Growth and development of juvenile spotted wolffish (*Anarhichas minor*) fed microalgae incorporated diets

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Preface

This dissertation is submitted in fulfillment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University. The presented original research was performed as part of the Stipendiatprogram Nordland and with financial support from Nord University, Aminor AS and Nordland Fylkeskommune.

The PhD project team consisted of the following members:

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Helene Rønquist Knutsen

Bodø, December 18., 2018

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Abstract

Spotted wolffish (*Anarhichas minor*) is a promising candidate species for cold-water aquaculture, and has been shown to be highly suitable for cultivation. As both present and future diets for farmed fish are expected to become more reliant on alternatives to fishmeal and –oil, future aquaculture of wolffish will also have to use these ingredients in the production. Therefore, there is a need to investigate the capacity of spotted wolffish to utilize alternative feed resources such microalgae. The main objectives of this thesis were to evaluate i) the capacity of wolfish to utilize microalgae in fishmeal-based or mixed diets consisting of marine and plant based protein ingredients, (ii) the dietary effect on muscle development and growth and iii) the dietary effects on liver, intestine and muscle proteome including intestinal health indices. This thesis and the papers included are based on results from two experiments. In the first experiment juvenile spotted wolffish were fed graded inclusion levels of the microalgae *Scenedesmus obliquus* replacing 0, 4, 8 and 12% of the fishmeal in the diets. In the second experiment juvenile spotted wolffish were fed graded inclusion levels of the microalgae *Nannochloropsis oceanica* replacing 0, 7.5 and 15% of a mix of fish- and plant-based ingredients in the diets. Wolffish fed both *S. obliquus* and *N. oceanica* had comparable growth rates to what has previously been reported for wolffish of similar size; growth was not different in the fish fed *S. obliquus* and *N. oceanica* compared to the control-groups in the respective experiments. Hepatosomatic index decreased in all groups and muscle crude fat increased in both the *S. obliquus* and N. *oceanica* growth trials, indicating the wolffish incorporates more lipid in the muscle as they grow. Incorporation of *N. oceanica* in the diet of spotted wolffish altered the fatty acid composition of the fish. Polyunsaturated fatty acids and especially linoleic acid (C18:2n-6) increased in all groups reflecting the use of about 50% plant-based ingredients in all diets; the ω-3 fatty acid eicosapentaenoic acid was increased in whole body of the algae-fed fish. Tendencies of increased fast muscle hypertrophy were observed in the fish fed 12% *S. obliquus*; for fish fed *N. oceanica* there was no difference in fast muscle growth parameters between the treatment groups. The assessment of fast muscle cellularity in the *N. oceanica* growth trial indicate that wolffish have a similar growth pattern as other

fishes with a sub-arctic and benthic life history, by producing relatively large fibers and a modest maximum fiber number. Incorporation of *S. obliquus* in the diet of spotted wolffish affected muscle, liver and intestinal proteome; identified proteins indicate that *S. obliquus* diet affected protein synthesis and cell growth in muscle and intestine, liver gluconeogenesis as well as the antioxidant system of the fish. From the morphological assessment of intestine there was no indications of intestinal inflammation, but the decreased size of goblet cells may indicate a compromised mucous barrier in the fish fed *S. obliquus*.

Abstract in Norwegian – Sammendrag på norsk

Flekksteinbit (*Anarhichas minor*) er en lovende fiskeart for oppdrett i kalde farvann, er blitt vist å ha mange gunstige egenskaper for akvakulturproduksjon. Ettersom det forventes at både dagens og fremtidens fôr for oppdrettsfisk kommer til å bli økende avhengig av alternativer til fiskemel og –olje som fôrressurser vil det forventes at disse fôrressursene også må benyttes i produksjon av flekksteinbit i fremtiden. På grunn av dette er det et behov for å undersøke flekksteinbitens kapasitet for å utnytte alternative fôrressurser som mikroalger. De overordnede målene ved denne avhandlingen var å evaluere i) flekksteinbitens kapasitet for å utnytte mikroalger i et fiskemels-basert eller blandet fôr bestående av marine og plantebaserte protein-ingredienser, ii) fôrets påvirkning på muskelutvikling og vekst og iii) fôrets påvirkning på lever, tarm og muskel proteom inkludert tarm-helse indikatorer. Denne avhandlingen og artiklene som er inkludert er basert på resultater fra to forsøk. I det første forsøket ble flekksteinbityngel fôret graderte mengder av mikroalgen *Scenedesmus obliquus* som erstattet 0, 4, 8 og 12% av fiskemelet i fôret. I det andre forsøket ble flekksteinbityngel fôret graderte mengder av mikroalgen *Nannochloropsis oceanica* som erstattet 0, 7.5 og 15% av et blandet fôr bestående av marine og plantebaserte protein-ingredienser. Steinbit fôret både *S. obliquus* og *N. oceanica* hadde vekstrater tilsvarende det som er rapportert i andre studier av steinbit av tilsvarende størrelse; det var ingen forskjell i vekst mellom fisken som var fôret *S. obliquus* eller *N. oceanica* og de respektive kontrollgruppene i hvert av forsøkene. Hepatosomatisk indeks avtok over tid i alle gruppene i både *S. obliquus* og *N. oceanica* forsøket og muskelfettet økte, noe som indikerer av steinbiten inkorporerer mer fett i muskel etterhvert som de vokser. Bruk av *N. oceanica* I fôret til flekksteinbit endret også fettsyresammensetningen i fisken; flerumettede fettsyrer og særlig linolensyre (C18:2n-6) økte i alle grupper og reflekterer bruken av omtrent 50% plante-baserte ingredienser i fôret; ω-3 fettsyren eikosapentaensyre økte i hel-kropp til den alge-fôrede fisken. Tendenser til økt hypertrofi i hvit muskel ble også observert i fisken som var fôret 12% *S. obliquus*; for fisken fôret *N. oceanica* var det ingen forskjell i vekstparametere for hvit muskel mellom forsøksgruppene. Evaluering av cellularitet for hvit muskel i *N. oceanica* vekstforsøket indikerer at steinbit har et lignende

vekstmønster for muskel som er observert for andre fiskearter med sub-arktisk og bentisk livshistorie, ved at de produserer relativt store fibre og potensielt har et mer moderat maksimalt fiberantall. Bruk av *S. obliquus* som fôringrediens for flekksteinbit påvirket proteomet i muskel, lever og tarm; identifiserte proteiner indikerte at *S. obliquus* påvirket proteinsyntese og cellevekst i muskel og lever, lever glukoneogenese samt antioksidant systemet i fisken. Fra den histologiske evalueringen av tarm var det ingen indikasjoner på betennelsesrespons i tarmen, men en reduksjon i størrelsen på gobletceller kan muligens indikere en kompromittert slim-barriere i tarmen hos fisken som var fôret *S. obliquus*.

1. Introduction

1.1 Feeding a growing population: Global aquaculture production and food security

The global population is expected to exceed 9 billion by 2050, and with a rapidly growing population, fish will be increasingly important for food security and proper nutrition (Béné et al., 2015). The world's production of fish reached a peak of 171 million tons in 2016, where aquaculture production represented 47 percent of the total and 53 of fish used for human consumption (FAO, 2018). Aquaculture is the fastest growing food production sector in the world, producing 80 million tons of seafood in 2016, whereof 54.1 million tons were finfish (FAO, 2018). Presently, the world's wild fish stocks are either over-exploited or fished to their carrying capacity; capture fishery production has been stable since the late 1980s (FAO, 2018). Consequently, aquaculture has been responsible for the majority of the increase in global fish production in the last decades, and future demands for fish must be provided from aquaculture (FAO, 2018).

A beneficial composition of essential fatty acids, protein, vitamins and minerals make fish and other seafood uniquely healthy (Lund, 2013). The Norwegian Health Directorate recommends the consumption of 300-450 g of fish per week, of which at least 200 g should be fatty fish such as salmon, trout, mackerel or herring (Helsediktoratet, 2011). In a large-scale review of the scientific evidence for adverse and beneficial health effects of fish consumption, benefits of seafood consumption clearly outweighed the potential risks involved, for example related to accumulation of environmental toxins and heavy metals (Mozaffarian and Rimm, 2006). Fish, and especially fatty fish, is our main dietary source of essential polyunsaturated fatty acids (PUFA), particularly omega-3 (ω-3) fatty acids (Calder, 2014). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the most important ω-3 fatty acids, and a daily consumption of a minimum of 250 mg EPA+DHA is the current recommendation (FAO, 2010, Helsedirekoratet, 2011). DHA and EPA are vital for human health and have been linked to prevention of cardiovascular disease, cancer and inflammation and are essential for normal function, development and disease prevention in the brain and

other neural tissues (Calder, 2014). Fish is also an important protein source for humans; in 2015 fish accounted for 17% of the animal protein consumed globally and provided 3.2 billion people with 20% of their average animal protein intake (FAO, 2018). Providing not only essential fatty acids and protein, but also other important micronutrients such as vitamins, iron and zinc, fish is an important food-source for humans all over the world, and especially in developing countries (Golden et al., 2016).

1.2 Feed ingredients and sustainability

Expanding food production from the aquaculture sector has created an increasing demand for high quality and cost-effective feed ingredients. Traditionally, fishmeal and oil has provided the basis for cost-efficient diets with a complete nutritional profile and high digestibility for cultured fish (FAO, 2018, Olsen and Hasan, 2012, Tacon and Metian, 2015). However, the use of these ingredients has raised important issues, both due to negative impacts on the marine ecosystems but also because the fish could instead be used directly for human consumption (Béné et al., 2015). Presently, about 12% of the world's total fish production (about 20 million tons) is reduced to fishmeal and oil, of which the majority is used to feed both aquatic and terrestrial farmed animals (FAO, 2018). Although this is a substantial decrease from the peak production of fishmeal of 30 million tons in 1994 and a growing share (25-35%) of the fishmeal production is sourced from fish by-products, 90% of the wild-captured fish used for producing fishmeal are food-grade fish that could have been used as human food (Cashion et al., 2017, FAO, 2018). The steadily increasing price of fishmeal also makes it necessary for the aquaculture sector to look for alternative resources (FAO, 2018). Fish feed is also the highest cost factor in intensive aquaculture, accounting for up to 50-70% of production costs (Rana et al. 2009). Hence, to ensure sustainable and economically feasible growth, future aquaculture production needs to rely more on both fishmeal produced from non-food by-products as well as alternatives to fishmeal and –oil such as terrestrial plants, insect meal, marine invertebrates, macro- and microalgae.

Several alternatives to fishmeal and –oil have been suggested and evaluated. These alternative ingredients include by-products from terrestrial animal production such as meat, blood, bone and even feather meal (e.g. Breck et al., 2003, Bureau et al., 2000, Hamed et al., 2017, Hatlen et al., 2015, Yang et al., 2004); single-cell protein from bacteria and yeast (e.g. Grammes et al., 2013, Kiessling and Askbrandt, 1993, Perera et al., 1995, Øverland and Skrede., 2017); insect meal (e.g. Devic et al., 2018, Magalhães et al., 2017), marine invertebrates (e.g. Wan et al., 2017) and marine macroalgae (e.g. Sphigel et al., 2017). Terrestrial plant proteins are probably among the most highly studied fishmeal alternatives (e.g. Minjarez-Osorio et al., 2016, Valente et al., 2016). Use of plant proteins such as soybean, beans, peas and wheat in aquaculture diets have been evaluated for many species, e.g. for European sea bass (*Dicentrarchus labrax*) (Kaushik et al., 2004, Torrecillas et al., 2017), Atlantic salmon (*Salmo salar*) (Johnsen et al., 2011, Ytrestøyl et al., 2015), Atlantic cod (*Gadus morhua*) (Hansen and Hemre, 2013) and Senegalese sole (*Solea senegalansis*) (Cabral et al., 2011). Plant ingredients are also used commercially in Norway for farmed Atlantic salmon and were gradually increased from 10% in 1990 to more than 70% in 2013 (Ytrestøyl et al., 2015).

Although replacement of fishmeal and oil with terrestrial plant-ingredients has proven successful for many species, challenges do exist. It could be argued that terrestrial plants can be used more efficiently, in terms of food security, as human food. As land-based protein sources use land-area and fresh water, both of which are limited resources, the sustainability of using these resources to feed fish is also debatable (Boissy et al, 2011, Pahlow et al., 2015). Imbalanced amino acid composition, high carbohydrate content and potential presence of anti-nutritional factors may have adverse effects on gut health, digestion and utilization of nutrients (Bakke et al. 2014, Krogdahl et al., 2010, Marjara et al. 2012). Finally, due to the increased use of terrestrial plant-ingredients in salmon farming, the content of n-6 fatty acids in salmon has increased while levels of DHA and EPA are decreasing (Sprague et al., 2016). To ensure that the nutritional value of the final product consumed by humans is not compromised, future aquaculture production cannot rely on land-based plant ingredients alone.

Microalgae and other photosynthetic or heterotrophic single cell organisms, such as bacteria and yeast often recognized as microalgae, pose an interesting alternative to replace, at least partly, both fishmeal, fish oil and terrestrial plant-ingredients (Ruiz et al., 2016). Microalgae contain high levels of protein with balanced amino acid profiles, essential PUFAs, antioxidants and vitamins (Brown et al., 1997; Schüler et al., 2017, Yaakob et al., 2014). They can also be produced in salt- and wastewater or be cultivated on land areas which are unsuited for other types of agriculture (Collotta et al., 2016; Marjakangas et al., 2015). Several species of microalgae such as *Nannochloropsis* sp. and *Phaeodactylum tricornutum* have been successfully tested as feed ingredients in aquaculture showing benefits for growth and final product traits (Sørensen et al., 2016; 2017). Atlantic salmon fed *Schizochytrium* sp. showed an improved fillet quality and retention efficiency of omega-3 fatty acids (Kousolaki et al. 2016). Nile tilapia (*Oreochromis niloticus*) fed the microalgae *Nannochloropsis salina* gave improved protein retention efficiency and higher ratio of n-3 polyunsaturated and n-3/ n-6 fatty acids in the fish (Gbadamosi and Lupatsch, 2018). Post-smolt Atlantic salmon fed 30% *N. salina* also showed promising results in terms of feed digestibility (Gong et al., 2018). However, there are still bottlenecks that need to be solved before microalgae can be used commercially. With the production technology available today, biological productivity of microalgae is still too low and production cost too high for microalgae to compete with fishmeal and -oil (Chauton et al., 2015). Such decrease in production costs may be feasible within the next decade by improving the biological productivity, evaluating optimal geographical locations for production and developing novel production technology (Chauton et al., 2015). Thus, as microalgae likely will be commercially available at competitive prices in the future, evaluation of microalgae as feed ingredients in aquaculture production is important.

1.3 Spotted wolffish

Species diversification is another factor that is expected to play an important role in sustainable future expansion of food production from aquaculture (FAO, 2018). Production of a wide range of native species is not only expected to contribute to

reduced pressure on wild fish stocks, but also to a more robust industry, enabling new markets and hence also promoting economic gains (Pascal et al., 2009). Norway is a good example of a country where the aquaculture production is nearly dominated by a single species; Atlantic salmon accounted for 94.5% of the total aquaculture production in Norway in 2017 (SSB, 2018). Another salmonid, rainbow trout (*Onchorynchus mykiss*), made up the majority of the remaining, accounting for 5.1% of the total Norwegian aquaculture production (SSB, 2018). Governmental strategic documents are pointing to the need to expand the number of species in Norwegian aquaculture (Nærings- og fiskeridepartementet, 2015). Optimal environmental conditions for fish farming and a wealth of expertise and technological advances give Norway a vast potential for production expansion through increased species diversification.

Spotted wolffish (*Anarhichas minor*) is a marine fish with promising qualities for cold-water fish farming. The spotted wolffish is not only considered to be excellent food-fish but also displays a unique and curious biology. It belongs to the family Anarhichadidae (Order: Perciformes, Suborder: Zoarcodei), also known as the sea wolfs or wolffishes (Le François et al. 2010). The wolffish family are primarily benthic fish found from shallow to deep cold water in both the Pacific and Atlantic Ocean (Le François et al. 2010). The family comprise two genera, *Anarrhichthys* and *Anarhichas*, where the spotted wolffish belong to the latter. The word *Anarhichas* originated from Greek meaning “to climb up”, as it was believed from legends that they could climb up onto rocks and cliffs (Rountree, 2002). The genus *Anarhichas* includes four species: the common or Atlantic wolffish (*Anarhichas lupus*), the northern wolffish (*A. denticulatus,* formerly known as *A. latifrons*), spotted wolffish and the Bering wolffish (*A. orientalis*) (Figure 1). The genus *Anarrhichthys* includes only one species: the wolf eel (*Anarrhichthys ocellatus*) (Figure 1). Generally, wolffish have a compressed and moderately elongated body-shape, with large, blunt heads and characteristic powerful jaws and teeth adapted to a diet of hard-shelled animals such as crustaceans, echinoderms and mollusks (Albikovskaya, 1982). These powerful jaws and teeth along with theird unsightly appearance and aggressive temperament when captured, have given wolffishes a reputation among fishermen, and they have been known to bite through wood and even steel (Rountree, 2002).

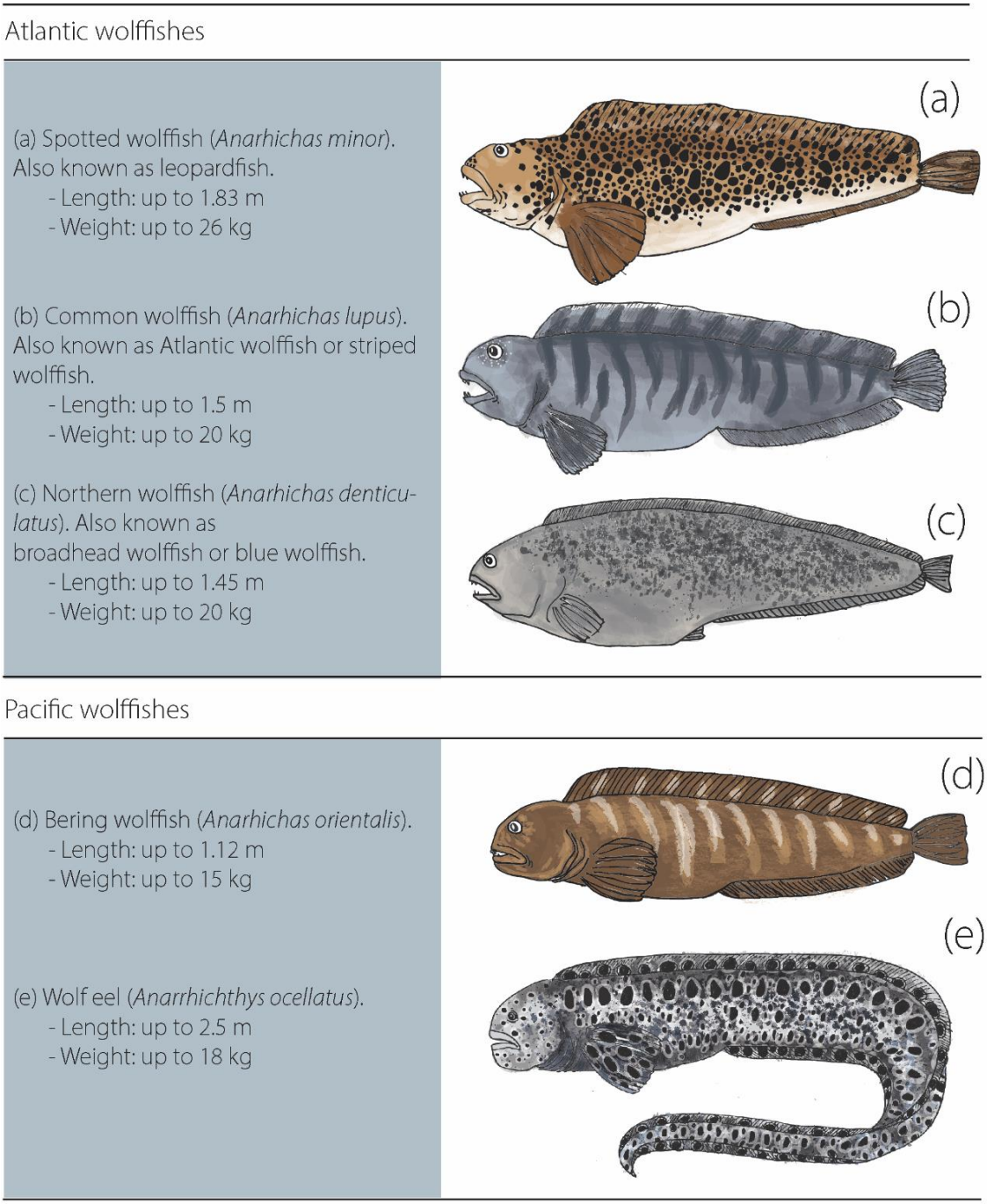


Figure 1: The wolffish family (Anarhichadidae). (a) Spotted wolffish (*Anarhichas minor*). (b) Common wolffish (*Anarhichas lupus*). (c) Northern wolffish (*Anarhichas denticulatus*). (d) Bering wolffish (*Anarhichas orientalis*). (e) Wolf eel (*Anarrhichthys ocellatus*). Illustration: Helene Rønquist Knutsen, Nord University.

The common, northern and spotted wolffishes are found in the North-Atlantic Ocean, while the wolf eel and Bering wolffish are native to the North Pacific Ocean (Le François et al. 2010). The spotted wolffish inhabits the arctic-boreal North Atlantic Ocean, from

Spitsbergen and Bear Island to the coast of Northern Norway to the Barents Sea (Østvedt, 1963). All three Atlantic wolffishes have somewhat overlapping distributions on both sides of the Atlantic Ocean and Barents Sea, but are found in different depth zones (Le François et al. 2010).

The spotted wolffish is the largest of the Atlantic wolffishes and can achieve sizes of up to 1.8 m and 26 kg (Nedraas, 2016, Rountree, 2002). Sometimes referred to as leopard-fish, the spotted wolffish is usually distinguishable from its Atlantic relatives from its leopard-like skin pigmentation, which has given it its name. The northern wolffish has however also been reported to in some cases have a spotted appearance and the two species are sometimes confused (Templeman, 1986a). However, the greater body depth, shorter and broader head (sometimes named broadhead wolffish), smaller caudal and pectoral fins and more jelly-like flesh distinguishes the northern wolffish from both *A. minor* and *A. lupus* (Templeman, 1986a). The spotted wolffish shares many similar characteristics with the closely related common wolffish and they have even been found to produce viable hybrids (Gaudreau et al., 2009). The name *lupus* of the common wolffish originates from Latin and means wolf, and it is the most studied of all the wolffish species. It can reach sizes up to 1.5 m and 18 kg, although this is considered unusual (Rountreee, 2002). Its pigmentation can vary, but it usually has dark bar-like stripes along the back which sometimes gives it the name striped wolffish (Rountree, 2002).

