing much less of both fatty acids is that the treatment diet had significantly higher levels of stearidonic acid (SDA, C18:4n-3) (Figure 21). The copepod *Calanus finmarchicus* and North Atlantic mesopelagic and pelagic fish species normally contain substantial amounts of SDA (Pedersen, Vang, & Olsen, 2014), which is again indicative that the treatment diet contained oil from North Atlantic fish or *Calanus finmarchicus*. SDA is a precursor of n-3 long chain polyunsaturated fatty acids (n-3 LC PUFA) such as EPA and DHA, and is derived by desaturation of ALA (Tocher, 2003). It has been suggested that the delta-6 desaturation from ALA to SDA is the limiting step in this bioconversion process (Guil-Guerrero, 2007). In Atlantic salmon parr, dietary SDA potentially bypasses the initial rate-limiting delta-6 desaturase step and enables greater biosynthesis of EPA and DHA. When fed a diet containing SDA and only trace amounts of n-3 LC PUFA, fish maintained their concentrations of EPA and DHA, highlighting SDAs potential as a dietary precursor in aquafeeds (Miller, Nichols, & Carter, 2007). It is therefore possible that SDA contributed to the EPA/DHA synthesis after broodstock consumed the treatment diet in the present study. There is also a third possibility explaining the equal amount of EPA and DHA in eggs and larvae, and that is the ability of female cod to allocate different lipids classes and fatty acids to different batches of eggs throughout the spawning period (Bachan et al., 2012). Oocyte development begins 8–9 months before spawning, and nutrients are allocated to the eggs all the way to the final maturation of the different batches (Kjesbu, 1991). Because the artificial spawning in the present study took place over a period of 4 days, it is feasible that females were able to allocate differentially greater amounts of EPA and DHA to eggs to compensate for the lower amounts in the treatment diet. Although it is not possible to draw any conclusions from these findings, it is of particular interest to the sustainability of the aquaculture industry and would benefit from further investigation. One of the biggest challenges facing the growth of the aquaculture industry is access to a larger sources of feed ingredients that are rich in omega 3 fatty acids. If the amount of marine based fish oils added to cod aquaculture feeds could be reduced by using plant-based substitutes without compromising growth, reproduction or performance, then the industry could become more sustainable.

5.1 Limitations of the study

The limitations discussed below were outside of the researcher's control due to the nature of the study being undertaken in collaboration with Nofima and being part of another project. It

was therefore not possible to alter the experimental design or diets in any major way. The most important limitation was the use of two very different diets in terms of their composition and ingredients. This made it difficult to quantify what impact the substitution of nutrients in the experimental diet had on spawning success and egg and larval quality. Additionally, it would not have been possible to separate the effects of astaxanthin, taurine and higher water content if any significant differences were found. Ideally, two or more identical diets should have been tested with the treatment diets containing one or a combination of the additional nutritional substitutes should have been used.

Calculating the initial number of eggs can often be a difficult task. Egg diameter should have been measured accurately for each family in order to avoid the issue of undercalculated initial egg numbers. Which in turn led to overestimations of egg survival and hatching success. Egg diameter decreases with time throughout the spawning period (Hansen & Puvanendran, 2010), therefor, egg numbers could have been underestimated as egg size was recorded as the same for all females.

Initially biochemical analysis was intended to include antioxidative capacity (AOC) measured by ORAC assay (oxygen radical absorbance capacity) in order to investigate the effect of the antioxidant activity of astaxanthin and taurine. However, due to insufficient volumes of egg and newly hatched larvae samples being taken, everything was used for the fatty acid analysis, so AOC had to be omitted. This information would have been useful for showing any possible differences in the effects of additional astaxanthin and taurine on lipid peroxidation. Additionally, amino acid composition analysis on eggs and larvae to compare the levels of taurine across the samples could have yielded useful information. Without this information, it would not be possible to distinguish between the effects of taurine and astaxanthin. However, due to budget and time restraints this was not possible.

Finally, the sample sizes for the majority of variables were relatively small. This was due to the resources required for biochemical analysis and housing large numbers of larvae and the high cost involved. By increasing the number of families for each dietary treatment and ultimately the number of eggs and larvae for biochemical analysis a more representative distribution of the population would have been achieved.

5.2 Conclusion

This experiment aimed to test the effects of an Atlantic cod broodstock conditioning diet with added astaxanthin, taurine and dietary water contents when compared to a standard conditioning diet. The results of this study were inconclusive and found no differences in the performance of the two diets. This was partly due to the genetic effect of different families and limitations to the experimental design. While the lack of an experimental effect in this study made it not possible to make any generalizations, it was however informative because it opens up the possibility of future work relating to the use of alternatives to marine oils in cod broodstock diets. Some of the previously stated limitations of this study could be minimized or eliminated in a revised experimental design. To improve the issues of genetic differences between families, larger sample sizes should be used, preferably with crosses of genetically similar males with similar females from different bloodlines. The effects of the different nutritional supplements should be investigated on their own, using similar diets and amino acids analysis to support the data in future studies. Due to the issues of mortality, egg quality and larval rearing associated with the cod aquaculture industry, broodstock nutrition still requires further research in order to improve the quality of gametes and create a more sustainable industry.

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