1.4 Wolffish aquaculture

Norway is presently the only country with a commercial production of spotted wolffish, but several other countries such as Sweden, Chile, Canada and Iceland are also interested in production of the species. In Norway, the first spotted wolffish eggs were artificially fertilized in 1993, at Norges Fiskerihøgskole using broodstock captured in the Barents Sea (Falk-Petersen et al., 2003). These fish and the generations after them were later incorporated in the production of wolffish at Troms Steinbit AS. Through the late 1990's and the early 2000's spotted wolffish aquaculture production in Norway was

developed. The major bottlenecks of the production appeared to be solved and everything was ready for upscaling of the production. Today, spotted wolffish is re-emerging as a promising species for aquaculture with renewed efforts to bring the species back in the spotlight, among others through the financing of this doctoral project. The world's only current producer of spotted wolffish, Aminor AS, was established in 2013. They have a concession to produce 500 tons of wolffish in their land-based aquaculture systems located at Halsa at Meløy in Nordland (Aminor, 2018). Aminor had 953 broodstock fish and 24 000 juvenile fish in 2016 and 31 000 individual fish in the ongrowing phase in 2017 (Aminor, 2018).

Males and females form pairs during summer or autumn and make nesting holes where spawning occurs (Keats et al., 1985). The spawned fertilized eggs are surrounded by ovarian fluid, which makes them stick together as they swell during contact with water (Pavlov, 1994). The female will coil around the eggs for several hours after spawning, shaping the sticky mass into a ball which is guarded by the male during the whole incubation (Keats et al., 1985). As for most fishes, the natural reproductive behavior of wolffish is disrupted in captivity and the eggs have to be artificially fertilized. A curious aspect of wolffish biology is their reproductive strategy. Unlike the majority of teleost fish, morphological and behavioral evidence indicate that both common and spotted wolffish have internal fertilization of eggs (Pavlov, 1994; Johannessen et al., 1993; Le Francois and Archer, 2007). The male only produces a small quantity of sperm, around 1.0-1.5 mL, and the sperm is activated immediately, in contrast to other fish species where the sperm is activated in contact with water. Due to the low volume of milt, it is usually necessary to collect sperm from several males in order to fertilize the eggs of one female in captivity (Johannessen et al., 1993).

Broodstock females from 3.6 to 13 kg are reported to produce between 8000 and 30,000 eggs per season (Falk-Petersen and Hansen, 2003, Foss et al., 2004). The eggs are large, 5.4-6.5 mm diameter, and take between 800-1000 d^{o} from fertilization to hatching (Falk-Petersen and Hansen, 2003, Hansen and Falk-Petersen, 2001, Pavlov, 1986). At hatch, the larvae are 22-25 mm long, with well-differentiated organs and a juvenile-like morphology (Falk-Petersen and Hansen, 2003). The only larval characteristics at hatch is

a small yolk-remnant and a cartilaginous skeleton. The larvae will switch between swimming to the surface and resting on the bottom, where the larvae with the least yolk left are the most active (Falk-Petersen and Hansen, 2003). The newly hatched larvae have a well-developed digestive system, and, within a few days, the majority will start feeding (Falk-Petersen and Hansen 2002, Falk-Petersen and Hansen, 2003). However, some larvae never initiate exogenous feeding and die after 4-5 weeks when the yolk reserves are exhausted (Falk-Petersen and Hansen, 2003, Strand et al., 1995). Spotted wolffish can be fed formulated diets directly from start-feeding, which is a great advantage of wolffish culturing as it avoids the labor intensive and costly use of live-feeds that is usually required for early rearing of marine fish (Hansen and Falk-Petersen, 2002, Strand et al., 1995). Hansen and Falk-Petersen (2002) calculated an optimum temperature of 10.3^{o}C for ensuring high growth and survival during the weaning phase of spotted wolffish. The newly hatched wolffish are pelagic and display positive photoreaction (Pavlov, 1999). After the yolk sac is absorbed, the majority of the skeleton will be ossified and the wolffish are characterized as juvenile; they also lose their positive photoreaction, which marks the beginning of their benthic life stage (Pavlov, 1999).

Spotted wolffish have been shown to display very husbandry-suited behavior, and do not appear to be stressed by culture conditions (Foss et al., 2004, Øiestad, 1999). When held in tanks they are non-aggressive towards each other, even when densities are high (Lachance et al., 2010, Le François et al., 2013, Tremblay-Burgeois et al., 2010, Øiestad, 1999). Providing shelters in the culture system can also reduce potential aggression between the fish (Lachance et al., 2010). Spotted wolffish seem to be a social species and prefer to be densely packed in the culture system, and will usually cluster together when densities are low (Foss et al., 2004, pers. obs). However, too high densities may compromise growth; Jonassen (2002) demonstrated reduced growth when spotted wolffish were reared at 40 kg m^{-2} compared to 25 kg m^{-2}. The optimal rearing density does however appear to be influenced by fish size. Tremblay-Burgeois et al. (2010) suggested from their studies that the optimal rearing densities is below 40 kg m^{-2} for smaller wolffish (50-100 g), and that larger fish (100-160 g) probably can be reared at ≥ 40 kg m^{-2}. Temperature is the most important environmental factor influencing fish

growth (Brett, 1979). For spotted wolffish, the temperature requirements vary between the different life stages. The optimum temperature for growth of juveniles is believed to be 6-8 ^{o}C, but as they grow larger this optimum is believed to decrease (Foss et al. 2004). Spotted wolffish tolerate and adapt to a wide range of environmental parameters. Foss et al. (2001) demonstrated the strong osmoregulatory capabilities of spotted wolffish: no differences were observed for wolffish reared at salinities from 12 to 34‰. Growth performance and food intake was not reduced for wolffish reared under hyperoxic conditions, and although reduced growth was found under hypoxic conditions the fish still obtained acceptable growth rates (Foss et al., 2002). Under hyperoxic conditions wolffish have also demonstrated increased tolerance for ammonia (Foss et al., 2003). This ability to tolerate variation in certain environmental parameters is an advantage for intensive rearing conditions, though it is still important to keep the rearing conditions close to the optimum to ensure best possible fish growth and welfare in captivity.

Compared to the common wolffish, spotted wolffish is considered the best suited for farming. In the comparative study of spotted and common wolffish reported by Moksness (1994), spotted wolffish reached more than four times the weight of common wolffish over a period of 2 years. In the same study, spotted wolffish was also reported to mature after a size of 2.5 kg, while the common wolffish matured already after 0.5kg (Moksness, 1994). Fillet yield was also slightly higher in spotted wolffish compared to the common wolffish, with an average of 50% for spotted wolffish compared to 45% for common wolffish (Moksness, 1994). This late maturation (after 7-9 years) and fast growth to market size is another clear advantage for production of spotted wolffish.

To summarize, key traits making the spotted wolffish well suited for cold-water aquaculture includes late sexual maturation, high growth rates compared to the related species, husbandry suited behavior and tolerance for a variety of environmental parameters (Foss et al., 2004). Low fisheries of the wild wolffish and higher fillet quality of the farmed product ensures limited competition for the production. The large larvae that hatches at an advanced stage of development make the captive rearing of the early life stages easier compared to other species, even salmonids (Pavlov and Moksness,

1994). The skin of wolffish could also be an interesting by-product. Wolffish skin is stronger than all other leathers except for kangaroo-tail and can be used in the production of clothes, shoes, handbags and furniture (RUBIN, 2001; Falk-Petersen et al., 2003).

1.5 Feeding habits and dietary requirements

One of the most distinct characteristics of wolffishes are their powerful jaws and peculiar teeth, although there are some species-specific differences. All three Atlantic wolffishes have well-developed teeth on the intermaxillae, mandibles, palate bones and vomer (Albikovskaya, 1983). On the front part of the maxillae and mandibles are hook-shaped teeth that the wolffish use to tear food organisms from the bottom (Figure 2) (Albikovskaya, 1983). Further back in the vomer and palate bones of common and spotted wolffish are conic and round teeth that are used for crushing hard-shelled benthic organisms (Figure 2). Due to the nature of the wolffish diet, the teeth wear down quickly and are replaced annually; wolffish stop feeding during the spawning season and their teeth are replaced (Albikovskaya, 1983, Keats et al., 1985). The northern wolffish have slightly different teeth, with smaller and sharper fangs that do not wear down as quickly (Albikovskaya, 1983). The teeth of the northern wolffish are not adapted to tearing and crushing benthic hard-shelled animals, but rather feed on pelagic and demersal free-living animals (Albikovskaya, 1983). This has also been confirmed by stomach content, where the stomach of northern wolffish often are full of ctenophores and jellyfish, while spotted and common wolffish have primarily benthic organisms in their stomach (Albikovskaya, 1982, Albikovskaya, 1983). Stomach contents have shown that echinoderms are the most important food for wild spotted wolffish, followed by molluscs, crustaceans and fishes (Albikovskaya, 1982, Albikovskaya, 1983, Templeman, 1986b). Polychaetes, cnidarians and even algae are also reported in their stomach in smaller amounts (Templeman, 1986b). While the common and spotted wolffish are primarily benthic fishes, the northern wolffish has been reported in open waters and even occasionally at the surface in the search for food (Albikovskaya, 1982). Fish size also influences diet preference; stomach content of medium-sized fish contains more

echinoderms, crustaceans and gastropods, and stomachs from larger fish (above 75 cm) contain more bivalves and fish (Falk-Petersen et al., 2010).

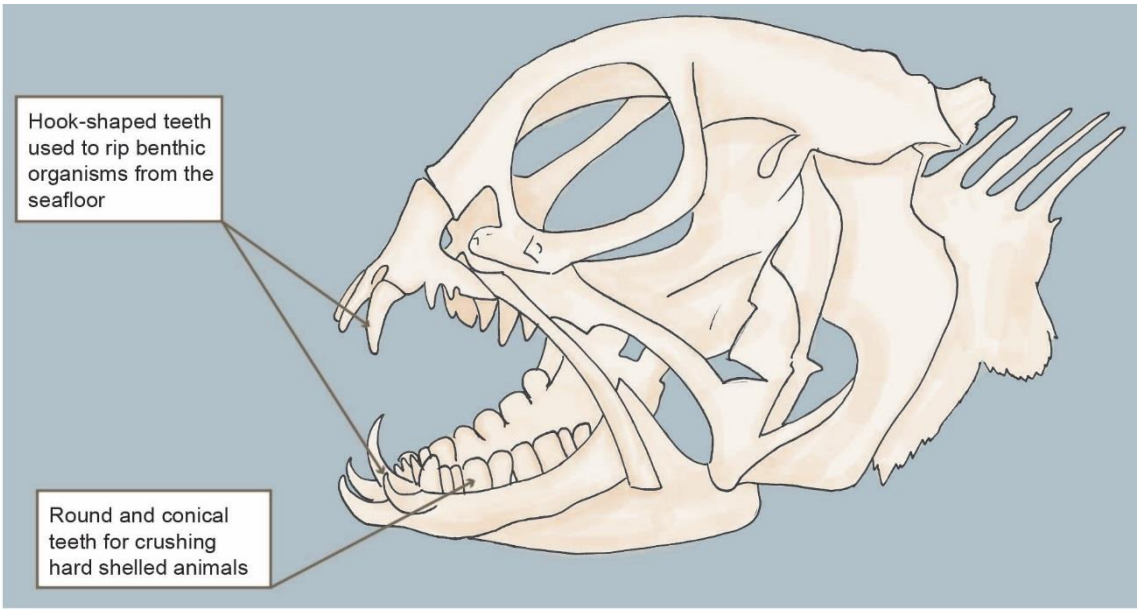


Figure 2: Teeth on the intermaxillae, mandibles, palate bones and vomer of spotted and common wolffish. Illustration: Helene Rønquist Knutsen, Nord University.

Although some research efforts has been devoted to elucidating the dietary requirements of captive reared spotted wolffish, the majority of the existing literature is concentrated on the early life stages (e.g. Hansen and Falk-Petersen, 2002, Savoie et al., 2006, Savoie et al., 2008). The nutritional requirements of juvenile spotted wolffish have not been thoroughly investigated, and there are still knowledge gaps that need to be covered. However, protein-rich feeds (55-62%) are generally used for spotted wolffish, although good growth rates have been obtained also when using lower protein content (45-50%) (Foss et al., 2004). Growth has also been found to be affected by dietary lipid levels, where growth was reduced when increasing dietary lipid levels from 15 to 20% (Jonassen, 2002). Papoutsoglou and Lyndon (2006) found no difference in growth of spotted wolffish when comparing high fat (35%) /low protein (38%) and low fat (28%) /high protein (45%) diets. However, the number of animals used in this study was very low (15 in total per treatment) and the feeding trial lasted only 18 days. For common wolffish, fishmeal quality, pellet moisture and feed composition have been

found to affect fish growth, where sufficiently high dietary protein (>50%) also appeared to be a critical factor (Moksness et al., 1995, Stefanussen et al., 1993).

In the reported studies on spotted wolffish standard commercial diets formulated for other marine fish species such as turbot, cod and halibut are used. The ingredients of these diets are not listed for the majority of these reports, but they are presumably fishmeal based. There are no studies evaluating the use of different feed ingredients for juvenile spotted wolffish. As both present and future diets for farmed fish are expected to become more reliant on alternatives to fishmeal and –oil, future aquaculture of wolffish will also have to use these ingredients in the production. Therefore, it is necessary to investigate the capacity of spotted wolffish to utilize alternative feed resources such as microalgae.

1.6 Structure, development and growth of fish muscle

1.6.1 Structure and organization

Understanding the growth and development of fish muscle is important for aquaculture as it impacts the quality of the final product which is eventually sold to and consumed by humans (Johnston, 1999a, Johnston et al., 2000a). Muscle tissue functions both in locomotion and energy storage and usually comprises around 40-60% of the total fish body mass, a higher proportion than in the majority of other vertebrates (Bone, 1978). The muscle is organized in a number of “V-shaped” or “W-shaped” identical units known as myotomes (Figure 3a). The myotomes are comprised of various cell types including muscle fibers, myogenic precursor cells (MPCs), nerve cells, connective tissue, fibroblasts, skeletal osteocytes, adipocytes and capillary endothelial cells (Johnston et al., 2011). Each myotome is separated from another by sheets of connective tissue, the myosepta or myocommata (Figure 3a) (Sänger & Stoiber, 2001). The myotomal blocks are arranged on each side of the fish body separated by a vertical septum; in many species, a collagenous horizontal septum also separates the dorsal and ventral compartments (Figure 3b) (Johnston et al., 2011). When the muscle contracts, force is

transmitted to the myosepta via the tendons to the skeleton and fins to create movement (Sänger & Stoiber, 2001).

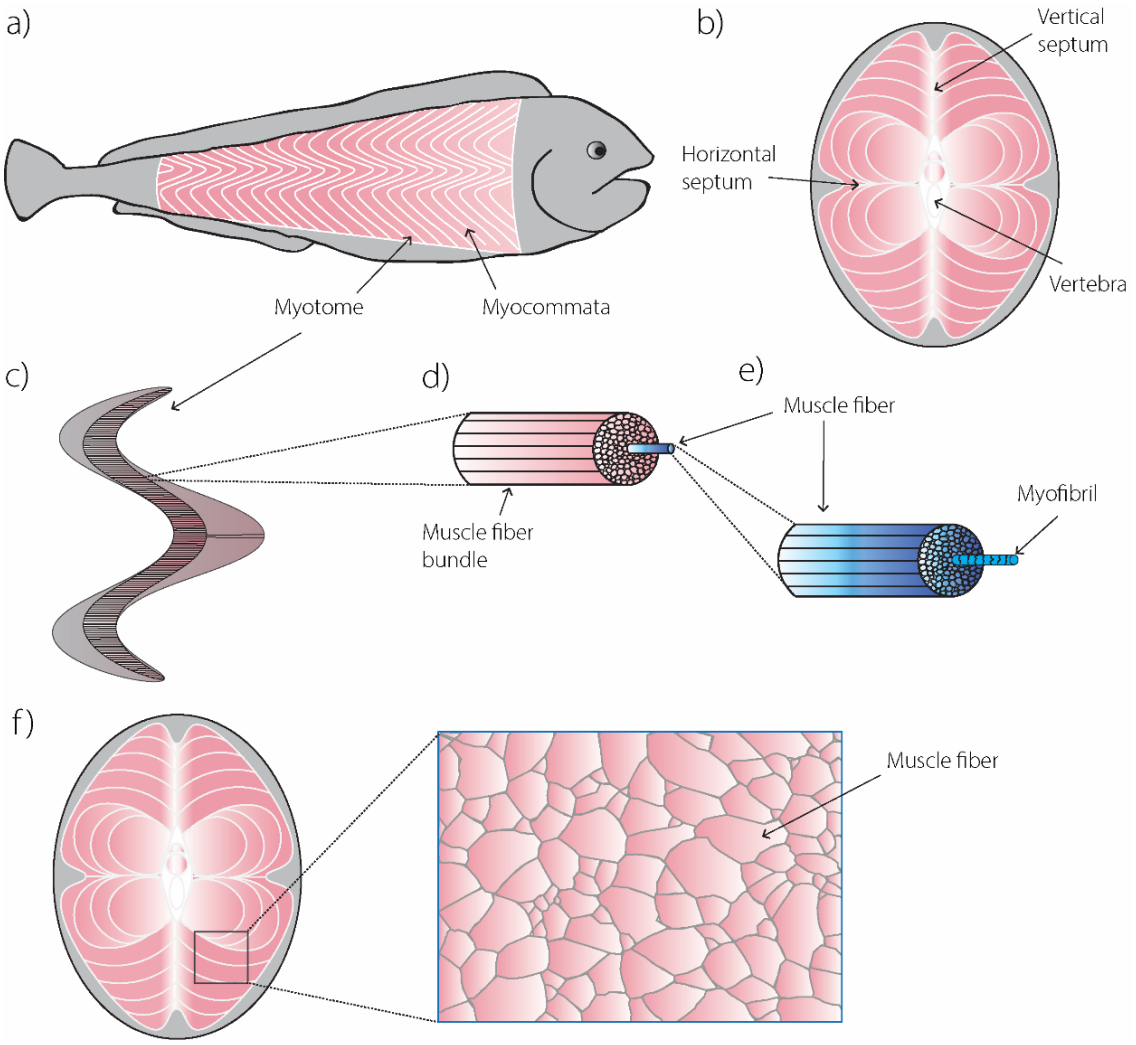


Figure 3: Structure and organization of fish muscle. (a) Organization of muscle in myotomes, separated by myocommata. (b) Cross section of fish showing the vertical and horizontal septum. (c) Myotome. (d) Organization of muscle fibers in bundles. (e) Muscle fiber. (f) Cross section of fish muscle tissue will show muscle fibers of different sizes and different stages of maturity Illustration: Helene Rønquist Knutsen, Nord University.

The number, size and shape of myotomes in a fish will vary both with the life-stage and position in the body of an individual fish, but also with morphology, phylogeny and style of locomotion of the species (Johnston et al., 2011). In the myotome, muscle fibers are bound together in bundles (Figure 3d) by sheets of connective tissue which contain proliferative populations of fibroblasts and adipocytes between the fibers (Johnston et

al., 2011). The muscle fiber itself is a multi-nucleated cell with multiple myofibrils arranged longitudinally (Figure 3e). The myofibrils are composed of repeating sarcomere units with thin and thick filaments which contain actin and myosin (and associated proteins), respectively. The sarcomere is the basic contractive unit of the muscle.

Fish muscle fibers consists of three distinct types: white, red and pink fibers. White, or fast, muscle is the main fiber type, making up 70-90% of teleost muscle, but with large variation between species (Love, 1988). Fast muscle fibers function during rapid bursts of movement, have anaerobic metabolism, poor vascularization and low content of mitochondria and myoglobin (Bone, 1978). The red, or slow, muscle function during normal slow movement, and is found as a thin superficial layer which thickens into a triangular shape around the horizontal myosepta just below the skin (Bone, 1978). Slow muscle fibers have aerobic metabolism, are well-vascularized and have high content of mitochondria and myoglobin (Bone, 1978). Fast muscle is also much larger than slow muscle and deliver 5-10 times more power (Altringham and Johnston, 1990). The pink fibers are an intermediate type between the red and white fibers. Although there are exceptions, the red and white fibers of fish are separated into distinct layers, while in mammals all the different fiber types occur in a complex mix (Bone, 1978).

1.6.2 Muscle development and growth

During the embryonic stage, muscle develops in cellular compartments called somites, which differentiate into a myotome with the different muscle fiber types (Johnston et al., 2011). In mammals and higher vertebrates, myogenesis is mostly completed during the embryonic stage. Post-embryonic muscle-growth of higher vertebrates happens through expansion in diameter and length of already existing fibers (hypertrophy); fish in addition continue to recruit new muscle fibers (hyperplasia) for an extended part of their post-embryonic life. Because of this, a transverse section of fish muscle tissue will show muscle fibers of different sizes and different stages of maturity, a phenomenon known as mosaic hyperplasia (Rowlerson and Veggetti, 2001). Postembryonic muscle growth happens as MPCs are destined either for proliferation or

differentiation; they either are absorbed into growing fibers or they can contribute to the formation of a new muscle fiber (also called a myotube) (Johnston et al., 2011). Hyperplastic growth dominates the earlier stages of development, but will gradually cease at a size of about 44% of the final body length in several teleost fish (Weatherly et al., 1988). Recruitment of new fibers stops once the maximum fiber number (FN_{MAX}) is reached, but can continue in case of muscle injury (Rowlerson et al., 1997). FN_{MAX} both within and between species is largely correlated to body size and environmental temperature (Johnston et al., 2011). Low temperature combined with reduced metabolic demands has been shown to produce reduced FN_{MAX} combined with increased mean and maximum fiber diameter (FD_{MAX}) in the Antarctic Nototheniodeii fish species (Johnston et al., 2003a). Hypertrophic growth of muscle fibers occurs also after hyperplastic growth has ceased until the individual fiber has reached its FD_{MAX}, which normally is from 100-300 µm for fast muscle. Some species produce even larger fibers; FD_{MAX} of 360 µm has been observed for Atlantic halibut (*Hippoglossus hippoglossus*) and gigantic fibers > 700 µm have been observed in common Dab (*Limanda limanda*) and Notothenioid fishes (Hagen et al., 2008, Hurling et al., 1996, Johnston et al., 2003a). Generally, FD_{MAX} is dependent on body mass, activity and metabolic demand; it increases with increasing body size and decreasing metabolism and activity (Johnston, 2006). This is why sedentary species living in cold environments such as Notothenioid fishes can achieve such large muscle fibers.

The relative contribution of hyperplasia and hypertrophy to increase in muscle mass will affect the muscle cellularity (distribution of muscle fiber sizes) which again is affected by a number of both biological and environmental factors such as species (e.g. Weatherly et al., 1988), sex (e.g. Hagen et al., 2006, Hagen et al., 2008), temperature (Alami-Durante et al., 2007, Johnston et al., 2000b), diet (e.g. Alami-Durante et al, 2010, Silva et al, 2009, Bjørnevik et al, 2003), feeding regime (Galloway et al., 1999, Kiessling et al., 1991), diploidy/triploidy (Johnston et al., 1999b) and photoperiod (Johnston et al., 2003b, Johnston et al., 2004).

2. Objectives

The main objectives of this thesis was to evaluate i) the capacity of wolfish to utilize microalgae in fishmeal-based or mixed diets consisting of marine and plant based protein ingredients, (ii) the dietary effect on muscle development and growth and iii) the dietary effects on liver, intestine and muscle proteome including intestinal health indices. This thesis and the papers included are based on results from two experiments. **Experiment I (Paper I + III):** Juvenile spotted wolffish were fed graded inclusion levels of the microalgae *Scenedesmus obliquus* replacing 0, 4, 8 and 12% of the fishmeal in the diets. **Experiment II (Paper II):** Juvenile spotted wolffish were fed graded inclusion levels of the microalgae *Nannochloropsis oceanica* replacing 0, 7.5 and 15% of a mix of fish- and plant-based ingredients in the diets. Specific objectives in the three papers of the study were:

1. **Paper I:** Investigate the effect of partial replacement of fishmeal with *S. obliquus* on (i) fast muscle growth dynamics, (ii) muscle proximate composition and (iii) overall biometric gain and somatic indexes.

2. **Paper II:** Investigate the effect of partial replacement of fishmeal and plant protein with *N. oceanica* on (i) overall biometric gains and somatic indexes, (ii) blood plasma biochemistry, (iii) fillet and whole body proximate chemical composition, (iv) fast muscle growth dynamics, and (v) fillet, liver and whole body fatty acid profile.

3. **Paper III:** Investigate the effect of partial replacement of fishmeal with *S. obliquus* on (i) muscle, liver and intestine proteome and (ii) fish welfare indices by histological evaluation of anterior and distal intestinal morphology.

3. Summary of papers: Main results

Paper I

Muscle growth and changes in chemical composition of spotted wolffish juveniles (*Anarhichas minor*) fed diets with and without microalgae (*Scenedesmus obliquus*)

Aquaculture Reports, 2019, 100175.

In this paper juvenile spotted wolffish were provided diets either containing fishmeal as the primary source of protein or microalgae (*Scenedesmus obliquus*) replacing 4%, 8% or 12% of the fishmeal. Although no definite conclusions could be made, evaluation of fast muscle cellularity showed indications of microalgae affecting the relative contribution of hyperplasia and hypertrophy for increasing muscle mass in spotted wolffish. Biochemical composition of muscle was affected by diet, as muscle mineral content was reduced in the fish fed *S. obliquus*. All dietary groups did also had reduced hepatosomatic index and increasing muscle crude protein and lipid over the course of the experiment. Dietary replacement of fishmeal with *S. obliquus* also affected skin coloration, where results from spectrophotometric analysis of skin showed increasing skin-yellowness with increasing dietary microalgae replacement.

Paper II

Fast muscle cellularity, fatty acid and body-composition of juvenile spotted wolffish (*Anarhichas minor*) fed a combination of plant proteins and microalgae (*Nannochloropsis oceanica*)

Manuscript submitted to Aquaculture.

In this paper we evaluated the effects of including graded levels (0. 7.5 and 15%) of the microalgae *Nannochloropsis oceanica* in the diet of juvenile wolffish. We used a diet with higher inclusions of plant based ingredients (about 50% of the feed) which is more in line with the present and future industrial standard of aquafeeds. No difference was found in growth and fast muscle cellularity between the treatment groups. Blood plasma

analysis showed no indications of stress in the fish fed *N. oceanica*. Hepatosomatic index did however decrease over the course of the experiment, and was also significantly reduced in the algae fed fish at the end of the trial compared to the control fish. Polyunsaturated fatty acids increased in muscle, liver and whole body of the fish fed diets containing *N. oceanica*.

Paper III

Proteomic profile of intestine, liver and muscle and evaluation of intestinal morphology of juvenile spotted wolffish (*Anarhichas minor*) fed the microalgae *Scenedesmus obliquus*

Manuscript.

In this manuscript juvenile spotted wolffish were provided diets either containing fishmeal as the primary source of protein or microalgae (*Scenedesmus obliquus*) replacing 12% of the fishmeal. We used two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) coupled with mass spectrometry to analyze the proteome of muscle, liver and intestine of the fish. Differentially expressed protein spots were identified using homology search. Muscle, liver and intestinal proteome was altered as an effect of the dietary treatment. Spots that were differentially expressed were identified as among others structural proteins, proteins involved in regulation of energy homeostasis and proteins with detoxification and antioxidant functions. From the evaluation of intestinal morphology the results showed that fish fed *S. obliquus* also had thinner muscularis and reduced mean size of acid mucins.

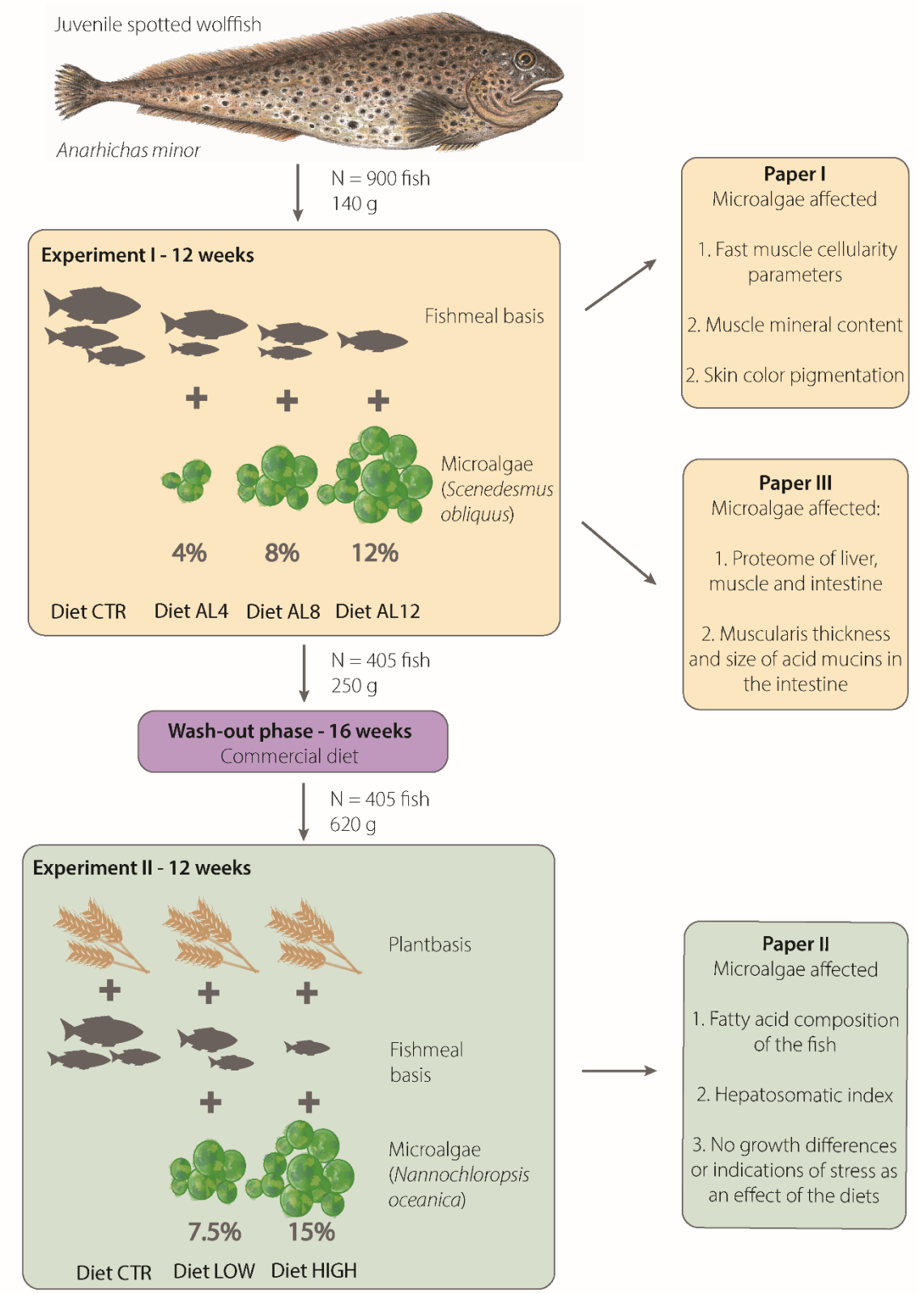


Figure 4: A simplified graphical overview of the experiments and research papers which this thesis is based on. Illustration: Helene Rønquist Knutsen, Nord University.

4. General discussion

4.1 Performance of spotted wolffish fed microalgae incorporated diets

This thesis represents the first evaluation of microalgae as a feed ingredient for spotted wolffish in aquaculture. Overall, spotted wolffish showed a promising potential for tolerating microalgae in the diet; growth performance was similar in the algae-fed groups and the control-groups for both the experiment evaluating incorporation of *Scenedesmus obliquus* and *Nannochloropsis oceanica* (**paper I** and **paper II**), though the 8% group of **paper I** showed consistently poorer performance compared to all other groups in the experiment. The discussion of this group is specifically treated in **paper I**, and will be considered to a lesser extent for the majority of the general discussion. The fish in the *S. obliquus* experiment grew from an average weight of 140 g to 250 g during the 12 week feeding trial. At termination of the trial, specific growth rate (SGR) was on average 0.66% day^{-1}, or 0.70% day^{-1} when excluding the 8% group, for wolffish fed 0, 4, 8 and 12% incorporation of *S. obliquus* replacing fishmeal in the diet (**paper I**). These values are comparable to what has previously been reported for wolffish of similar age (~1 year) and size class (140-250 g); SGR of 0.37-0.65% day^{-1} has been reported for 1-2 years old 150-700 g spotted wolffish (Falk-Petersen et al., 1999, Tremblay-Bourgeois et al., 2010). The fish in the *N. oceanica* experiment grew from an average weight of 625 g to 794 g during the 12 week feeding trial. At termination of the second trial, specific growth rate (SGR) was much lower with an average of 0.26% day^{-1} for wolffish fed 0, 7.5 and 15% incorporation of *N. oceanica* replacing a mix of fish- and plant-based ingredients in the diets (**paper II**).

Higher SGR has been reported for wolffish of smaller size groups (about 60-140 g); between 0.8-1.2% day^{-1} for wolffish reared at normoxic conditions (9.6 mg L^{-1}) and full strength seawater salinity (34‰) (Foss et al., 2001, Foss and Imsland, 2002, Foss et al., 2003). From the available literature, there seems to be an inverse relationship between size and SGR for spotted wolffish, which was also confirmed by Imsland et al. (2006). This tendency was also observed in this project. Due to the limited availability of fish, the same fish were used in both experiments, with a 4 month wash-out period between,

where the fish were fed a standard commercial diet for marine fish (Amber Neptun, Skretting, Stavanger, Norway). During this time period the fish grew from 250 g to 625 g with a mean SGR of about 0.58% day^{-1} (data not shown). Jonassen (2002) reported SGR of 0.47% day^{-1} for spotted wolffish reared at optimal density growing from about 450-850 g, which is a size group comparable to the fish in **paper II**. In both of the experiments, SGR was also reduced within the experimental period. In **paper I**, SGR was 0.90% day^{-1} at week 4, 0.87% day^{-1} at week 8 and finally 0.70% day^{-1} at week 12 (excluding the 8% group). In **paper II** SGR was 0.40% day^{-1} at week 6 and 0.26% day^{-1} at week 12. Hence, it appears that the growth in both experiments was within the normal range of spotted wolffish, although **paper II** probably was in the lower range of the normal growth rates.

In both **paper I** and **paper II** reduced hepatosomatic index (HSI) was observed over the course of the experiments. HSI of fish fed 8% *S. obliquus* and 7.5% *N. oceanica* was also reduced compared to the respective control treatments. Reduced HSI has been observed in comparative trials where fish are fed microalgae as a fishmeal protein substitute (Tulli et al., 2012, Vizcaíno et al., 2014, Walker and Berlinsky, 2011). Patterson and Gatlin (2013) reported lower HSI in red drum (*Sciaenops ocellatus*) fed non-extracted algae compared to those fed lipid-extracted algae; in both **paper I** and **paper II** we used non-extracted algae. However, in both **paper I** and **paper II** we related the decrease in HSI to a possible modulated lipid metabolism following an increased lipid deposition in the muscle. The wolffish had on average 4.15% muscle crude lipid at the start and 5% at the end of the first trial (**paper I**); at the start of the second trial this was increased to 8.4% and at the end to 9.6% (**paper II**). Whole body proximate composition was only analyzed in the second experiment where whole body crude lipid remained unchanged over the course of the trial (**paper II**). Hence, the results of both papers strongly indicate that the observed reduction in HSI is a consequence of wolffish incorporating more lipid in the muscle as they grow larger.

In **paper II** linoleic acid (C18:2n-6) increased over time in all treatment groups reflecting the higher proportion of plant based ingredients in the diet. Interestingly, although the 7.5 and 15% *N. oceanica* diets both had slightly higher linoleic acid, there

was no difference in the content of this fatty acid in the fish among the groups. Although all diets were formulated with the same amount of fish oil, the algae diets had higher crude fat and also higher content of the ω-3 fatty acid EPA compared to the control diet; this was not surprising as *N. oceanica* is an EPA-rich algae (Hulatt et al., 2017, Ma et al., 2014). The higher EPA of the algae-diet was reflected in the fatty acid profile of the whole fish, where increased EPA was found over time in whole body of the algae-fed fish. As whole body crude lipid was unchanged, it also indicates a selective retention of ω-3 fatty acids. Further, this also demonstrates that incorporation of microalgae may improve retention of ω-3 fatty acids in spotted wolffish fed diets high in plant based ingredients, which is promising for both fish health and final product quality traits as a source of ω-3 fatty acids. For future studies it would be of interest to investigate if the same effects are observed for incorporation of *N. oceanica* in isolipidic diets. In **paper II** we also observed most differences both over time and among treatments in whole body and liver. The main changes in muscle included reduced saturated fatty acids (C14:0 and C16:9) and unsaturated fatty acid (C22:1n-9) as well as increased PUFAs (sum PUFA and C18:2n-6). A longer feeding trial could potentially reveal if *N. oceanica* could increase muscle EPA as well. The results of **paper II** show that fatty acid composition of whole body, liver and muscle clearly were affected by diet in spotted wolffish and thus demonstrate the importance of considering the fatty acid composition of potential feed ingredients when tailoring diets for farmed wolffish.

4.2 Muscle development and growth

This thesis represents the first evaluation of fast muscle cellularity in spotted wolffish. To my knowledge, it is also the first time the effect of dietary incorporation of microalgae on muscle cellularity is investigated for fish overall. Muscle development and growth was evaluated from morphometric measurements of muscle fiber diameters on histological sections (**paper I** and **paper II**) as well as from proteomic analysis of muscle tissue (**paper III**); the choice of methods are discussed in section 4.4. When evaluating changes over time, morphometric measurements of muscle fiber diameters of fish fed *S. obliquus* indicated tendencies of increased fast muscle hyperplasia in the algae-fed

fish (**paper I**). However, the probability density distributions of muscle fibers were not different, which was consistent with the findings of wolffish fed *N. oceanica* (**paper II**). The altered muscle proteome of fish fed *S. obliquus* could further support the indications of increased fast muscle hyperplasia in the algae-fed fish (**paper III**). The upregulation of spots identified as structural proteins (vimentin and tropomyosin) together with an upregulation of spots identified as proteins involved in energy homeostasts (triosephosphate isomerase, beta-enolase, creatine kinase) could be explained by increased muscle fiber recruitment in the algae-fed fish (**paper III**).

All in all, the results from both experiments indicate no negative effects of incorporation of microalgae in the diet on muscle growth of spotted wolffish. The findings of unchanged or even decreasing fiber number over time in **paper II** was quite surprising; though the results may be explained by the life history of spotted wolffish. Spotted wolffish is a sedentary benthic fish species adapted to cold water temperature. Therefore, spotted wolffish muscle growth might be similar to other fish species with similar life histories, such as Atlantic halibut, Dab and Notothenioid fishes, producing relatively large fibers and a lower maximum fiber number (Hagen et al., 2008, Hurling et al., 1996, Johnston et al., 2003a). This is also supported by the observations of large fibers (diameter > 430 µm) as well as muscle growth over time being mainly dominated by hypertrophy in all treatment groups (**paper II**).

4.3 Changes in proteome of liver and intestine

Proteome of both liver and intestine of juvenile spotted wolffish was altered by incorporation of *S. obliquus* in the diet (**paper III**). In liver, downregulated spots in the algae-fed fish were identified as proteins involved in the pathway of gluconeogenesis (triosephosphate isomerase and glyceraldehyde-3-phosphate) as well as cathepsin D which is a lysosomal protease. Carbohydrate in the experimental diets was not directly measured, but by subtracting the measured percent of fat, protein, ash and water from a theoretical 100%, the algae diets had slightly higher carbohydrate (11.25%) compared to the control group (9.33%). Moderate dietary levels of carbohydrates can have a

protein-sparing effect in fish (Hemre et al., 2002). Therefore, the results could indicate a protein-sparing effect of *S. obliquus* through reduced use of non-carbohydrate substrates (i.e. protein and lipids) in glucose production in the liver. Aldolase and citrate synthase, both involved in carbohydrate metabolism, were also identified in upregulated spots in the intestine of fish fed *S. obliquus*. This might indicate increased digestion of carbohydrates which further supports the suggested protein sparing effect of a slightly more carbohydrate-rich diet. Fatty-acid binding protein and trypsin were identified from downregulated spots of intestine in algae-fed fish (**paper III**). Therefore, these results could also indicate lower digestibility of fat and protein in the *S. obliquus* diet. As we used whole algal cells it is possible that inefficient disruption of the algal cell wall during feed processing or in the digestive tract could cause reduced availability of protein and fat in the diets. Still, as seen in **paper I**, there was no difference in growth between the fish fed the control diet and the fish fed *S. obliquus*, so overall we observed no indications of insufficient utilization of nutrients and energy in the algae-diet.

Interestingly, several of the downregulated protein spots in liver were identified as proteins involved in mechanisms related to antioxidant and detoxification activity: the protection of cells against oxidative damage caused by reactive oxygen species (ROS). Specifically, proteins functioning in removal of peroxides were identified among the downregulated spots in liver. This included glutathione peroxidase (GPx) and peroxiredoxin. Heat shock cognate 71 kDa (HSC71) and tetratricopeptide repeat protein could also be involved in the endogenous antioxidation system of the fish (**paper III**). *S. obliquus* and related *Scenedesmus* species contain several antioxidant compounds, including the carotenoids astaxanthin, β-carotene and lutein (Chan et al., 2013, Macías-Sánches et al., 2010, Qin et al., 2008). Proteins with peroxidase activity (peroxiredoxin and thioredoxin domain-containing protein 17) as well as heat shock protein 90-beta (HSP90-beta) were also identified among downregulated spots in the intestine (**paper III**). Therefore, it can be argued that *S. obliquus* contributed with dietary antioxidants; hence there is less need for the fish to synthesize their own antioxidant enzymes, specifically enzymes with peroxidase activity.

As for muscle, proteins identified among the upregulated proteins in the intestine of fish fed *S. obliquus* could be generalized to proteins involved in regulation of energy homeostasis (creatine kinase) and structural proteins (tropomyosin alpha-1 chain, actin, keratin type I cytoskeletal 13 and tubulin alpha chain) (**paper III**). Although differing in expression pattern, genes correlating to these proteins have also been found to be altered in rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) fed different fishmeal and –oil replacements (Martin et al., 2003, Morais et al., 2012, Murray et al., 2010). The upregulation of structural proteins and proteins involved in regulation of energy homeostasis might be explained by increased energy demand for protein synthesis and cell-growth. Although not significant, we observed slightly longer villi length (VL) from the histological evaluation, which may support this theory (**paper III**). The intestine not only serves an important function in digestion and absorption of nutrients; it also has a vital role in immune regulation and protection against pathogens. Changes in structural proteins in the intestine could potentially affect contractile activity, nutrient absorption and the overall health of the fish.

From the histological evaluation of intestinal morphology, no indications of intestinal inflammation were observed in the fish fed *S. obliquus* (**paper III**). However, the observations of reduced thickness of distal intestinal muscularis and reduced mean size of acid mucin-containing goblet cells may indicate that algae negatively affected intestinal morphology. Reduced muscle layer thickness may affect the contractile activity in the distal intestine. Increased number of goblet cells is considered an indication of intestinal inflammation. In **paper III**, the number of goblet cells were not different between the algae-fed fish and the control group, but decrease in size of goblet cells could indicate reduced mucus production and hence a compromised mucous barrier.

4.4 Methodological aspects

4.4.1 Evaluating fast muscle growth dynamics

The rate of hyperplasia and hypertrophy in fish muscle is commonly studied by measuring the diameter of individual muscle fibers. As the fish increase muscle mass, the fibers grow both in diameter and length, but the diameter is more convenient to measure. Morphometric measurements of muscle fiber diameters can be done on histological sections within a representative cross-sectional area of the musculature; this was done in **paper I** and **paper II**. Replicate blocks (2-4 in **paper I** and 6 for the larger fish of **paper II**) of fast muscle were collected in order to cover the majority of the cross-sectional area of the musculature and making sure to avoid areas of slow muscle. A minimum of 800 fibers were measured per fish, which is consistent with what is used in comparative studies (e.g. Johnston et al., 2003c, Johnston et al., 2006, Periago et al., 2005). Because fish continue to recruit new muscle fibers also throughout the post-embryonic life stage, the population of muscle fibers will consist of a variety of fibers of different diameter; this is known as mosaic hyperplasia and is characteristic of muscle growth in juvenile fish. Due to the presence of newly recruited fibers, the mean fiber diameter alone is an unreliable measurement for assessing muscle growth dynamics. A more accurate approach is to look at the size distribution of muscle fibers. Traditionally, this was done by assessing size distribution-histograms. However, advances in statistical analysis have provided an improved the quantitative approach for comparing distributions of muscle fiber diameters.

The use of non-parametric smoothing techniques was developed by and described in detail by Johnston et al. (1999b). This method was used to analyze size distributions of muscle fibers of spotted wolffish fed diets with and without microalgae in **paper I** and **paper II**. In short, smooth non-parametric estimates of the probability density functions (PDFs) are constructed using the kernel method (Silverman, 1986) using the software R (R Core Team, 2017) and sm library (Bowman and Azzalini, 1997). A smooth kernel function is the basic building block used in the kernel estimator to create the PDF:

$$\hat{f}(y) = \sum_{i=1}^{n} w(y - y_i; h)$$

Here, $\hat{f}$ is the estimated probability density function, y_i is the i'th observation from the list of n fibers, w is the kernel function and h is the smoothing parameter which controls the variation of the kernel function (Johnston et al., 1999b). The mean PDF is created both for each group of interest of comparison as well as for the mean of all groups. Next, a visual indication of potential significant differences between muscle fiber populations are created using bootstrap techniques to create approximated variability bands of $\hat{f}$ using the mean smoothing parameter. This creates a shaded area around the PDFs which can be inspected to assess significant differences between the muscle fiber populations; if the PDF falls outside of the variability band, it is interpreted as a significant difference between the distributions. No significant differences were observed from the visual inspection of variability bands in **paper I** and **paper II**. However, when the use of non-parametric smoothing techniques was first introduced, 100 bootstrap replicates were recommended (e.g. Johnston et al., 1999b, Johnston et al., 2003c, Johnston et al., 2006) ; in more recent papers this has increased to 1000 bootstrap replicates (e.g. Campos et al., 2013, Johnston et al., 2014). In both **paper I** and **paper II**, 1000 bootstrap replicates were used to create the variability bands; however, when 100 bootstrap replicates were used we did observe significant differences (data not shown). Increasing the number improves the accuracy of the variability bands; therefore 1000 bootstrap replicates were used, as is consistent with newer literature (e.g. Campos et al., 2013, Johnston et al., 2014). Finally, a Kolmogorov-Smirnov two-sample test is used to test if the PDF of the treatment groups are equal over all diameters. This visual inspection of the variability bands around smoothed PDFs provides a powerful tool for assessing the variation between muscle fiber distributions as well as in which areas of the distribution potential differences occur.

In **paper I** and **paper II** additional parameters for analysis of muscle growth were also calculated; these included fiber number (FN), fiber density (FD), mean fiber diameter, maximum fiber diameter as well as the more traditional approach of dividing the fiber

diameters into size categories. These additional parameters are useful measurements to supplement the analysis using non-parametric smoothing techniques, but should be weighted less for the discussion of the results. However, they do provide a useful measure to compare differences between muscle fiber distributions over time, which is why these additional parameters were used for comparing muscle growth of the groups between the start and end of the two experiments (**paper I** and **paper II**). Muscle fiber size distribution is strongly dependent on fish size (Weatherly et al. 1988); therefore, the use of non-parametric smoothing techniques to compare differences both between groups and within groups over time is not an accurate approach. To reduce the effect of fish size, which could potentially mask the effect of dietary treatment, fish of equal total length (TL) were selected for analysis using non-parametric smoothing techniques (28.0 ± 1.0 cm in **paper I** and 40.14 ± 0.24 cm in **paper II**). The additional parameters were also normalized based on fish TL for the statistical analysis. The normalization was done as described by Alami-Durante et al (2010): parameters increasing with increasing TL were divided by ln TL and parameters decreasing with increasing TL were multiplied by ln TL.

4.4.2 Proteomic analysis

Proteomic analysis of muscle, liver and intestine was used in **paper III** of this dissertation. The term proteome refers to the entire protein set of a cell, tissue or an organism at a given time under defined conditions. Proteomics is hence the large-scale study of these proteins as well as their temporal expression, structures, functions and interactions (Graves and Haystead, 2002). Modifications occurring all the way from transcription to translation as well as post-translation make the number of proteins in an organism much higher than the number of genes (Graves and Haystead, 2002). Proteins are also, after all, the real mediators of physiological function. This makes proteomic profiling and comparisons a powerful tool for obtaining a large amount of information from the complex and dynamic physiological state of a cell, tissue or organism.

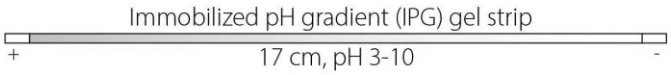
The technique of high-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of proteins was established in 1975 by the pioneering work of Patrick H. O'Farrell (O'Farrell, 1975). In short, proteins are first separated by charge and then by molecular weight (Figure 4). In the first dimension proteins are subjected to isoelectric focusing (IEF) where they are resolved in the gel according to their net charge (isoelectric point, pI). Use of polyacrylamide gels with immobilized pH gradients (IPG) in the first dimension has greatly improved both the resolution of proteins and the reproducibility of the method (Cho, 2007). When subjected to high voltage, proteins in the IPG strip migrate through the gel until they reach a pH that matches their pI where they become neutralized and stop migrating. In the orthogonal second dimension, proteins are further resolved according to their molecular weight (MW) in a two-dimensional polyacrylamide gel. After separation, proteins can be visualized using either a visible stain such as silver or Coomassie blue or by fluorescent stains such as Sypro Ruby™, Lava or Deep Purple (Abdallah et al., 2012). Through the introduction of fluorescent stains, the sensitivity of 2-D PAGE protein detection has greatly improved. Enabling resolution of up to 10 000 protein spots per gel makes the 2-D PAGE technique highly effective in terms of resolving power which makes the method a good choice for resolving complex mixtures of protein (Cho, 2007, Graves and Haystead, 2002). The primary application of 2-D PAGE is protein expression profiling, where the appearance, disappearance and intensity of the protein spots provide information about differentially expressed proteins as well as their expression levels (Graves and Haystead, 2002).

However, there are still some limitations in both the number and type of proteins that can be resolved using 2-D PAGE (Graves and Haystead, 2002). Proteins with pI's outside the range of the IPG gel will not be well represented in the final gel (Abdallah et al., 2012). In addition, many large and hydrophobic proteins will not enter the gel during the first dimension (Graves and Haystead, 2002). The separation of proteins in the second dimension is also dependent on the concentration of the polyacrylamide gel where more concentrated gels will resolve smaller proteins and less concentrated gels larger proteins. A typical 2-D PAGE will only resolve 30-50% of the total proteome (Baggerman et al., 2005). In the case of **paper III**, IPG strips with a pH range of 3-10 were

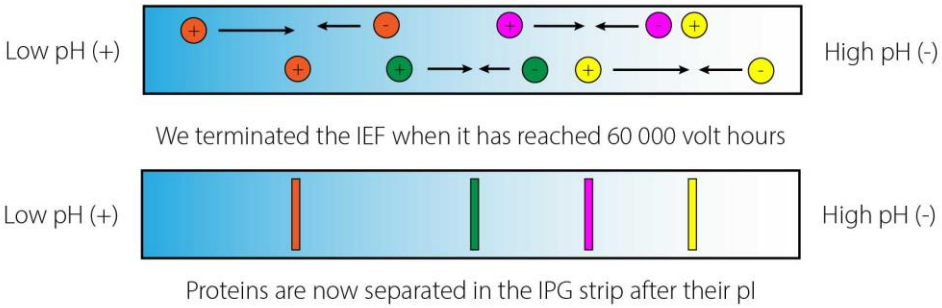
used in the first dimension and a 12.5% polyacrylamide gel in the second dimension. It could have been possible to cover a broader range of proteins in the study by using IPG gels with different pH gradients and by using different resolution conditions in the second dimension. However, as 2-D PAGE remains a slow, labor-intensive and relatively costly analysis, this was not realistic to do for **paper III**. As we still produced gels with high number of differentially expressed spots, this was not considered a problem, but it is important to keep in mind that we cannot cover the full proteome of the investigated tissues using 2-D PAGE approach.

Although it is a well-established technique, limitations of 2-D PAGE also include low-dynamic range and high gel-to-gel variation causing concern for the quantitative reproducibility of the technique (Abdallah et al., 2012, Cho, 2007). Because of this, developments in the field of proteomics in the recent years have been focused on gel-free approaches. Gel-free proteomic techniques are still not a complete alternative to the gel-based techniques and the two approaches are currently considered complimentary rather than competing (Abdallah et al., 2012). Gel-based proteomic techniques still have an important role in research of organisms with no or incomplete genome sequence data available, as it enables the use of homology searches using peptide sequences from single proteins (Rogowska-Wrzeinska et al., 2013). As spotted wolffish is a quite novel research organism, 2-D PAGE was considered the best choice for our study.

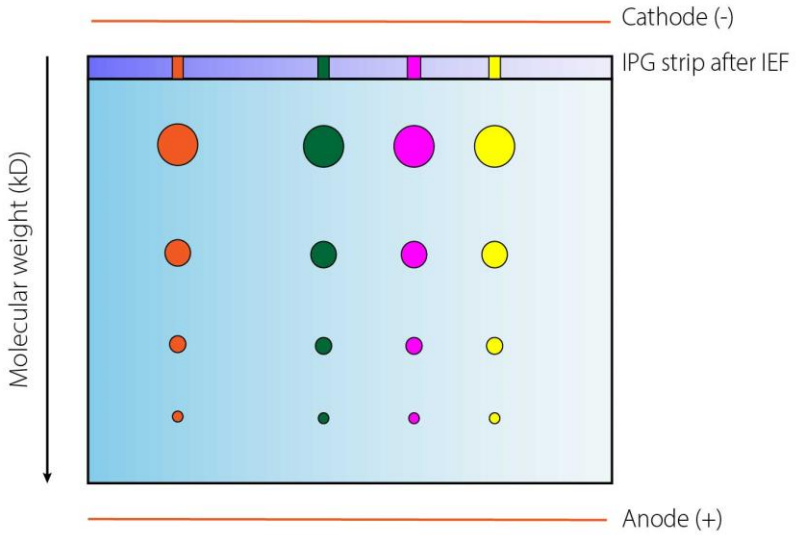
First dimension: Isoelectric focusing



Isoelectric focusing (IEF): The IPG strip is subjected to voltage. Proteins separate according to their isoelectric point (pI). The voltage makes the protein migrate through the gel until it meets a pH that matches its pI. The charge of the protein then becomes neutral and it stops migrating.



Second dimension: SDS PAGE



The IPGis placed on top of the polyacrylamide gel. Voltage is applied and proteins will migrate from negative to positive end (downwards). Proteins are now separated by their molecular weight. Proteins with high molecular weight travel slow and are "trapped" higher up in the gel, while smaller proteins travel faster and end up further down in the gel.

Figure 5: Principle of two-dimensional polyacrylamide gel electrophoresis. Illustration: Helene Rønquist Knutsen, Nord University.

6. Conclusion

The main conclusion from the work making up this PhD thesis is that the microalgae *Scenedesmus obliquus* and *Nannochloropsis oceanica* can be included in the diet of spotted wolffish. Based on the main objectives, the following conclusions are made:

(i) A) For wolffish in the experiment evaluating use of *S. obliquus* in a fishmeal-based diet (140 g start weight), no significant differences in weight gain were observed between fish fed the diet with the highest incorporation (12%) of microalgae *S. obliquus* and fish fed the control diet without microalgae.

(i) B) For wolffish in the experiment evaluating the use of *N. oceanica* in a mixed diet consisting of marine and plant based protein ingredients (start weight 625 g), no significant differences in weight gain were observed between the fish fed 7.5 and 15% of microalgae *N. oceanica* and fish fed the control diet without microalgae.

(ii) Tendencies of increased fast muscle hypertrophy was observed in the fish fed 12% *S. obliquus*; for fish fed *N. oceanica* there were no differences in fast muscle growth parameters between the treatment groups.

(iii) Incorporation of *S. obliquus* in the diet of spotted wolffish affected muscle, liver and intestinal proteome; identified proteins indicate that the *S. obliquus* diet affected protein synthesis and cell growth in muscle and intestine, liver gluconeogenesis and the antioxidant system of the fish. From the morphological assessment of intestine there were no indications of intestinal inflammation, but the decreased size of goblet cells may indicate a compromised mucous barrier in the fish fed *S. obliquus*.

In addition, the following concluding remarks are also made:

a) HSI decreased in all groups over time in both the *S. obliquus* and *N. oceanica* growth trials and muscle crude fat increased, indicating the wolffish incorporates more lipid in the muscle with increasing body mass.

b) Incorporation of *N. oceanica* in the diet of spotted wolffish altered the fatty acid composition of the fish. PUFAs and especially linoleic acid (C18:2n-6)

increased in all groups reflecting the use of about 50% plant-based ingredients in all diets; the ω-3 fatty acid EPA was increased in whole body of the algae-fed fish.

c) The assessment of fast muscle cellularity in the *N. oceanica* growth trial indicate that wolffish have a similar growth pattern as other fishes with a benthic and sub-arctic origin, producing relatively large fibers and potentially having a moderate maximum fiber number.

7. Future perspectives

The ability of spotted wolffish to tolerate fishmeal alternatives such as terrestrial plant proteins and microalgae is promising for the future prospects for wolffish aquaculture. The results from the two experiments making up this thesis show that wolffish are able to tolerate incorporation of microalgae in the diet without negative effects on growth. However, this thesis represents only the first attempts to evaluate fishmeal alternatives for spotted wolffish. Future research continuing this work should be focused on evaluation of other species of microalgae and also on other available fishmeal alternatives, such as for example insect meal. As we have shown that whole body, liver and muscle fatty acid composition is influenced by diet, special care should be taken with evaluation of fatty acid composition of feed ingredients when tailoring diets for spotted wolffish. Further research efforts are also necessary to elucidate the dietary requirements of spotted wolffish, in order to tailor diets ensuring that the maximum growth potential of the fish is utilized. For future research it is important to characterize muscle development and growth throughout the whole life cycle of spotted wolffish, especially in order to reveal stages of development which are sensitive to environmental and dietary influence in terms of muscle cellularity. It is also necessary to further evaluate the effects of microalgae on muscle growth and development. Inclusion of microalgae in the diet of spotted wolffish at strategic developmental stages for muscle growth might improve the overall fish growth, size and final product quality traits; this would be an interesting hypothesis to investigate for future research.

The results of this thesis also indicate that incorporation of microalgae in the diet might improve the antioxidant status of the fish. Incorporation of selected microalgae species in the diet during sensitive stages of development and production could potentially be beneficial in terms of robustness and stress-tolerance of the fish. For future research on this topic, my recommendation would be to perform a microalgae-feeding trial with specific focus on evaluation the antioxidant-status of the fish, combined with a stress-experiment where the performance of fish fed diets with or without microalgae is evaluated. Today, the productivity of microalgae is still too low and production cost too high for microalgae to compete with fishmeal and –oil.

However, as demonstrated in this thesis, incorporation of microalgae in the diet may have beneficial effects on fatty acid composition of the wolffish and potentially also muscle growth, fish health and antioxidant capacity. Therefore, microalgae do not only pose an interesting alternative to fishmeal for spotted wolffish, it could possibly also be incorporated at strategic times in the production to ensure optimal growth, produce robust and healthy fish and manipulate the fatty acid composition of the final product. Finally, it is worth mentioning that spotted wolffish represents a relatively new species for aquaculture; thus there is a need for selective breeding in order to develop strains of robust and healthy fish with maximized and homogenous growth potential and to establish a national broodstock program.

8. References

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Paper I

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Muscle growth and changes in chemical composition of spotted wolffish juveniles (*Anarhichas minor*) fed diets with and without microalgae (*Scenedesmus obliquus*)

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ABSTRACT

Spotted wolffish (*Anarhichas minor*) is a promising new candidate for cold-water fish farming, but knowledge is needed about its physiology and its capacity to utilize alternative feed ingredients. The aim of the study was to investigate fast muscle growth dynamics, changes in chemical composition as well as growth performance of spotted wolffish when fed diets with or without the microalgae *Scenedesmus obliquus* incorporated. Juvenile spotted wolffish were fed four diets containing fishmeal as the primary source of protein (CTR diet) or microalgae (*Scenedesmus obliquus*) replacing 4% (AL4 diet), 8% (AL8 diet) or 12% (AL12 diet) of the fishmeal. During the 12 week experiment, fish grew from an average weight of 140 g to 250 g. The results showed indications of fast muscle cellularity of spotted wolffish being affected by dietary algae inclusion as the control and AL4 groups appeared to be more strongly favored by hypertrophic growth compared to the AL8 and AL12 groups. The CTR and AL4 groups tended towards increased muscle fiber diameters and higher proportions of larger muscle fibers, while the AL8 and AL12 group tended towards similar or increased proportions of smaller muscle fibers at the end of the trial. Probability density functions showed no difference in fast muscle fiber size distributions between dietary groups. Muscle crude protein and fat content tended to increase with growth in all treatment groups and muscle mineral content was reduced in all groups fed diets containing *Scenedesmus*. At the end of the trial, hepatosomatic index was reduced in all treatment groups. Dietary replacement of fishmeal with *Scenedesmus* also affected skin coloration, with increasing yellowness observed with increasing microalgae replacement. This study indicates that spotted wolffish has the potential to use microalgae as an alternative to fishmeal in the diet.

1. Introduction

Spotted wolffish (*Anarhichas minor*, Olafsen) is a North Atlantic fish species with promising potential for aquaculture production. Good growth performance at low temperatures, tolerance for high stocking density and robustness towards stress and disease make the spotted wolffish favorable for diversification of aquaculture in the North-Atlantic (Foss et al., 2004). The edible portion of fish consists primarily of muscle, which is also the largest tissue mass, comprising 60% or more of the fish body (Sänger and Stroiber, 2001). Fish increase their muscle mass by both hyperplastic and hypertrophic growth. As opposed to other vertebrates such as mammals and birds, fish can recruit new fibers for an extended part of their post-embryonic life. Hypertrophic growth occurs in fish throughout their life until the muscle fibers have reached their maximum diameter. The relative contribution of hyperplasia and hypertrophy to increases in muscle mass can affect the muscle cellularity. Muscle cellularity is affected by a number of factors including temperature (Johnston et al., 2000) and photoperiod (Johnston et al., 2003). Diet has also been shown to influence muscle cellularity (e.g. Alami-Durante et al., Bjørnevik et al., 2003, 2010, Silva et al., 2009). However, to date, little is known regarding the effect of alternative feed ingredients on fish muscle cellularity. The only study to our knowledge is the report by Alami-Durante et al. (2010) investigating how changes in dietary plant protein sources and amino acid profiles affect muscle growth in rainbow trout (*Onchorynchus mykiss*).

Fishmeal has historically provided a cheap and high quality source of dietary protein for both farmed aquatic animals and terrestrial livestock (Olsen and Hasan, 2012, Tacon and Meitan, 2015). However, the wild fish stocks that fishmeal production depend on are being

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Table 1
Ingredients (g 100 g^{-1} diet) and proximate composition [%] of the experimental diets containing different levels of microalgae (*Scenedesmus obliquus*) as a replacement for fish meal.

	Treatment diet			
	CTR	AL4	AL8	AL12
Ingredients (g 100 g^{-1} diet)				
Scenedesmus obliquus[1]	-	4.00	8.00	12.00
Fish meal LT70[2]	80.00	76.00	72.00	68.00
Fish oil[3]	7.00	7.00	7.00	7.00
Wheat meal[4]	12.30	12.30	12.30	12.30
Micro-ingredients[5]	0.70	0.70	0.70	0.70
Proximate composition of diets (g 100 g^{-1} ± S.E.M)				
Crude fat	16.12 ± 0.03	15.88 ± 0.04	16.01 ± 0.07	16.37 ± 0.28
Crude protein	52.12 ± 0.22	52.33 ± 0.30	52.91 ± 0.06	51.92 ± 0.45
Ash	13.48 ± 0.41	13.30 ± 0.37	12.20 ± 0.20	12.17 ± 0.20
Water	8.97 ± 0.07	9.50 ± 0.05	8.56 ± 0.05	8.29 ± 0.05
Energy (KJ g^{-1})	20.61	20.69	21.01	21.10
Amino acid composition (g 100 g^{-1})				
Asparagine	4.99	4.65	4.73	4.70
Serine	2.28	2.13	2.30	2.30
Glutamic acid	7.08	7.02	7.44	7.67
Proline	2.51	2.71	2.79	2.90
Glycine	3.98	3.84	3.88	3.91
Alanine	3.44	3.35	3.42	3.46
Valine	2.56	2.49	2.56	2.59
Isoleucine	2.14	2.03	2.05	2.08
Leucine	3.85	3.67	3.76	3.82
Tyrosine	1.72	1.60	1.66	1.67
Phenylalanine	2.18	2.00	2.11	2.25
Histidine	1.12	1.06	1.08	1.08
Lysine	4.02	3.76	3.74	3.68
Arginine	3.12	3.02	2.95	2.97
Tryptophan	0.584	0.558	0.580	0.604
Cysteine	0.466	0.442	0.464	0.502
Methionine	1.62	1.49	1.51	0.824
Hydroxyproline	0.676	0.457	0.610	0.561
Ornitine	< 0.05	< 0.05	< 0.05	< 0.05
Treonine	2.32	2.14	2.26	2.24

Treatment diets: CTR: control. AL4: 4% Scenedesmus inclusion. AL8: 8% Scenedesmus inclusion. AL12: 12% Scenedesmus inclusion.
1 Protein: 45.7%; lipid: 9.1%; carbohydrates: 15.6%; dietary fiber: 15.8%; ash: 8.3%; moisture: 5.6%; energy: 1.5 MJ g-1; pigments: 2.056% chlorophyll, 0.607% total carotenoids (Allma, Lisbon, Portugal).
2 Protein: 70%; lipid: 5.8% (Sopropeche, France)
3 SAVINOR UTS, Portugal
4 Protein: 11.7%; lipid: 1.6% (Casa Lanchinha, Portugal).
5 Vitamin & Mineral Premixi: Vitamins (IU or mg kg-1 diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg-1 diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings (PREMIX Lda, Portugal.

depleted. As such, the supply of fishmeal cannot keep pace with the demand resulting in a tripling of the cost of fishmeal since the early 2000's. Fishmeal is increasingly becoming both environmentally and economically unsustainable (FAO, 2016). Therefore, fishmeal as a dietary protein source cannot supply the world's growing aquaculture industry alone. To reduce the industry's dependence on fishmeal and fish oil, evaluation of novel feed ingredients is necessary.

Microalgae are potential feed ingredients in aquacultural diets. Some strains of microalgae contain important nutrients, including protein with a balanced amino acid profile, polyunsaturated fatty acids, vitamins and health promoting compounds (Becker, 2007, López et al., 2010, Yaakob et al., 2014). An increasing number of reports demonstrate that microalgae can partially replace fishmeal for several commercially important species of cultured fish. For example, moderate levels (10%) of the microalgae *Nannochloropsis oceanica* could be included in the diet of Atlantic salmon (*Salmo salar*) without adverse effects on fish performance (Sørensen et al., 2017). Other species of microalgae incorporated in diets for Atlantic salmon also showed positive effects on fillet quality and retention of essential fatty acids (Kiron et al., 2012, Kousolaki et al., 2016, Kousolaki et al., 2015). *Tetraselmis suecica* and *Isochrysis* sp. could replace 20% of the fishmeal in European seabass (*Dicentrarchus labrax*) diets without adverse effects on performance (Tibaldi et al., 2015, Tulli et al., 2012). Replacement of fishmeal with microalgae has also been successful for other cultured fish species such as sea bream (*Sparus aurata*), red drum (*Sciaenops ocellatus*) as well as for terrestrial livestock (Patterson and Gatlin, 2013, Vizcaíno et al., 2014, Yaakob et al., 2014). The freshwater green algae *Scenedesmus obliquus* may be a potential ingredient for marine fish. *S. obliquus* has a relatively high protein content (50-56% of dry matter) and contains all essential amino acids as reported by Becker (2007). The closely related *Scenedesmus almeriensis* significantly increased intestinal absorptive capacity and could replace up to 20% of the fishmeal in the diet of gilthead sea bream (*Sparus aurata*) without adverse effects on fish performance (Vizcaíno et al., 2014). Bawdy et al. (2008) reported that *Scenedesmus* sp. could replace up to 50% of fish meal in Nile tilapia (*Oreochromis niloticus*) diets. To date, some of the largest challenges in using microalgae as feed ingredients in aqua diets are high production cost and low available volumes (Benemann, 2013). One of the advantages of *S. obliquus* is the possibility for large scale production (Basu et al., 2014, Becker, 2007).

Replacement of fishmeal with alternative feed ingredients for spotted wolfish has not yet been investigated. The aim of the present

study was to investigate the effect of partial replacement of fishmeal with *S. obliquus* as a source of dietary protein on (i) fast muscle growth dynamics, (ii) muscle proximate composition as well as (iii) overall biometric gain and somatic indexes.

2. Materials and methods

The experiment was carried out at Mørkvedbukta research station at Nord University (Bodø, Norway) following the Guidelines of the European Union (Directive 2010/63/UE) for the use of laboratory animals. The study was approved by the Animal Welfare Committee at FBA, Nord University and carried out in strict accordance with the Norwegian animal welfare act (LOV-2009-06-19-97) and the regulation on the use of animals in research (FOR-2015-06-18-761). Animal sacrifice was limited to the minimum required to conduct the trial.

2.1. Fish rearing facility and husbandry

Juvenile spotted wolffish (mean weight 60 g) was provided by Aminor AS (Halsa, Norway). Fish were randomly distributed into 12 circular tanks (1 m^3) with $n = 75$ fish per tank and acclimatized to the laboratory conditions for 7 weeks. During acclimatization, fish were fed a commercial diet (Amber Neptun, Skretting, Stavanger, Norway). The fish were adapted to the experimental diets over a 15 day period, starting with a mix of experimental/commercial feed in the ratio 1/3 for 5 days, 2/3 for 5 days and finally experimental diets only for 5 days. Fish were provided seawater from a flow through treatment system. Filtered (200 µm) and aerated seawater with stable salinity (34 ‰), temperature (7.7 °C ± 0.005) and oxygen (86.7 ± 0.11 %) was supplied from 250 m depth in Saltfjorden. Water flow was at 1400 L/h throughout the trial. Fish were kept under continuous light and with continuous meal-based feeding (in excess) between 08:00 and 21:00 during the experiment. Feeding rate was set to 1.6% and gradually reduced to 1.4% of total biomass towards the end of the trial based on appetite and accumulated feed waste.

2.2. Experimental diets and feeding trial

Feed ingredients and proximate composition of the experimental diets are shown in Table 1. Four diets were formulated to contain 0% (CTR), 4% (AL4), 8% (AL8) and 12% (AL12) of microalgae substituting fishmeal on an equal weight basis. Diets were otherwise identical in composition, as they were balanced based on crude chemical composition and formulated to be isonitrogenous, isolipidic and isocaloric. The microalgae (*Scenedesmus obliquus*) was cultured at Allma (Lisbon, Portugal) in closed photobioreactors and spray dried prior to inclusion in the diets. The experimental diets were extruded with a pellet diameter of 2-3 mm produced at Sparos Lda. (Olhão, Portugal). Diets were stored at room temperature, in air-tight containers and protected from light during the trial. Following the acclimatization period of the fish, the four diets were randomly allocated to triplicate tanks. At the start of the experiment fish were about 1 year old, with a body weight of 139.87 ± 1.08 g and length of 22.70 ± 0.05 cm (mean ± SEM). The feeding experiment was terminated after 12 weeks.

2.3. Sampling

Fish (n = 10 per tank, altogether n = 120 fish) were randomly sampled with four week intervals: at week 0, 4, 8 and 12 of the trial. The fish were euthanized with an overdose of the anaesthetic MS-222 (tricaine methanesulfonate, 0.14 g/L) buffered with equal parts sodium bicarbonate followed by mild cranial concussion. Weight, total length and liver weight was individually recorded for all sampled fish. Weight of fish was recorded to the nearest 0.5 g, total length to the nearest 0.1 mm and liver weight to the nearest 0.01 g. At week 0 and 12 all fish were anaesthetized with a non-lethal dose of buffered MS-222 (0.0875 g/L) prior to weighing and length measurements. At termination of the experiment (week 12), skin color was measured using a portable spectrophotometer (CM-700d, Konica Minolta Sensing Inc., Singapore). At each sampling point, individual fillets were taken for analysis of proximate biochemical composition and samples were frozen at −40 °C until further analysis. From four of the euthanized fish per tank, samples were taken for histological analysis of muscle (see section 2.4). Feed samples were collected at the beginning of the experiment and stored at −40 °C for analysis of proximate chemical composition and energy content.

2.4. Proximate composition

All proximate composition analyses were performed in duplicates. Muscle samples were thawed, pooled (n = 3 fish per tank) and analyzed for crude protein, ash, moisture and crude fat content. Moisture content was determined by drying samples (5 g) to a constant weight (20 hours at 105 °C) in a drying cabinet. Ash (mineral) content was obtained by burning samples (5 g) in a crucible in a muffle furnace (16 hours at 540 °C). Crude protein content was determined using the Kjeldahl titration method. Samples (1 g) were weighed out and hydrolyzed for 45 min at 420 °C with 15 mL sulfuric acid (H_2SO_4, 98%, VWR chemicals) and Kjeldahl catalyst tablets (3.5 kg K_2SO_4 and 0.4 g $CuSO_4$, Foss, Sweden). Room temperature samples were diluted in distilled water (75 mL) were then analyzed in a Kjeldahl titrator (Kjeltec™ 2300, Foss, Sweden). Crude fat content was determined by the diethyl ester extraction method. Samples (10 g) were measured and water free sodium sulfate (20 g, Na_2SO_4, VWR chemicals) was added and mixed to a dry powder. Ethyl acetate (50 mL, $C_4H_8O_2$, VWR chemicals) was added to the samples and stirred for an hour. The solution was then filtered and the solution (20 mL) was placed in steam bath for 15 min in an evaporation cup to remove the solvent. After evaporation of the solvent, the evaporation cup was dried at 105 °C for 15-20 min and cooled in a desiccator. The proximate composition of the feed was determined using the same methods as for the muscle samples. Gross energy was determined using a bomb calorimeter (IKA C200 bomb calorimeter, Staufen, Germany).

2.5. Fast muscle cellularity

Myotomal steaks (5 mm thickness) were cut anterior to the posterior ventral fin. A photograph of the steak was taken together with a scale. The total cross-section area (TCA) was then measured in these images using the software ImageJ (NIH, USA). Blocks (n = 2-4 depending on fish size, 5 × 5 x 5 mm) of fast muscle were prepared from each steak from the left side of the body to cover the steak area. Muscle blocks were mounted on small pieces of cork sheet (1.5 x 1.5 cm), covered in Cryomatrix (Shandon Cryomatrix, Thermo Scientific) and cooled in liquid nitrogen for 45 s in 2-methyl butane (isopentane, VWR chemicals) to near the freezing point (−159 °C). Frozen blocks were wrapped in pre-labeled tinfoil and stored at −80 °C. Prior to sectioning, blocks were acclimated to −20 °C for 30 min. Sections (7 µm) were prepared using a cryostat (Cryostar NX50, Thermo Scientific), mounted and air-dried on poly-L-lysine coated slides and stained with Hematoxylin (Meck Chemicals) for 12 min. Sections were then rinsed for 10 min under running tap water and a cover-glass was mounted on the section using glycergel (Glycergel mounting medium, Dako). Area of muscle fibers were measured on images at 10x magnification using a microscope (Axioskop2, Carl Zeiss) and camera (Axiocam HRC, Carl Zeiss) using the Carl Zeiss software, AxioVision 4.8. An illustrative photo of a muscle cross section is shown in Fig. 1. A minimum of 800 fibers were measured in total per fish.

2.6. Calculations

Weight gain (WG, %) was calculated from WG = ((final mean

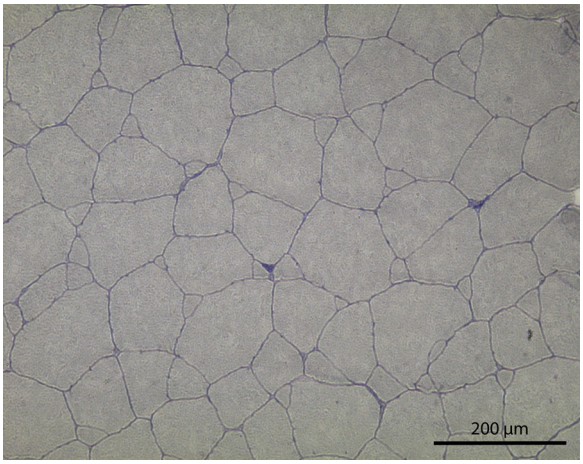

Fig. 1. Cross section (10x) of fast skeletal muscle of spotted wolffish showing smaller newly recruited fibers scattered among older larger fibers typical for the mosaic hyperplastic growth phase in juvenile fish.
Color online only

weight – initial weight)/initial weight) * 100. Specific growth rate (SGR, % day^{-1}) was calculated as 100 x ln[final mean weight (g) / initial mean weight (g)] / days. Condition factor (CF) was calculated as [fish weight (g) / total length $(cm)^3$] x 100. Hepatosomatic index (HSI) was calculated as [liver weight (g) / fish weight (g)] x 100.

Percent moisture was calculated according to the following formula: Moisture (%) = ((WW-(DW-ECW))/WW) x 100%, where WW is the sample wet weight, DW is the sample dry weight and ECW is the empty cup weight for the cup used during drying. Percent ash was calculated according to the following formula: Ash (%) = ((FCW-ECW)/SW) x 100, where FCW is the fired crucible weight, ECW is the empty crucible weight and SW is sample weight. Fat (g) was calculated according to the following formula: Fat (g) = ECF – ECE, where ECF is the weight of the evaporation cup with fat and ECE is the empty evaporation cup. Percent Fat was calculated according to the following formula: Fat (%) = (10,300 x F)/(40 – 2.17 x F x SW), where 10,300 is a constant and 40-2.17 is a calibration factor, F is the fat content (g) and SW is the sample weight.

Diameter and number of muscle fibers was calculated according to Johnston et al. (1999). Diameter of muscle fibers were calculated from the measured area using the formula 2 x (Square root(Muscle fiber area/π)).. Muscle fiber number (FN) was calculated from the formula FN = 10^6 x (TCA x number of counted fibers / sum area). Muscle fiber density (FD) was calculated from the formula FD = 10^6 x (number of counted fibers / sum area). Fiber recruitment per day was calculated from the formula (FN_1 – FN_0)/days, where FN_0 is the group-mean muscle fiber number at week 0 and FN_1 is the group-mean muscle fiber number at the end of the experiment. As fast muscle cellularity is dependent on fish size (Weatherly et al., 1988) the data was also normalized based on fish size according to the normalization reported by Alami-Durante et al (2010). The parameters that were increasing with increasing standard length were divided by the natural logarithm of the standard length (ln SL). Parameters that were decreasing with increasing standard length were multiplied by ln SL. All data are presented as mean ± SEM.

2.7. Statistical analysis

All data were tested for normality by the Shapiro-Wilk test and homogeneity of variance by Levene's mean test before being analyzed with a one-way ANOVA using the software Sigmaplot 12.0 (Systat Software, San Jose, CA). When the ANOVA showed significant differences, the Holm-Sidak method of multiple comparisons was used to compare individual means. When the data did not meet the ANOVA assumptions, a Kruskal-Wallis one-way analysis of variance on ranks was used. When the Kruskal-Wallis test showed significance, Dunn's method of multiple comparisons was used to compare individual medians. Means of replicate tanks were also compared with a one-way ANOVA.

To compare the distribution of muscle fiber sizes, nonparametric statistical techniques were used to fit smoothed probability density functions (pdfs) to the measurements using a kernel function (Bowman and Azzalini, 1997) as described by Johnston et al. 1999. This was done using the software R-3.4.1 (R Core Team, 2017) and package sm 2.2-5.4 (Bowman and Azzalini, 2014). Altogether, six fish of equal total length (28.0 ± 1.0 cm) were selected from each treatment group and smoothed pdfs were fitted to the fish in each group as well as the group mean. Bootstrap techniques were then used to plot approximate variability bands around the group pdfs using the mean smoothing parameter. This provided a visual indication of which areas of the muscle fiber distribution that was potentially significantly different. In addition, a Kolmogorov-Smirnov two-sample test was used to test if the pdf of the treatment groups were equal over all diameters. In all tests, significant differences were considered when $p \leq 0.05$.

3. Results

3.1. Biometrical data

Biometrical data is presented in Table 2. No mortality was noted during the trial and the fish weight was nearly doubled for most groups. However, at termination of the experiment, fish fed the AL8 diet had lower mean weight, total length, %WG and SGR compared to all other dietary groups. No differences in mean weight, length, %WG and SGR was observed among the other treatment groups ($p > 0.05$). At week 12 the AL8 and AL12 had lower condition factor compared to both the start of the experiment ($p < 0.001$) and to the control group ($p < 0.001$ and $p = 0.001$ respectively).

Mean hepatosomatic index (mean ± SEM) at the start of the experiment was 4.76 ± 0.04. Interestingly, all groups had lower hepatosomatic index at week 12 compared to week 0, but only the AL8 group (3.64 ± 0.07) was different from the control (3.82 ± 0.07) at week 12 ($p = 0.003$). The hepatosomatic index was lower for all groups already from week 4 ($p < 0.05$). The AL8 group was also the only group that showed no increase in mean liver weight at week 12 compared to week 0 (not shown in table, $p > 0.05$).

3.2. Fast muscle cellularity

Fast muscle cellularity is shown in Table 3. The results showed no difference ($p > 0.05$) in fast muscle cellularity between the different treatment groups for any of the assessed parameters at the end of the trial. Differences were observed when comparing the treatment groups to the means at the start of the trial. All treatments had increased fiber number at week 12, but AL12 was the only group that was different from week 0 ($p = 0.023$). Reduced fiber density at week 12 was also observed in all groups. The D mean and D median was increased for all groups at week 12, but D mean was only different for the CTR and AL4 group ($p = 0.027$ and $p = 0.007$ for AL4 and CTR respectively) and D median only for the AL4 group ($p = 0.011$). The CTR group was also the only group that showed increased D max ($p = 0.018$) at week 12. D mean of the upper 95 percentile was also increased in all groups ($p = 0.001$, 0.010 and 0.028 for the CTR, AL12 and Al4 groups respectively) with the exception of AL8 that was not different from week 0 ($p > 0.05$). Compared to the start of the trial, the proportion of fast muscle fibers with diameter ≤ 20 µm was decreasing in all treatment groups, but was only significant for the CTR group ($p = 0.035$). In all groups, there was no difference in proportion of fibers with $20 < D \leq 40$ µm and $80 < D \leq 120$ µm. Proportion of fibers in the range $40 < D \leq 80$ µm was decreasing in all groups, but was lower

Table 2
Survival, weight, length, condition factor (CF), hepatosomatic index (HSI), total weight gain (WG) and daily growth (DG) of spotted wolffish fed diets with different level of inclusion of microalgae (*Scenedesmus obliquus*). Weight, length, WG, SGR and CF for week 0 and week 12 are based on measurements of all fish. All other values are based on sampled fish. Values are means ± SEM. Means in the same column at the same time point with different superscript letters differ significantly ($P < 0.05$). Means in the same column with superscript * differ significantly from the start of the trial ($P < 0.05$).

Time	Diet	Survival [%]	Body weight [g]	Body length [cm]	CF	HSI	WG [%]	SGR [% day^{-1}]
Week 0	Start	n.a	139.87 ± 1.08	22.70 ± 0.05	1.18 ± 0.004	4.76 ± 0.04	n.a	n.a
Week 4	CTR	100	194.88 ± 6.74^{*a}	25.45 ± 0.26^{*a}	1.17 ± 0.01^{a}	4.21 ± 0.05^{*a}	39.11 ± 3.84^{a}	1.06 ± 0.09^{a}
	AL4	100	182.13 ± 6.18^{*ab}	24.86 ± 0.27^{*ab}	1.17 ± 0.02^{a}	4.02 ± 0.05^{*ab}	29.31 ± 6.58^{a}	0.82 ± 0.17^{a}
	AL8	100	164.47 ± 7.22^{*b}	24.23 ± 0.28^{*b}	1.14 ± 0.02^{a}	3.98 ± 0.06^{*b}	18.22 ± 1.12^{a}	0.54 ± 0.03^{a}
	AL12	100	181.47 ± 7.52^{*ab}	25.09 ± 0.31^{*ab}	1.13 ± 0.02^{a}	3.94 ± 0.07^{*b}	30.20 ± 3.75^{a}	0.84 ± 0.09^{a}
Week 8	CTR	100	231.95 ± 11.69^{*a}	27.03 ± 0.38^{*a}	1.15 ± 0.01^{a}	3.85 ± 0.07^{*ab}	65.70 ± 3.45^{a}	0.87 ± 0.04^{a}
	AL4	100	237.98 ± 10.85^{*a}	27.12 ± 0.34^{*a}	1.17 ± 0.02^{a}	4.06 ± 0.08^{*a}	68.55 ± 9.41^{a}	0.89 ± 0.10^{a}
	AL8	100	217.98 ± 10.01^{*a}	26.66 ± 0.35^{*a}	1.13 ± 0.02^{a}	3.64 ± 0.08^{*b}	56.70 ± 6.42^{a}	0.77 ± 0.07^{a}
	AL12	100	230.23 ± 7.91^{*a}	26.84 ± 0.30^{*a}	1.18 ± 0.02^{a}	3.75 ± 0.06^{*b}	65.70 ± 3.45^{a}	0.86 ± 0.04^{a}
Week 12	CTR	100	262.63 ± 5.56^{*a}	27.98 ± 0.18^{*a}	1.18 ± 0.01^{a}	3.82 ± 0.07^{*a}	87.63 ± 1.33^{a}	0.72^{a} ± 0.01^{a}
	AL4	100	265.34 ± 6.44^{*a}	28.13 ± 0.19^{*a}	1.16 ± 0.01^{ab}	3.73 ± 0.06^{*a}	88.20 ± 4.55^{a}	0.73^{a} ± 0.03^{a}
	AL8	100	221.59 ± 4.85^{*b}	26.94 ± 0.17^{*b}	1.11 ± 0.01^{*c}	3.64 ± 0.07^{*b}	59.31 ± 3.19^{b}	0.53^{b} ± 0.02^{b}
	AL12	100	247.10 ± 5.73^{*a}	27.72 ± 0.18^{*a}	1.13 ± 0.01^{*bc}	3.74 ± 0.07^{*a}	77.25 ± 2.26^{a}	0.66^{a} ± 0.01^{a}

only for the AL4 and CTR groups ($p = 0.005$ and 0.018 for AL4 and CTR respectively). The AL8 group was the only group that did not show any increase in number of fibers in the range $120 < D > 200$ µm.

Number of fibers recruited per day increased with increasing algae replacement. Fiber recruitment was 471, 322, 174 and 48 fibers day^{-1} for the AL12, AL8, AL4 and CTR groups, respectively. The probability density functions for muscle fiber size distributions showed no differences between the treatment groups (Fig. 2).

3.3. Proximate composition of muscle

The muscle proximate composition is shown in Table 4. Except for crude protein, there was no difference in muscle proximate composition among treatment groups at the end of the experiment ($p > 0.05$). Compared to the start of the experiment, muscle crude protein content and fat content tended to be slightly increased for all groups (Table 4). The spotted wolffish also had a subcutaneous fat layer (Fig. 3). At termination of the experiment, compared to week 0, all algae treatment groups had reduced muscle mineral content ($p = 0.007$, $p < 0.001$, $p = 0.004$ for AL4, AL8 and AL12 respectively) and the CTR and AL4 had reduced muscle moisture ($p < 0.001$ and $p = 0.008$ respectively).

3.4. Skin color

No difference was observed between treatments for skin color L* value (lightness, Fig. 4A, $p = 0.566$) and a* value (red/green, Fig. 4B, $p = 0.414$). However, the skin color b* value differed significantly among feeding groups (blue/yellow, Fig. 4C, $p < 0.001$), where the skin of the fish was increasingly more yellow with increasing algae inclusion of the diet. AL12 had higher b* value than AL4 ($p = 0.002$). Although not measured, no visual change in fillet color was observed.

4. Discussion

Overall, the fish performed well during the current experiment, displaying similar or even better growth performance compared to previously published data from juvenile spotted wolffish. The SGR calculated at termination of the trial varied between 0.53 and 0.73% for the different treatment groups and was in accordance with other reports of spotted wolffish. Falk-Petersen et al. (1999) reported that specific growth rates of 150-700 g (1-2 year old) wolffish ranged between 0.37-0.50% day^{-1}. Tremblay-Bourgeois (2010) also reported SGR of 0.65% day^{-1} for 160 g juvenile wolffish reared at optimal density. Imsland et al. (2006) found an inverse relationship between size and growth rate for spotted wolfish, a tendency also observed in the present trial. The lower growth of the AL8 treatment group contradicts findings in other

Table 3
Fast muscle cellularity of spotted wolffish fed diets with different levels of inclusion of microalgae (*Scenedesmus* sp.). Values are means ± SEM. Means in the same row with different superscript letters differ significantly ($P < 0.05$). Significance is presented from analysis of data normalized by total length (TL). For this normalization, parameters increasing with increasing TL were divided by ln TL and parameters decreasing with increasing TL were multiplied by ln TL.

Time	Week 0	Week 12			
	Start	CTR	AL4	AL8	AL12
Fiber number	125834 ± 3865^{a}	145866 ± 4634^{ab}	140651 ± 7492^{ab}	150185 ± 6096^{ab}	155686 ± 7065^{b}
Fiber density [fibers mm^{-2}]	188.02 ± 5.17^{a}	139.10 ± 9.46^{b}	138.24 ± 8.29^{b}	158.75 ± 7.96^{b}	144.03 ± 7.60^{b}
D mean	75.47 ± 1.18^{a}	87.36 ± 2.56^{b}	88.44 ± 2.84^{b}	81.53 ± 2.03^{ab}	85.89 ± 2.62^{ab}
D median	73.34 ± 1.41^{a}	85.59 ± 3.38^{ab}	87.59 ± 3.18^{b}	79.63 ± 2.52^{ab}	84.42 ± 2.88^{ab}
D max	194.70 ± 2.51^{a}	227.73 ± 6.92^{b}	227.05 ± 10.31^{ab}	218.28 ± 9.20^{ab}	224.92 ± 7.91^{ab}
D mean of upper 95th percentile	156.58 ± 1.78^{a}	182.81 ± 4.73^{b}	176.40 ± 4.29^{b}	170.37 ± 4.74^{ab}	179.21 ± 4.39^{b}
Proportion [%] of white muscle fibers with					
$D \leq 20$ µm	4.28 ± 0.33^{a}	2.47 ± 0.30^{b}	2.82 ± 0.48^{ab}	3.30 ± 0.63^{ab}	2.94 ± 0.49^{ab}
$20 < D \leq 40$ µm	14.43 ± 0.84^{a}	13.91 ± 1.07^{a}	12.31 ± 1.17^{a}	13.87 ± 1.08^{a}	12.83 ± 1.42^{a}
$40 < D \leq 80$ µm	38.55 ± 1.35^{a}	29.02 ± 2.65^{b}	27.67 ± 1.72^{b}	33.78 ± 2.61^{ab}	30.79 ± 1.17^{ab}
$80 < D \leq 120$ µm	30.70 ± 1.03^{a}	32.20 ± 1.84^{a}	34.85 ± 1.15^{a}	32.00 ± 1.30^{a}	33.54 ± 1.00^{a}
$120 < D \leq 160$ µm	10.16 ± 0.52^{a}	16.13 ± 1.34^{b}	17.36 ± 1.59^{b}	13.32 ± 1.30^{ab}	14.39 ± 1.29^{b}
$160 < D \leq 200$ µm	1.77 ± 0.20^{a}	5.29 ± 0.82^{b}	4.37 ± 0.74^{b}	3.28 ± 0.60^{ab}	4.85 ± 0.74^{b}
$D > 200$ µm	0.11 ± 0.04^{a}	0.98 ± 0.25^{b}	0.62 ± 0.19^{b}	0.45 ± 0.18^{ab}	0.66 ± 0.20^{b}

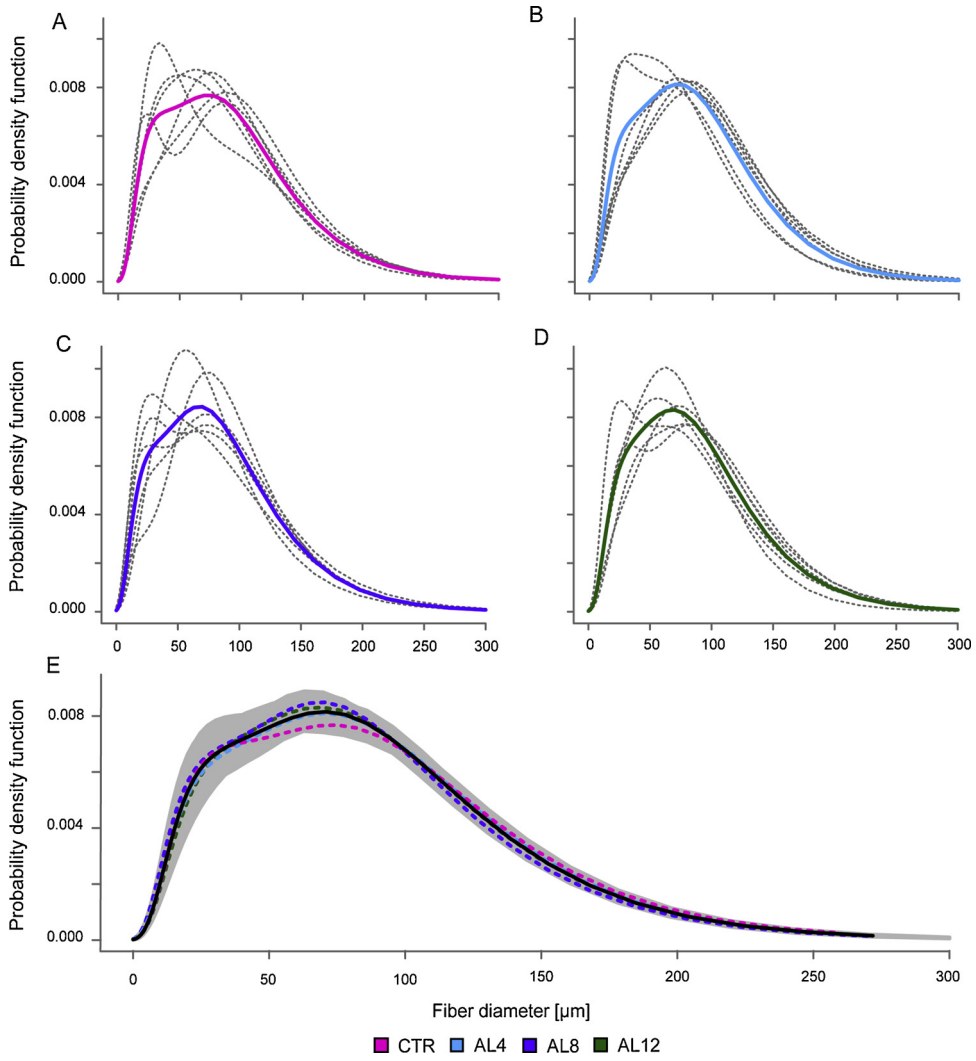

Fig. 2. Muscle fiber diameter probability density functions of *A. minor* fed increasing levels of *S. obliquus* meal in the diet. For A-D solid line is group mean and dotted line is individual fish. A: Control treatment. B: Treatment group AL4. C: Treatment group AL8. D: Treatment group AL12. E: Bootstrapping analysis comparing the four treatment groups over all fast fiber diameters using Kolmogorov-Smirnov statistics.
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Table 4
Muscle proximate composition [%] of spotted wolffish fed diets with different level of inclusion of microalgae (*Scenedesmus obliquus*). Values are means ± SEM. Means in the same column at the same time point with different superscript letters differ significantly ($P < 0.05$). Means in the same column with superscript * differ significantly from the start of the trial ($P < 0.05$).

Time	Diet	Crude protein	Crude lipid	Ash	Moisture
Week 0	Start	17.10 ± 0.07	4.15 ± 0.16	1.29 ± 0.01	76.95 ± 0.14
Week 4	CTR	17.35 ± 0.22^{a}	4.19 ± 0.32^{a}	1.32 ± 0.03^{a}	77.11 ± 0.28^{a}
	AL4	17.58 ± 0.17^{a}	3.32 ± 0.26^{a}	1.25 ± 0.02^{a}	77.43 ± 0.17^{a}
	AL8	17.43 ± 0.10^{a}	3.67 ± 0.22^{a}	1.29 ± 0.01^{a}	77.51 ± 0.24^{a}
	AL12	17.65 ± 0.15*^{a}	3.84 ± 0.26^{a}	1.26 ± 0.01^{a}	77.14 ± 0.20^{a}
Week 8	CTR	17.82 ± 0.08*^{a}	4.03 ± 0.33^{abc}	1.25 ± 0.02^{b}	77.33 ± 0.35^{a}
	AL4	17.72 ± 0.12*^{a}	4.47 ± 0.28^{b}	1.19 ± 0.02*^{a}	76.59 ± 0.24^{a}
	AL8	17.88 ± 0.14*^{a}	3.36 ± 0.27^{c}	1.26 ± 0.01^{b}	77.48 ± 0.24^{a}
	AL12	17.79 ± 0.12*^{a}	3.71 ± 0.21^{abc}	1.22 ± 0.01*^{ab}	77.24 ± 0.16^{a}
Week 12	CTR	17.51 ± 0.27^{abc}	5.72 ± 0.46*^{a}	1.24 ± 0.02^{a}	75.14 ± 0.50*^{a}
	AL4	18.00 ± 0.15*^{b}	4.94 ± 0.17^{a}	1.22 ± 0.01^{a}*	75.73 ± 0.33*^{a}
	AL8	17.71 ± 0.18*^{abc}	4.66 ± 028^{a}	1.20 ± 0.01^{a}*	76.29 ± 0.23^{a}
	AL12	17.25 ± 0.07^{a}	5.03 ± 0.38^{a}	1.21 ± 0.02^{a}*	76.28 ± 0.35^{a}

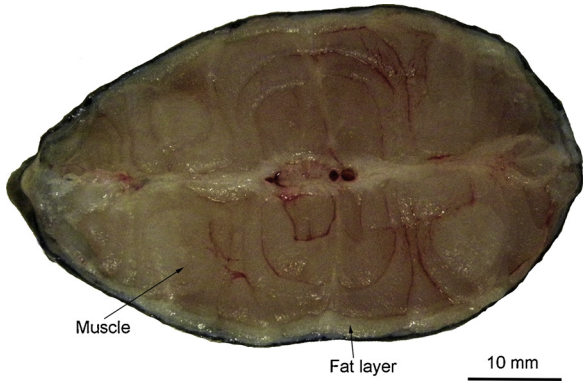

Fig. 3. Cross-section (cut anterior to the posterior ventral fin) showing the subcutaneous fat layer of juvenile *A. minor*.
Color online only.

studies. Usually lowest growth is found at the highest inclusion levels (e.g. Bawdy et al., 2008, Patterson and Gatlin, 2013, Sørensen et al., 2017, Vizcaíno et al., 2014). This also conflicts with other trials using *Scenedesmus* sp. where no negative effects on growth performance were observed (Bawdy et al., 2008, Vizcaíno et al., 2014). This unexpected result may be explained by larger variation (mean weights 205, 221 and 239 g) among tank means at week 12, resulting in significant differences in among-tank means ($p = 0.027$). However, no tank effects were noted for any other study parameters.

The probability density functions showed no indication that diet influenced the distribution of fast muscle fibers. However, a few significant differences were observed when comparing the individual changes from the start to the end of the trialSpotted wolffish can reach a maximum body length of 180 cm and the fish used in this experiment had a final mean standard length of about 27 cm. Recruitment of new fibers cease at a size of about 44% of the final body length in several teleost fish (Weatherly et al., 1988). If this is true also for spotted wolffish, they are expected to continue increasing muscle mass through hyperplastic growth. Recruitment fibers (fibers ≤ 20 µm) were present in all treatment groups, but tended to be reduced compared to the start of the experiment. However, there seemed to be a correlation between the rate of hyperplasia and algae inclusion. Reduced recruitment was found at the end of the trial for all groups, but it was only significant for the control group. The fast muscle fibers of the control and AL4 groups had in general larger diameter compared to the other groups. In addition, larger fibers appeared to compose an increasing proportion of the total muscle fiber distribution, indicating a favoring of hypertrophic growth. The population of muscle fibers in the AL8 and AL12 groups consisted in general of fibers with smaller diameter; in addition, there was a similar size distribution compared to the start of the trial. This may indicate minimal changes in the ratio between hyperplastic and hypertrophic growth. Increased fiber number at the end of the trial was noted only for the AL12 group. Consequently, the results indicated an increased muscle fiber recruitment as an effect of algae inclusion. The inverse relationship between algae inclusion and daily fiber recruitment also further supports this. Although only significant for the AL8 treatment, both fish fed the AL8 and AL12 diets had numerically lower mean weight at the end of the trial compared to fish fed the AL4 and CTR diets. The favoring of hyperplasia with the higher algae inclusions was most likely associated with the growth dynamics, as fish white muscle growth is influenced both ration level (Kiessling et al., 1991) and dietary composition (Silva et al., 2009; Alami-Durante et al., 2010). Kiessling et al. (1991) also reported a favoring of muscle hypertrophy for rainbow trout in periods of rapid fish growth and a corresponding favoring of muscle hyperplasia in periods of slow growth. At 75-100% substitution of fishmeal with plant protein for juvenile rainbow trout, Alami-Durante et al. (2010) observed a reduced median diameter of white muscle fibers and explained the observation with reduced growth performance and increased expression of cathepsin D, an enzyme involved in lysosomal proteolysis in muscle. The diets used in the present experiment with wolfish were nearly identical in composition, suggesting that more research is needed to explain the mechanism as well as the long-term effects of microalgae on fish growth and performance. However, the probability density functions are much stronger statistical tools for studying muscle fiber populations compared to individual measurements (Johnston, 1999). The results from this analysis should hence be weighted stronger compared to the individual measurements and further studies are necessary to confirm this hypothesis.

Existing reports on the effect of algae inclusion in fish diets report conflicting findings of muscle chemical composition (Bawdy et al. 2008, Dallaire et al., 2007, Nandeesha et al., 2001, Patterson and Gatlin, 2013, Sørensen et al. 2017, Vizcaíno et al. 2014). Vizcaíno et al. (2014) reported no change in muscle proximate composition for sea bream fed *Scenedesmus* in the diet. Bawdy et al. (2008) reported higher dry matter and protein content, but lower lipid content, in the carcass of Nile tilapia fed 50% *Scenedesmus* in the diet. With an intramuscular fat content found to be around 5%, the present juvenile spotted wolffish are classified as intermediately fatty fish (Hocquette et al., 2010). Fat is deposited in the muscle, liver and in the subcutaneous fat layer (shown in Fig. 3). The control treatment was the only treatment with significantly higher intramuscular fat at the end of the trial. It could be hypothesized that the observed tendency for favoring hypertrophic muscle growth in the control group could be connected to lipids being stored in the muscle rather than being metabolized for muscle fiber recruitment. As intramuscular fat positively affects the flavor and quality of fish fillets, increased intramuscular fat could affect the organoleptic properties, and hence the quality of the final product (Hocquette et al., 2010). The significantly higher muscle lipid content in fish fed control diet at the end of the experiment could also indicate higher digestibility and utilization of nutrients and energy compared to

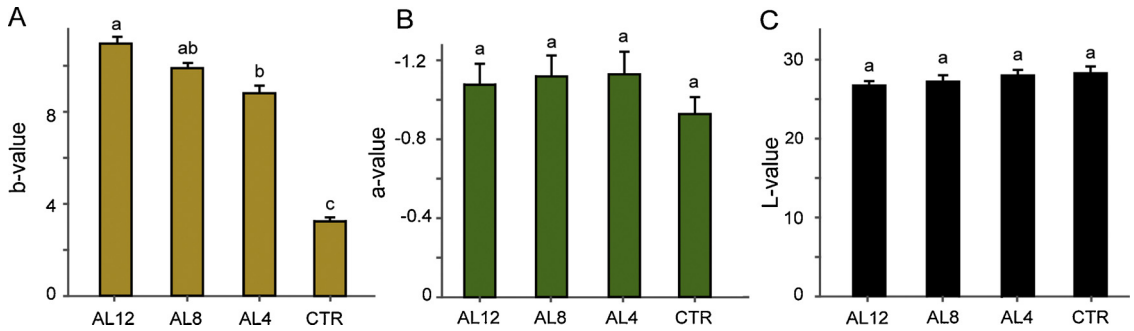

Fig. 4. Spectrophotometric measurements of skin color differences at week 12. Values are means ± SEM. Means with different superscript letters differ significantly *($P < 0.05$)*. A: b-value (blue-yellow, > 0: yellow, < 0: blue. B: a-value (green-red, < 0: green, > 0: red). C L-value (lightness, L = 100: white, L = 0: black).
Color online only.

the algae-fed fish. The higher muscle lipid content may also be explained by the slightly higher body weight of fish fed the control diet. This is in line with Moksness et al. (1995), who reported increased muscle lipid content in groups of common wolffish (*Anachichas lupus*) with the highest growth rate. Moksness et al. (1995) reported no differences in muscle crude protein of common wolffish with different growth rate. Compared to the start of the present trial, increased muscle crude protein content at week 12 was observed for the AL4 and AL8 groups. The muscle protein content increased from 17% in the initial population to approximately 18% at week 8 and 12, respectively. Though the results indicate some minor differences among the dietary groups the main trend was that protein content increased slightly with growth. The reduction in muscle mineral content (ash) from the start to the end of the trial found for the algae groups reflects the lower mineral content in these diets.

Carotenoids are used in health foods, food coloring, cosmetics, vitamin supplements and feed additives (Yaakob et al., 2014). The yellow-pigmented carotenoid lutein is produced by several species of microalgae including *Scenedesmus* sp. (Chan et al., 2013). The change in skin pigmentation observed for the algae-fed wolffish could be caused by deposition of this carotenoid. High lutein was also found in rainbow trout (*Oncorhynchus mykiss*) with yellow flesh discoloration (Welker et al., 2001). Yellow discoloration of flesh will reduce the market value of rainbow trout (Skonberg et al., 1998), but it is not known how skin discoloration would affect the market value of spotted wolffish if skin is present in the product. Changed skin pigmentation with increasing algae inclusion have also been reported for similar trials with other algal species (Tulli et al., 2012, Walker and Berlinsky, 2011), but is not reported from other experiments using *Scenedesmus* sp. (Bawdy et al. 2008, Vizcaíno et al., 2014).

All dietary groups seemed to perform within the normal range for juvenile spotted wolfish, but the reduced condition factor observed for the two highest algae inclusion groups may indicate a negative effect of algae replacement. Though condition factor was reduced in fish fed algae diets, the values were higher than previously reported for juvenile spotted wolffish. Foss et al. (2001) reported condition factors of 1.08 for 206 g wolffish reared at 34‰ salinity, similar to other reports (Foss et al., 2003, Tremblay-Bourgeois et al., 2010). However, as the diets were nearly identical in composition, it can also be hypothesized that the lower CF can be explained by poorer digestibility of the microalgae diets. The cell walls of microalgae can be difficult to digest for fish, and if these are inefficiently disrupted during feed processing or in the digestive tract it can lead to reduced availability of the nutrients and energy in the diet. Reduced digestibility has been reported from similar studies with microalgae replacing fishmeal (e.g. Tibaldi et al. 2015, Tulli et al., 2012). This may also explain the observed differences in muscle proximate composition in the present experiment.

Reduced HSI was observed for all treatments, which correlates with other reports of algae-fed fish (Tulli et al., 2012, Vizcaíno et al., 2014, Walker and Berlinsky, 2011). Patterson and Gatlin (2013) reported lower HSI in fish fed non-extracted compared to those fed lipid-extracted algae. Walker and Berlinsky (2011) suggested that the lower HSI observed in their study was a result of starvation of the fish. As reduced hepatosomatic index at the end of the trial was found also for the control treatment it could indicate that the feed composition of the diets used in the present experiment was suboptimal for the fish. Presently, no tailored feed for cultured wolffish exist and the knowledge about their nutritional demands is still quite undescribed. The macronutritional profile of the diets of the present study were formulated based on earlier feeding studies with wolffish. Jonassen (2002) indicated reduced growth in juvenile wolffish fed high fat diets (20%) compared to low fat diets (15%). Papoutsoglou and Lyndon, 2006 reported no difference in growth between high (45%) and low (38%) protein inclusion in the diet. However, this trial lasted only 18 days, which may be too short to make conclusions about the performance of the fish. Protein rich diets (55-62%) are generally used for the spotted wolffish (Foss et al., 2004). Further investigation of the nutritional requirements of spotted wolffish will be necessary in future studies.

5. Conclusion

The present study investigated the effects of dietary microalgae on fast muscle cellularity in wolffish. The results indicated that diet affected fast muscle cellularity as fish fed the control and AL4 group had higher hypertrophic growth than those fed the AL8 and AL12 diets. The muscle protein and lipid increased for all the diet groups during the course of the experiment, while mineral content was reduced for the algae-fed groups. Reduced hepatosomatic index observed for all dietary groups indicate that the energy supply was suboptimal for the growing fish. A reduction in condition factor in fish fed the high algae diet indicated reduced utilization of energy. Investigations of nutrient composition as well as the capacity of wolffish to utilize microalgae is warranted in future studies.

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Paper II

Fast muscle cellularity, fatty acid and body-composition of juvenile spotted wolffish (*Anarhichas minor*) fed a combination of plant proteins and microalgae (*Nannochloropsis oceanica*)

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Figure captions

Figure 1: Workflow for sampling and analysis of fast muscle cellularity.

Figure 2: Probability density functions (pdf) of fast muscle fiber diameters for spotted wolffish fed diets with different levels of the microalgae *Nannochloropsis oceanica* included in the diet. A-C shows the mean pdf for each group (solid line) as well as pdf's for individual fish in each group (dashed line). A: Control, no algae. B: 7.5% microalgae (N7.5). C: 15% microalgae (N15). D shows the mean pdf for all groups (solid black line), mean pdf for each group (dashed lines) and approximate variability bands produced with bootstrapping techniques.

Abstract

Spotted wolffish (*Anarhichas minor*) is a promising candidate for diversification of cold-water aquaculture. An increased knowledgebase is needed concerning the capacity of spotted wolffish to utilize a variety of feed ingredients such as microalgae and terrestrial plants. The aim of the study was therefore to investigate the effect of incorporating graded levels of microalgae (*Nannochloropsis oceanica*) on fish welfare indices (growth, hepasomatic index, blood plasma metabolites), fast muscle cellularity, chemical and fatty acid composition. Three isonitrogenous and isocaloric diets were formulated; one control diet (CTR) and two diets with low (N7.5) and high (N15) levels of microalgae (*N. oceanica*) replacing fishmeal and wheat in the diets. After 12 weeks of feeding, there were no differences in growth and fast muscle cellularity between the three treatment groups and blood plasma analysis showed no indications of stress in the fish fed microalgae. Hepatosomatic index decreased over the course of the experiment for all treatment groups; a significantly larger reduction was noted in the algae-fed fish compared to the control. Polyunsaturated fatty acids increased in muscle, liver and whole body of the fish fed diets containing microalgae. The results suggest that spotted wolffish has potential to utilize inclusions of up to 15% of the microalgae *N. oceanica*.

Keywords: fish nutrition · sustainable feed alternatives · blood plasma biochemistry · muscle fiber size distribution · arctic aquaculture · novel aquaculture species

1. Introduction

The continuously increasing demand for fish and shellfish for human consumption has led to exploitation and even over-exploitation of wild fish stocks (FAO, 2018). Thus, a further increase in the harvest of wild fish populations for fish-feed is not sustainable and instead, the utilization of alternative feed ingredients need to be investigated. Ingredients such as terrestrial plants, marine invertebrates, macro- and microalgae as well as insects are examples of novel ingredients with potential as feed ingredients for cultured fish (Naylor et al., 2009). The use of plant proteins has allowed the aquaculture industry to grow without increasing the pressure on wild fisheries. However, the sustainability of land-based protein sources is debatable as these compete with land area for human food production and depend on the use of fresh water, both of which are limited resources. Also, complete replacement of fishmeal with plant protein has proven difficult for many species, particularly for marine fish (Gatlin et al., 2007, Hardy, 2010). Compared to fish-oil and fishmeal, challenges with terrestrial plant-ingredients include imbalanced amino acid composition, high levels of carbohydrates and potential presence of anti-nutritional factors that may have adverse effects on gut health, digestion and utilization of nutrients (Bakke et al. 2014, Krogdahl et al., 2010, Marjara et al. 2012). Omega-3 (n-3) fatty acids, particularly eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids, are essential nutrients for both fish and humans (Calder, 2014, Tocher, 2015). Fish, and especially fatty fish like salmon, are the main dietary source of these polyunsaturated fatty acids (PUFA's) for humans (Tocher, 2015). However, due to the increased use of terrestrial plant-ingredients in salmon farming, not only are terrestrial fatty acids in salmon significantly increasing but the DHA and EPA levels are simultaneously decreasing (Sprague et al., 2016). To ensure that the nutritional value of the final product consumed by humans is not compromised, future aquaculture production cannot rely on land based plant ingredients alone.

Microalgae are unicellular photoautotrophic organisms and are primary producers of the long-chain PUFA's in the marine environment, including DHA and EPA (Adarme-Vega et al., 2012). Microalgae are cultivated for their high content of lipids and PUFAs, balanced amino acid profile, antioxidants and vitamins, which are suitable not only for animal feeds, but are also used in health food products and cosmetics (Brown et al., 1997; Skjånes et al., 2013; Yaakob et al., 2014). They can be produced in salt- and wastewater or be cultivated on land areas unsuited for other types of agriculture, making microalgae very promising as feed ingredients for future aquaculture production (Collotta et al., 2016; Marjakangas et al., 2015). The microalgae *Nannochloropsis oceanica* is a good source of PUFA, especially EPA, making it a promising feed ingredient for fish (Hulatt et al., 2017, Ma et al., 2014). Its is commonly used to grow rotifers for finfish hatcheries (Hemaiswarya et al., 2011). Inclusions of defatted *N. oceanica* have

been successfully tested in the diet of post-smolt Atlantic salmon at a modest inclusion level of 10 % with no adverse effects on fish health (Sørensen et al., 2017). Dietary inclusion of *N. oceanica* at a level of 30% in the diet of post-smolt Atlantic salmon has also shown promising results in terms of feed digestibility (Gong et al., 2018). Gbadamosi and Lupatsch (2018) found improved protein retention efficiency and a beneficial fatty acid profile in Nile tilapia (*Oreochromis niloticus*) fed *Nannochloropsis salina* replacing both fish and soybean products.

Spotted wolffish (*Anarhichas minor*) is a bottom-dwelling marine finfish, native to the North Atlantic and Barents Sea, with promising potential for cold-water aquaculture production (Foss et al., 2004). Countries like Canada, Norway, Iceland, Sweden and even Chile have been interested in production of spotted wolffish, but Norway is currently the only country with commercial production of wolffish, at Aminor AS (Halsa, Norway) with a current yearly concession of 500 tons (Aminor, 2018). Favorable traits of the spotted wolffish include high growth-rate, husbandry-suited behavior, late sexual maturation and ability to tolerate and adapt to a wide range of certain water quality parameters, such as salinities between 7 to 35‰, high unionized ammonia, hyperoxic conditions (100.5 mg O_2 kg^{-1} h^{-1}) and hypercapnia (Foss et al., 2001, Foss et al., 2003, Foss et al., 2004). The possibility to use formulated diets directly at start-feeding, reduces the costly and labor intensive use of live-feeds in the hatchery phase of many other marine species (Falk-Petersen et al., 1999). Spotted wolffish have greater potential in aquaculture compared to the closely related common wolffish (*Anarhichas lupus*) because it reaches slaughter weight of 3-4 kg after 3 years and has higher fillet-yield (Hansen & Falk-Petersen, 2001, Foss et al., 2004). As an added-value product, their skin can be tanned and used as exclusive leather that is stronger than most other leathers (RUBIN, 2001).

Spotted wolffish have been shown to tolerate replacement of up to 12% of fishmeal with the microalgae *Scenedesmus obliquus* (Knutsen et al., 2019). The results of the study showed that spotted wolffish had potential to tolerate alternative feed ingredients. However, the diets in the study of Knutsen et al. (2019) contained high amounts of fishmeal (68-80%). In the present study, we aimed to evaluate another microalgae species for spotted wolffish, *N. oceanica*, and also used a diet with higher inclusion of plant based ingredients in order to better mimic the present and future industrial standard for aquadiets. *N. oceanica* may have a greater potential in feed for marine species because of the high content of PUFA's. The aim of the present study was to investigate inclusion of graded levels (0, 7.5 and 15%) of the microalgae *N. oceanica* in a mixed plant- and fish-based diet for juvenile spotted wolffish. The diets were evaluated through (i) overall biometric gains and somatic indexes, (ii) blood plasma biochemistry (iii) fillet and whole body proximate chemical composition, (iv) fast muscle growth dynamics, and (v) fillet, liver and whole body fatty acid profile.

2. Materials and methods

The experiment was approved by the Animal Welfare Committee at FBA, Nord University and was conducted in accordance with the Norwegian animal welfare act (LOV-2009-06-19-97) and the regulation on the use of animals in research (FOR-2015-06-18-761). Animal sacrifice was limited to the minimum required to conduct the experiment in accordance with the principle of the 3R's (Russel et al., 1959).

2.1 Experimental diets and feeding experiment

Three isonitrogenous and isocaloric diets were formulated; one control diet (CTR) and two diets with low (N7.5) and high (N15) levels of microalgae (*N. oceanica*) replacing fishmeal and wheat in the diets. All three diets were formulated to have 40% of the protein sourced from fishmeal/algae and with the remaining 60% of the protein being of plant origin; wheat gluten, soy protein concentrate, pea protein concentrate and potato concentrate (Table 1). The diets were extruded into 5 mm pellets by Sparos Lda. (Olhão, Portugal). Prior to use, diets were stored at 4^{o}C and in the dark. During the course of the experiment, diets were stored at room temperature, in air-tight and light protected containers. The current study was carried out at Nord University research station, Mørkvedbukta (Bodø, Norway). Juvenile spotted wolffish (*Anarhichas minor*) with an initial mean weight of 625 ± 7.44 g, were provided by Aminor AS (Halsa, Norway). For each treatment, fish were randomly distributed into triplicate groups in 9 circular glass fiber tanks (45 fish per tank, 1 m^3). Initial stocking density of fish was 23 kg m^{-2} and this decreased to 21 kg m^{-2} towards the end of the experiment and was hence below the upper stocking density threshold of 40-60 kg m^{-2} for spotted wolffish suggested by Jonassen (2002). Water quality parameters was also within the recommendation from the literature for this species (Foss et al., 2001, Foss et al., 2003, Foss et al., 2004). Fish were supplied with flow through, filtered (200 µm) and aerated seawater (34 ‰) at stable temperature (7.49 ± 0.01 ^{o}C), oxygen (87.29 ± 0.13 %) and flow 1500 L/h, from 250 m depth in Saltenfjorden. Fish were kept under continuous light and fed the experimental diets in excess through automatic feeders at a feeding rate of 1.3 % body weight per day between 08:00 and 21:00 during the experiment. The feeding experiment lasted 12 weeks, from January 4th to April 4th 2017.

2.2 Sampling, growth data and biometrical data collection

Individual weight and length was recorded for all fish at week 0, 6 and 12 of the experiment to determine weight and length gain, growth rate and condition factor which was calculated following the formulas:

- Weight gain (WG, %) = ((final mean weight – initial weight)/initial weight) * 100;
- Specific growth rate (SGR, % day^{-1}) = 100 x ln[final mean weight (g) / initial mean weight (g)] / days;
- Condition factor (CF) = [fish weight (g) / total length $(cm)^3$] x 100.

At each sampling point, randomly selected fish from each tank (N = 13 per tank) were anaesthetized with MS222 (tricaine methanesulfonate, 0.14 g/L) and then sacrificed by cranial concussion. Blood samples were collected from all sacrificed fish immediately. Four of the fish per treatment were frozen whole, at -20°C, and later homogenized using a meat grinder for analysis of whole body proximate composition. For the other nine fish, the liver was removed, weighed for determination of hepatosomatic index and frozen at -20°C for later analyses of fatty acid profiles. Hepatosomatic index (HSI) was calculated as [liver weight (g) / fish weight (g)] x 100. From five of these fish, muscle fillets were removed, frozen and later homogenized using a meat mincer and subsequently analyzed for muscle proximate composition and fatty acid profiles. From the four remaining fish, muscle was sampled for analysis of white muscle cellularity (described in section 2.6). Feed samples were stored at -20°C and later homogenized for analyses of proximate composition, energy, fatty acid and amino acid profiles.

2.3 Blood sampling and plasma analysis

Blood samples were obtained by puncture of the caudal vessels using heparinized syringes and immediately centrifuged (4°C, 6000 rpm, 10 minutes) to obtain plasma. The plasma was subsequently snap-frozen and stored at -80°C until further analyses.

Plasma cortisol was analysed, in un-extracted plasma, using a radioimmunoassay described by Young, (1986), modified by Sundh et al. (2011) and using cortisol antibodies (code: S020; Lot: 1014-180182) purchased from Guildhay Ltd (no longer in business). Plasma glucose and lactate levels were measured using commercially available enzymatic kits (Sigma-Aldrich, St Louis, USA and Instruchemie, Delfzijl, The Netherlands), with protocols adapted to a 96-well microplate reader (Schram et al., 2010). Plasma osmolality was measured using a cryoscopic osmometer Advanced Model 3320 Micro-Osmometer4 (Advanced Instruments Inc., Norwood, USA). Deionized water (0 mOsmol/kg) and a standard solution (290 mOsmol/kg) were used as reference. Plasma pH and concentrations of electrolytes (Na^+, Cl^-, K^+ and Ca^+) were determined from 100 µl plasma samples using an electrolyte analyzer based on ion selective electrode technology (Convergys® ISE comfort Electrolyte Analyzer, Convergent Technologies, Cölbe, German) (Brijs, 2018).

2.4 Proximate chemical composition

All analyses of proximate chemical composition of feed, fish whole body and fillets were performed in duplicates. In short, moisture was determined by drying sample (5 g) to constant weight (105^{o}C, 20 hours). Ash was determined by burning sample (5 g) to constant weight (540^{o}C, 16 hours). Crude protein was determined using the Kjeldahl titration method (N x 6.25, Kjeltec^{TM} 2300, Foss, Sweden, Bradstreet, 1954), where protein is estimated from the nitrogen in the sample (Bradstreet, 1954, Kjeldahl, 1883). Crude fat was determined using the diethyl ester extraction method, according to the Norwegian Standard Association (1994). Gross energy of feed was determined using a bomb calorimeter (IKA C200 bomb calorimeter, Staufen, Germany).

2.5 Fatty acid and amino acid profile

Prior to fatty acid analysis, samples from feed, muscle, whole fish and liver were freeze dried (VirTis benchtop K Mod. 2KBTXL-75 with D2.5E Vac Pump, SP Industries, Warminster, USA) and homogenized. Of the freeze dried homogenate, N = 5 fish per tank for liver and muscle and N = 4 fish per tank for whole fish was pooled (100 mg per fish). All analysis was in addition performed in duplicates. Extraction of lipids was done according to Bligh & Dyer (1959) and hydrolysis of lipid as described by Metcalfe et al. (1966). After sample preparation all samples were frozen at -80 °C. Gas Chromatography analysis of fatty acids was automatically performed using a Scion 436-GC (Agilent Technologies, USA) and by reference to a known standard (Fame mix2, Absolute standards, Inc., USA). Fatty acids were measured by peak integration and expressed as relative area percentage on the total fatty acid area by using the software Compass CDS Bruker Co-operation chromatography data system (Scion Instruments, UK).

Amino acid profiles of the feed were analyzed at Eurofins Food & Feed Testing (Moss, Norway). In short, samples for tryptophan analysis were subjected to alkaline hydrolysis in barium hydroxide followed by separation by reversed phase C18 column HPLC. Free/bound tryptophan was determined by fluorescence detection (280/356 nm). Samples for cysteine and methionine analysis were oxidized with hydrogen peroxide and formic acid. Samples for analysis of the remaining amino acids were hydrolyzed in aqueous hydrochloric acid. Amino acids were then separated in an amino acid analyzer with an ion-exchange column. Detection of amino acids was performed employing a post column derivatization with ninhydrin reagent and measured at 440 and 570 nm.

2.6 Fast muscle cellularity

Workflow for sampling and analysis of fast muscle cellularity is illustrated in Fig. 1. Myotomal steaks (5 mm thickness) were cut prior to the posterior ventral fin and photographed for image analysis (ImageJ, NIH, USA) to measure white muscle total cross-sectional area (TCA). From the left side of each steak blocks of white muscle (n = 6, 5x5x5 mm) were prepared, covering the whole cross-sectional area. Muscle blocks were mounted on cork sheet (1.5x1.5 cm), covered in Cryomatrix (Shandon Cryomatrix, Thermo Scientific, USA) and frozen in liquid nitrogen for 45s in 2-methyl butane (isopentane, VWR chemicals, USA) to near the freezing point (159^{o}C) and thereafter stored at -80^{o}C. Sections of muscle (7 µm) were prepared from the blocks at -20^{o}C using a cryostat (Cryostar NX50, Thermo Scientific, USA) and stained with Hematoxylin (Merck Chemicals, Germany). Sections were imaged using a microscope (Axioskop2, Carl Zeiss, Germany) and camera (Axiocam HRC, Carl Zeiss, Germany). Area of a minimum of 800 individual fast muscle fibers was measured using the software AxioVision 4.8 (Carl Zeiss, Germany) and individual fast muscle fiber diameter was calculated.

From the distribution of muscle fibers, recruitment of new fibers (hyperplasia) was evaluated from the presence of fibers with diameter < 20 µm. Hypertrophy was evaluated from the average diameter of the 95th percentile of the muscle fiber population. Muscle fiber number (FN) was calculated from the formula FN = 10^6 x (TCA x number of counted fibers / sum area counted fibers). Muscle fiber density (FD) was calculated from the formula FD = 10^6 x (number of counted fibers / sum area counted fibers). Daily fiber recruitment was calculated from the formula ($FN_1 – FN_0$)/days, where FN_0 is the group-mean muscle fiber number at week 0 and FN_1 is the group-mean muscle fiber number at the end of the experiment.

2.7 Statistical analysis

Data were tested for normality by the Shapiro-Wilk test as well as from visual inspection using density histograms and qq-plots. Homogeneity of variance was tested using Levene's test. A one-way ANOVA was used for the statistical analysis of differences between groups of the whole dataset. When the ANOVA showed significant differences, the Tukey method of multiple comparisons was used among means. A Kruskal-Wallis one-way analysis of variance on ranks was used when the data did not meet the ANOVA assumptions. When the Kruskal-Wallis test showed significance, Dunn's method of multiple comparisons was used among medians. To study the changes taking place during the course of the experiment, individual groups were compared with week 0 using a t-test, if the assumptions for the test were met. A Welch test was used if the data did not have homogeneity of variance and a Mann-Whitney U test was used if the data was not normally distributed.

Distributions of white muscle fiber diameters were analyzed using nonparametric statistical techniques fitting smoothed probability density functions (pdfs) to the measurements using a kernel function (Bowman and Azzalini, 1997), as described by Johnston et al. (1999). N = 6 fish of equal total length (40.14 ± 0.24 cm) were selected from each treatment group and smoothed pdfs were fitted to the fish in each group as well as the group mean. A visual indication of potential significant differences in the white muscle fiber populations was created using bootstrap techniques for plotting approximate variability bands around the group pdfs using the mean smoothing parameter. In addition, a Kolmogorov-Smirnov two-sample test was used to test if the pdf of the treatment groups were equal over all diameters. Statistical analysis was performed using R-3.4.1 (R Core Team, 2017). Significant differences were considered when $p \leq 0.05$. Data are presented as means ± SEM.

3. Results

3.1 Experimental diets

All three experimental diets were isonitrogenous and isocaloric (Table 1). The diets were also balanced in terms of fiber and total phosphorous. The algae diets had higher ash and lower starch than the control diet. Water content was balanced between the control and N7.5 treatment, but was lower in the N15 treatment. Amino acid profiles of the three experimental diets (Table 2) were similar among diets, except for methionine and hydroxyproline which was higher in the algae diets than the control, and highest in the N15 diet. Fatty acid composition varied slightly among diets (Table 3). The algae diets especially N15, had higher amount of PUFAs compared to the control diet, likely due to the higher content of linoleic acid (C18:2n-6) and EPA. Fatty acids were overall well balanced among the diets, but the algae diets had slightly higher content of C16:1 and C16:0.

3.2 Biometrics

Biometrical data are presented in Table 4. The three treatment groups had overall similar performance throughout the experiment. At the end of the experiment the ANOVA showed no difference in mean body weight, length, CF, WG and SGR between the treatment groups ($p > 0.05$). The CF and HSI was significantly lowered for all groups at week 12 compared to the start of the experiment ($p < 0.05$). HSI was also significantly lower in the N7.5 group compared to the control group at week 12 ($p = 0.01$).

3.3 Plasma biochemistry

Plasma biochemistry is presented in Table 5. There were no differences in plasma cortisol or glucose levels either between sampling-points or between treatment groups ($p = 0.12$). Plasma lactate levels was not different between groups within each sampling point, but was lower for all groups at week 12 compared to week 6 ($p < 0.0001$). Osmolality was not different between groups at week 6, but at week 12 it was significantly higher in the control and N15 group as compared to the N7.5 group.

3.4 Proximate composition

The proximate composition of whole fish and muscle samples is presented in Table 6. When comparing with the start of the experiment, whole body crude protein was lower for the control and N7.5 group ($p = 0.02$ for both) at week 6. At week 12 there were no differences in whole body crude protein among dietary groups, compared to week 0, but the N7.5 group had lower whole body crude protein compared to the control group ($p = 0.02$). Whole body crude lipid was unaltered both among treatment groups and over time of the experiment. Interestingly, muscle crude lipid increased for all groups at the end of the experiment although no difference among treatments was observed. Whole body ash was also lower in the N7.5 group compared to the N15 group at week 12 ($p = 0.004$). No differences were observed in muscle ash or moisture nor in whole body moisture.

3.5 Fast muscle cellularity

Fast muscle cellularity is presented in Table 7. There was no difference in TCA among treatments at week 12, but both algae-groups had significantly higher TCA at termination compared to the start of the experiment ($p = 0.02$ for both). No differences in fiber number among treatment groups were observed, but the control group had lower fiber number at week 12, compared to week 0 ($p = 0.048$). Although not significant, the N7.5 group also had a slightly lower fiber number compared to week 0 while those fed N15 had higher fiber number compared to week 0. Fiber densities were similar among groups at week 12, but all groups had significantly lower fiber density at week 12 compared to week 0 ($p = 0.01$, $p = 0.01$ and $p = 0.04$ for the control, N7.5 and N15 group, respectively). Mean diameter of the 95^{th} percentile, 90^{th} percentile and 75^{th} percentile had increased for all feeding groups at week 12 ($p < 0.05$), but mean diameter increased only for the N7.5 and N15 groups ($p = 0.01$ and $p = 0.04$ respectively). All feeding groups showed decreased proportion of fibers with $D \leq 20$ µm and increased proportion of fibers with $D > 200$ µm at the end of the experiment ($p < 0.05$). The

probability density function (Figure 2) showed no difference in muscle fiber distributions among the feeding groups.

3.6 Fatty acid composition of whole body, liver and muscle

Fatty acid composition of muscle, liver and whole body are presented in Table 8. The fatty acid profile differed slightly among the different tissues. Fatty acid composition of whole fish was dominated by monounsaturated fatty acids, of which C18:1n-9 accounted for 73-74%. The sum of saturated fatty acids was slightly reduced from 18% to approximately 16%; sum PUFA's increased, while EPA+DHA were in the same range during the course of the experiment. Compared to whole body, muscle fillet contained less mono saturated fatty acids and they were less dominated by C18:1n-9. Moreover, the sum of PUFAs was 50-60% higher, and sum of EPA and DHA were in the range 43-59% higher in fillet compared to the whole body. Fatty acid profile of the liver was more similar to the fillet, but with lower content of the sum of PUFAs as well as the sum of EPA and DHA. An increased content of C18:2n-6 was observed in muscle ($p = 0.02$, $p = 0.01$ and $p = 0.01$ for CTR, N.7.5 and N15 respectively), liver ($p = 0.01$, $p = 0.0003$ and $p = 0.001$ for CTR, N.7.5 and N15 respectively) and whole fish ($p = 0.02$, $p = 0.0005$ and $p = 0.003$ for CTR, N.7.5 and N15 respectively) during the course of the experiment.

At termination of the experiment, there were only marginal differences in muscle fatty acid composition among the different feeding groups. The saturated fatty acid C14:0 decreased over time in both the algae groups ($p = 0.02$ and $p = 0.04$ for N7.5 and N15 respectively) and C16:0 also decreased with time in N7.5 ($p = 0.045$). The monounsaturated fatty acid C22:1n-9 also decreased in the muscle of the algae groups with time ($p = 0.03$ and $p = 0.01$ for N7.5 and N15, respectively).

In the liver, sum of saturated fat was reduced in all treatment groups at termination of the experiment. Liver content of the saturated fatty acid C14:0 was reduced for all groups at week 12 ($p = 0.004$, $p = 0.001$ and $p = 0.005$ for CTR, N.7.5 and N15 respectively). The saturated fatty acid C16:0 was reduced in liver for all groups at week 12 ($p = 0.002$, $p = 0.0004$ and $p = 0.01$ for CTR, N.7.5 and N15 respectively) and was also lower in N7.5 compared to N15 at week 12 ($p = 0.02$). The saturated fatty acid C19:0 was higher in liver of the algae-fed fish compared to the control at week 12 ($p = 0.039$ and $p = 0.046$ for N7.5 and N15 respectively). The monounsaturated fatty acid C16:1 was higher in the control and N7.5 groups at week 12 compared to the start of the experiment ($p = 0.04$ for both treatments). The sum of monounsaturated fatty acids in the liver were only lower at the terminal sampling compared to the start of the experiment, in the N7.5 group ($p = 0.049$). The monounsaturated fatty acids C20:1n-9 and C22:1n-9 were reduced in the liver of all groups during the course of the experiment

($p < 0.05$). In the N7.5 group, the sum of PUFA's ($p = 0.03$) as well as the fatty acid C22:5n-3 ($p = 0.04$) had increased in the liver at week 12 compared to the start of the experiment.

Whole body C14:0, C16:0 and C18:0 was reduced for all groups at termination of the experiment compared to week number 0 ($p < 0.05$). Although no difference was observed among groups, whole body C16:1 was reduced in both algae groups at week 12 compared to week 0 ($p = 0.023$ and $p = 0.049$ for N7.5 and N15 respectively). Whole body C18:1n-7 was also higher in N7.5 compared to the control group ($p = 0.02$) and C22:1n-9 increased with time for both algae groups ($p = 0.001$ and $p = 0.02$ for N7.5 and N15 respectively). The PUFA 22:5n-3 increased over time in whole body samples for all treatment groups ($p = 0.04$, $p = 0.01$ and $p = 0.03$ for CTR, N7.5 and N15 respectively) and whole body 20:5n-3 was increased in the algae groups at the end of the experiment ($p = 0.015$ and $p = 0.046$ for N7.5 and N15 respectively).

4. Discussion

In the present experiment, all three experimental groups had similar overall performance. Total weight increase was on average 26% (625– 794 g) during the course of the experiment with an average SGR of 0.26%. This is slightly lower than the growth rates reported by Jonassen (2002), who found SGR values of 0.41-0.47% in wolffish growing from 450-850 g. Knutsen et al (2019) reported an average SGR of 0.66% in wolfish growing from 140-250 g. Within a species, growth rates are expected to decrease with size (Ricker, 1979), so the smaller fish (140-250 g) used in the study of Knutsen et al. (2019) are not directly comparable to growth rate observed in the present experiment.

The plasma levels of cortisol in the experimental fish were within the range considered normal for spotted wolffish of this size (~15-30 nmol/L) (Lays, 2009, Le François, 2013). In this species, stress have been reported to elicit lower plasma cortisol responses than shown in most other teleosts. Acute and severe disturbances such as air exposure or hypoxia, elicit increases of up to 80-95 nmol/L in the spotted wolffish (Lays, 2009, Le François, 2013), whereas in other teleosts, rapid responses above 276 nmol/L are generally considered to reflect an acute and severe stress response and increases up to 166 nmol/L are referred to as a mild stress response (Wendelaar Bonga, 1997). Similarly, basal plasma glucose levels in spotted wolffish are relatively low (0.3-0. 4 mmol/L (Lays, 2009) and comparable to species with a sedentary lifestyle and low locomotor activity (< 1 mmol/L) (Vijayan, 1994). The stress response is known to differ considerably between teleost species, depending on the ecotype and lifestyle. Benthic, sedentary species with a low metabolic rate, such as the spotted wolffish, tend to have a lower

response than pelagic and active species such as salmonids (Barton, 1991, Vijayan, 1994). The low levels of plasma cortisol and glucose observed in our study and the absence of differences between treatments both suggest that the partial substitution of a fish-based diet with microalgae did not impose stress.

We did not observe any major differences between the three treatments at weeks 6 and 12 regarding plasma pH, osmolality and ion concentrations of Na^+, K^+, Cl^- and Ca^{2+}. Plasma pH, osmolality and ionic concentrations of Na^+, Cl^- and K^+ are within the range found for this species (Foss, 2001, Imsland, 2009, Magnussen, 2008, Tremblay-Bourgeois, 2010). Plasma concentrations of Ca^{2+} have not been measured to date to our knowledge for wolffish species but seem to be relatively low compared to other marine species (Abbink, 2004, Person-le Ruyet, 2003, Sala-Rabanal, 2003). We observed a slight decrease of plasma Ca^{2+} with increasing inclusion levels of microalgae. We also observed an increase in osmolality, Na^+ and Cl^- for each treatments between weeks 6 and 12. Nevertheless, these values are still within the normal range for this species in similar rearing conditions (Na^+: 127-186 mM, Imsland, 2009, Magnussen, 2008, Tremblay-Bourgeois, 2010; Cl^-: 140-164 mM, Foss, 2001, Magnussen, 2008; K^+: 2,60-4,56 mM, Imsland, 2009, Tremblay-Bourgeois, 2010; osmolality: 320-356 $mOsmolkg^{-1}$, Foss, 2001, Magnussen, 2008, and pH: 7,12-7,42, Imsland, 2009) and those differences are probably not biologically relevant. Similarly, we observe lower plasma lactate at week 12 in all groups, compared to week 6 and no difference between treatments at week 6 and 12 are seen. These differences in plasma concentration between weeks 6 and 12 could be linked to the differences in osmoregulatory capacity seen between the two sampling points, as lactate is used as an important metabolic fuel of the chloride cells, involved in osmoregulation (Perry and Patrick, 1989). To our knowledge, it is the first time plasma concentrations of lactate are measured in spotted wolffish. As for glucose and cortisol, the values obtained for lactate are once again very low in comparison with active pelagic fish, such as the yellowtail kingfish, *Seriola lalandi* (Blanco Garcia, 2015), and comparable to resting levels of benthic fish, such as turbot *Scophthalmus maximus* (Pichavant, 2002), and are consistent with the relatively quiet lifestyle of spotted wolffish.

The reduction in CF during the course of the experiment coincided with a reduction in HSI. Knutsen et al. (2019) also reported a reduction in HSI over time when feeding spotted wolffish the microalgae *Scenedesmus obliquus*. The reduction in HSI could be explained by modulated lipid metabolism and increased need for lipid to deposit in the muscle associated with growth of the fish. There were no changes in whole body crude lipid with the growth of the fish, while muscle crude lipid increased for all groups. These findings strongly suggest that lipids stored in the liver were mobilized and deposited in the muscle. Increased muscle crude lipid can be explained by the life stage of the fish, since no difference in crude lipid was observed between groups at week 12 of the

experiment. Spotted wolffish of 250 g have about 5% muscle crude lipid (Knutsen et al., 2019), while the 794 g fish in the present experiment had 9.6% muscle crude lipid; it is likely that spotted wolffish incorporate more lipid in the muscle as they increase body mass. The lower HSI observed for the algae-fed groups compared to the control treatment could also be explained by slightly lower fat content in the experimental diets, 16.6 and 17.3% fat in N7.5 and N15 respectively, compared to the commercial diet which the fish were provided prior to the experiment, 18% fat (Amber Neptun 5, Skretting, Norway). A similar pattern is seen in farmed Atlantic cod (*Gadus morhua*) where an increased lipid level in the feed resulted in increased HSI (Hansen et al., 2008; Kjær et al., 2009). The reduction in HSI may also be explained by the increasing incorporation of microalgae in the two experimental diets, per se. Reduced HSI has been found for Atlantic cod fed high inclusions of microalgae (30%) in the diet and for European seabass (*Sparus aurata*) fed the microalgae *Tetraselmis suecica* replacing 20% of the dietary fishmeal (Tulli et al., 2012, Walker and Berlinsky, 2011). Tulli et al. (2012) suggested that this could be related to the lowered apparent digestibility coefficient of the microalgae diet but also that it could be related to microalgae compounds with possible modulating effects on lipid metabolism. Other studies (e.g. Walker and Berlinsky, 2011) have suggested that a reduction in HSI was caused by depletion of stored hepatic lipids as a response to starvation. However, Walker and Berlinsky (2011) observed difference in HSI of 4.18 between the control treatment (HSI = 6.99) and highest algae treatment (HSI = 2.18). This was far greater than the observed difference of 0.21 between the control and highest algae treatment in the present experiment. It is also far greater than the observed difference of 0.85 between week 0 and the mean HSI of all groups at week 12 in the present experiment. Other experiments replacing fishmeal with microalgae observed no difference in HSI for red drum (Patterson and Gatlin, 2013) and Atlantic salmon (Sørensen et al., 2017). High protein feeds (>50%) have generally been used for spotted wolffish (Foss et al. 2004), and the present diets were formulated according to this. Papoutsoglou and Lyndon (2006) successfully reared spotted wolffish on a lower protein diet (38%). However, the diets in that study had higher energy (23 MJ/kg) compared to the present experiment (21 MJ/kg), providing the fish with more energy to efficiently utilize the protein.

All three diets were isoenergetic, isonitrogeneous and had nearly identical amino acid profiles. Despite this, muscle crude protein was reduced in both the algae-fed groups and whole body crude protein was also lower in the N7.5 group, at week 12, compared to the control. This could indicate a lower capacity of the fish to absorb and utilize microalgae-protein due to the complex carbohydrates in the cell-walls of microalgae that may be hard to digest for carnivorous fish (Domozych et al., 2012; Krogdahl et al., 2005, Teuling et al. 2017, Teuling et al. 2019).

The increased content of linoleic acid (C18:2n-6) during the course of the experiment in all dietary treatments and in both muscle, liver and whole body is most likely reflecting a general increase in lipid deposition. It is well known that replacement of fishmeal and fish oil with plant based ingredients is generally reflected in the composition of fish tissue lipids, resulting in reduced levels of ω-3 (PUFAs) and increased levels of linoleic acid (C18:2n-6) (Watanabe, 1982). Overall, there were few changes in the fatty acid composition among fish fed the different experimental diets, and the two nutritionally important fatty acids EPA and DHA seemed to be conserved in all tissues during the course of the experiment. The EPA content even significantly increased in the whole body samples from fish fed the microalgae diets during the course of the experiment. *N. oceanica* is an EPA-rich algae, probably explaining the increased retention of EPA (Hulatt et al., 2017, Ma et al., 2014). Improved retention of essential fatty acids are previously reported in studies with Atlantic salmon fed diets where fish oil was replaced by *Schizochytrium* sp. (Kousoulaki et al., 2015, Kousoulaki et al., 2016). The retention of PUFAs in fish tissues is important both for the health of the fish and for the nutritional quality of fish in the human diet (Bou et al., 2017, Mozaffarian & Rimm, 2006). Although traditionally considered less nutritionally important, ω-3 fatty acid, docosapentaenoic acid (DPA, C22:5n-3) was increased in whole body samples of all groups and in the liver of the N7.5 group. Though present in low concentration, literature has indicated that DPA may have important biological functions (Kaur et al., 2011). *N. oceanica* has high levels of EPA but has also high levels of saturated and monounsaturated fatty acids (Draaisma et al., 2013; Hulatt et al., 2017). This was not reflected in either muscle, whole body or liver samples, where the level of total saturated fatty and monounsaturated acids was either unchanged or decreased in all groups and tissues.

As only slight differences were observed between the dietary treatment groups, the results do not indicate any negative effects on fast muscle cellularity by replacing fishmeal with microalgae for spotted wolffish. Overall, the number of fast muscle fibers did not change over the duration of the experiment, apart from in the control group where the numbers decreased slightly with time. This was surprising as the growth pattern of juvenile fish is expected to be dominated by mosaic hyperplasia in addition to hypertrophy, as shown for several other teleost species (Stickland, 1983, Weatherly et al., 1988, Zimmerman and Lowery, 1999). In general, teleost fish cease recruiting new fibers at a size of about 44% of the final body length (Weatherly et al., 1988). Studies have also recorded a cessation of hyperplasia at a much earlier point than 44% of final length in Antarctic living species (Johnston et al., 2003). Therefore, it is not unlikely that the recruitment of new fibers in the present experiment could cease at mean length 40 cm, 22% of the spotted wolffish's maximal adult length of 180 cm (Bakketeig et al., 2017). However, despite the unexpected low fiber number, fibers

525 < 20 µm increased in all groups at week 12, indicating a small contribution of
526 hyperplastic growth.

527 Studies on Atlantic salmon have shown that within strains of the same species a large
528 variation can exist in fiber number and density (Johnston et al., 2000). Furthermore,
529 Atlantic halibut (*Hippoglossus hippoglossus*), have been shown to cease fiber
530 recruitment in periods of poor growth (Hagen et al., 2006). The relatively low weight
531 gain in the present study can thus contribute and partially explain the lack of apparent
532 mosaic hyperplasia. In addition, halibut has the ability to grow larger fast fibers that
533 other more pelagic species and is most likely linked with the activity level, fish swimming
534 behavior and diffusion constraints (Johnston et al., 2004; Johnston et al. 2005).
535 Unusually large muscle fibers have also been documented for another benthic fish
536 species, common Dab (*Limanda limanda*; Hurling et al., 1996) as well as for the
537 Notothenioid fishes (fibers > 700 µm) which is a group of fishes living in the cold waters
538 of the sub-Antarctic (Johnston et al., 2003). Spotted wolffish had on average a maximum
539 fiber diameter of 317 µm at the end of the experiment, but for individual fish fiber
540 diameters over 430 µm were observed. Overall, the fast muscle fiber growth was
541 dominated by hypertrophy for all groups, this was reflected in the absence of increase
542 in fiber number, increase in fibers with diameter > 200 µm and increased D mean of 95th
543 percentile. Thus, having a subarctic and benthic lifestyle, it is possible that wolffish also
544 produce relatively large fibers and lower maximum fiber number similar to that of
545 halibut, Dab and Notothenioid fishes.

546

547 # 5. Conclusion

548 No distinct negative effects were observed when spotted wolffish were fed diets where
549 up to 15% of the fishmeal and wheat was replaced with *N. oceanica*. From the plasma
550 analysis there were no indications of stress as an effect of the diets. The observed
551 reduction in HSI of the algae-fed fish as well as constant whole body lipid indicates a
552 possible modulating effect of microalgae on lipid metabolism of spotted wolffish. Level
553 of linoleic acid (C18:2n-6) as well as PUFAs in general increased in both muscle, liver and
554 whole body of all treatment groups, reflecting the use of about 50% plant-based
555 ingredients in the diets. The ω-3 fatty acids were either conserved over the course of
556 the experiment, or increased in the algae-fed groups, indicating a positive effect of *N.*
557 *oceanica* on retention of PUFA's in spotted wolffish. There was no difference between
558 groups in fast muscle cellularity, and growth in all groups were dominated by
559 hypertrophy. Surprisingly, the number of fast muscle fibers did not increase over the
560 duration of the experiment. The results indicate that wolffish might have a similar
561 muscle fiber growth pattern as other fishes with a sub-arctic and benthic life history,

such as halibut, Dab and Notothenioid fishes, by producing relatively large fibers and a lower maximum fiber number.

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788 **Figure 1**

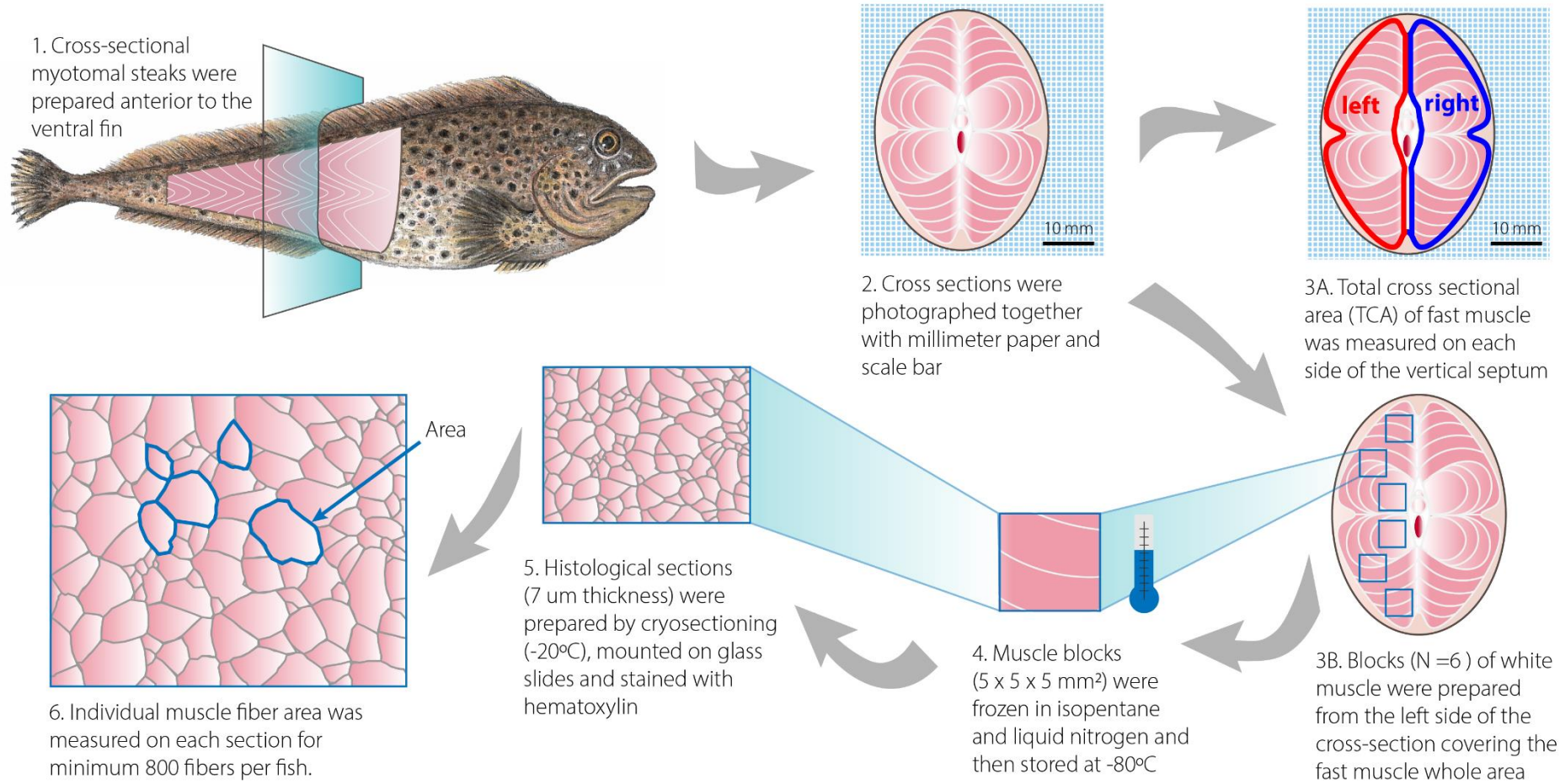

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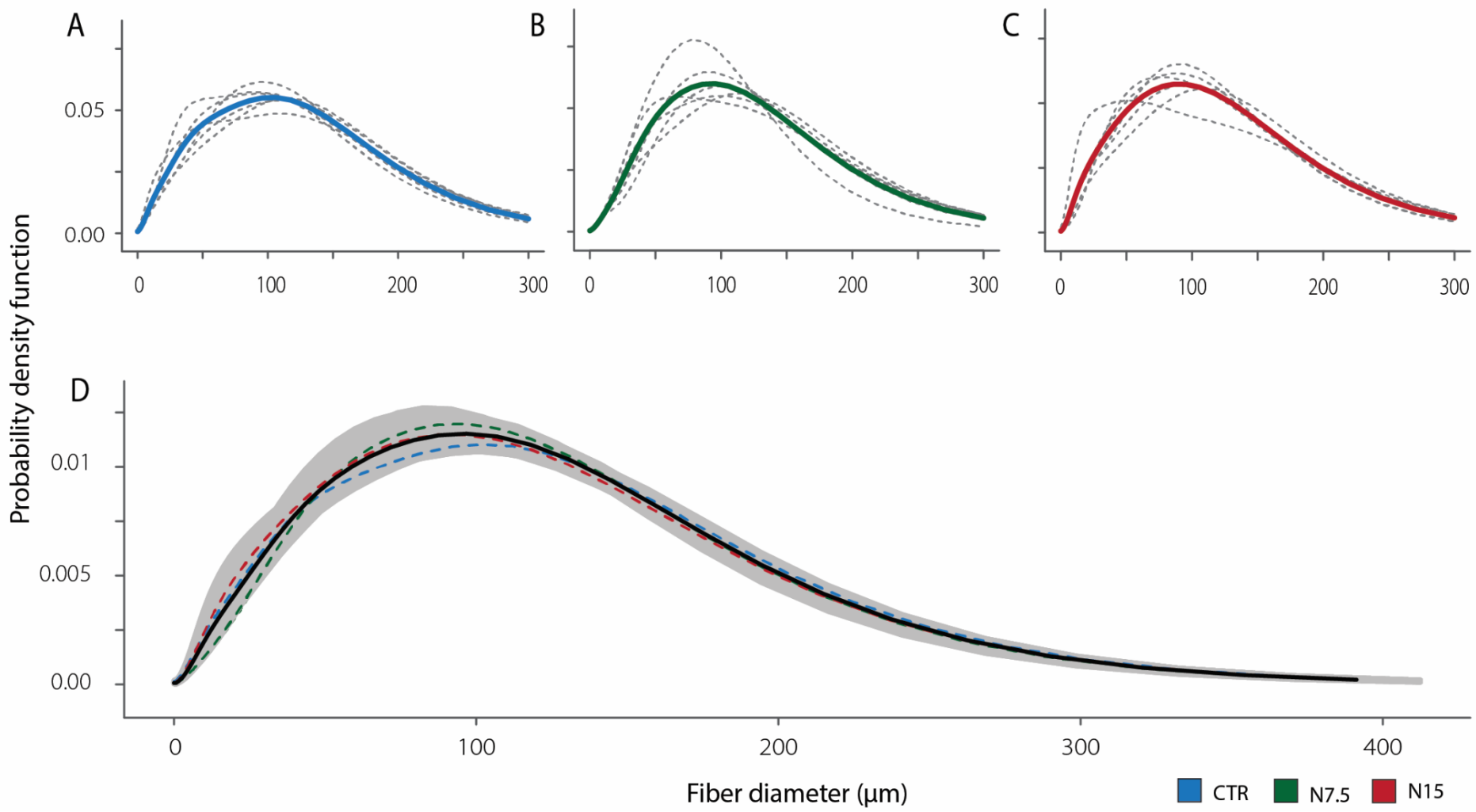
Figure 2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Probability density function
Fiber diameter (µm)
CTR
N7.5
N15

Table 1: Ingredients (g 100 g^{-1} diet) and proximate composition [%] of the experimental diets containing different levels of microalgae (*Nannochloropsis oceanica*) as a replacement for fishmeal.

	Treatment diet		
	CTR	N7.5	N15
Ingredients (g 100 g^{-1} diet)			
Nannochloropsis oceanica[1]	0.0	7.5	15.0
Fishmeal LT70[2]	30.0	26.3	22.5
Fish oil[3]	13.0	13.0	13.0
Wheat meal[4]	14.3	10.6	6.8
Soy protein concentrate[5]	12.0	12.0	12.0
Pea protein concentrate[6]	12.0	12.0	12.0
Potato concentrate[7]	12.0	12.0	12.0
Wheat gluten[8]	5.0	5.0	5.0
Antioxidant powder[9]	0.2	0.2	0.2
MCP[10]	0.5	0.5	0.5
Micro-ingredients[11]	1.0	1.0	1.0
Proximate composition of diets (g 100 g^{-1})			
Crude fat	15.9	16.6	17.3
Crude protein	53.3	52.9	52.5
Fiber	1.2	1.1	1.0
Starch	9.3	8.0	7.7
Ash	6.2	7.3	8.5
Total phosphorus	1.1	1.0	0.9

Water	8.4	8.8	5.4
Energy (KJ g^{-1})	21.7	21.1	21.3

Treatment diets: CTR: control. N7.5: 7.5% *N. oceanica* inclusion. N15: 15% *N. oceanica* inclusion.

[1] Protein: 45.7%; lipid: 9.1%; carbohydrates: 15.6%; dietary fiber: 15.8%; ash: 8.3%; moisture: 5.6%; energy: 1.5 MJ g^{-1}; pigments: 2.056% chlorophyll, 0.607% total carotenoids (Allma, Lisbon, Portugal).

[2] Protein: 70%; lipid: 5.8% (Sopropeche, France).

[3] SAVINOR UTS, Portugal.

[4] Protein: 11.7%; lipid: 1.6% (Casa Lanchinha, Portugal).

[5] Soycomil-P. Protein: 63%, lipid: 0.9% (ADM, The Netherlands).

[6] NUTRALYS F85F. Protein: 78%, lipid: 1% (ROQUETTE Frères, France).

[7] Prostar. Protein: 81%, Lipid: 3.1% (AVEBE, The Netherlands).

[8] VITAL. Protein: 83.7%, Lipid: 1.4% (ROQUETTE Frères, France).

[9] Paramega PX (KEMIN EUROPE NV, Belgium).

[10] Monocalcium phosphate. Phosphorus: 22%, Calcium: 16% (Fosfitalia, Italy).

[11] Vitamin & Mineral Premixi: Vitamins (IU or mg kg-1 diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg-1 diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings (PREMIX Lda, Portugal.

812 **Table 2:** Amino acids (g 100 g^{-1} diet) of the experimental diets containing different levels of microalgae (*Nannochloropsis oceanica*) as a replacement for
813 fishmeal.

	Treatment diet		
Amino acid	CTR	N7.5	N15
Asparagine	5.15	5.07	5.24
Serine	2.49	2.44	2.53
Glutamic acid	8.60	8.36	8.46
Proline	2.99	2.99	3.26
Glycine	3.14	3.06	3.16
Alanine	2.68	2.66	2.77
Valine	2.50	2.48	2.57
Isoleucine	2.19	2.17	2.21
Leucine	4.02	3.99	4.06
Tyrosine	1.85	1.82	1.89
Phenylalanine	2.54	2.45	2.52
Histidine	1.09	1.06	1.08
Lysine	3.54	3.40	3.40
Arginine	3.21	3.13	3.21
Tryptophan	0.516	0.557	0.584
Cysteine	0.591	0.577	0.589
Methionine	0.998	1.19	1.26
Hydroxyproline	0.278	0.336	0.634
Ornitine	<0.05	<0.05	<0.05
Treonine	2.15	2.12	2.19

814 **Table 3:** Fatty acids (g 100 g^{-1} diet) of the experimental diets containing different levels of microalgae (*Nannochloropsis oceanica*) as a replacement for
815 fishmeal.

	Treatment diet		
Fatty acid	CTR	N7.5	N15
C14:0	5.25 ± 0.08	5.43 ± 0.05	5.57 ± 0.05
C16:0	19.76 ± 0.12	20.49 ± 0.25	20.91 ± 0.05
C18:0	5.05 ± 0.02	5.14 ± 0.14	5.23 ± 0.07
C16:1	6.24 ± 0.08	7.23 ± 0.04	8.42 ± 0.06
C18:1n-11	15.87 ± 0.18	15.63 ± 0.20	15.98 ± 0.00
C18:1n-9	3.49 ± 0.11	n.a	n.a
C20:1n-9	5.08 ± 0.41	5.17 ± 0.45	n.a
C22:1n-9	5.46 ± 0.07	5.52 ± 0.03	5.43 ± 0.06
C18:2n-6	8.41 ± 0.03	9.42 ± 0.10	9.87 ± 0.11
C20:5n-3 (EPA)	10.19 ± 0.01	11.18 ± 0.19	13.44 ± 0.02
C22:6n-3 (DHA)	15.18 ± 0.14	14.80 ± 0.16	15.16 ± 0.10
∑ EPA+DHA	25.37 ± 2.49	25.98 ± 1.81	28.6 ± 0.86
∑ Saturated	30.06 ± 4.87	31.06 ± 5.02	31.71 ± 5.17
∑ Monounsaturated	36.14 ± 2.21	33.55 ± 2.46	29.83 ± 3.14
∑ Polyunsaturated	33.78 ± 2.03	35.4 ± 1.58	38.47 ± 1.56
n-6/n-3	1/3	1/3	1/3

816

Table 4: Survival, sex, weight, length, condition factor (CF), hepatosomatic index (HSI), total weight gain (WG) and daily growth (DG) of spotted wolffish fed diets with different level of inclusion of microalgae (*Nannochloropsis oceanica*). Weight, length, WG, DGI and CF for week 0 and week 12 are based on measurements of all fish. All other values are based on sampled fish. Values are means ± SEM. Means in the same column at the same time point with different subscript letter differs significantly ($p < 0.05$). Means in the same column with subscript * differ significantly from the start of the experiment ($p < 0.05$).

Time	Diet	Survival [%]	Sex [♂/♀]	Body weight [g]	Body length [cm]	CF	HSI	WG [%]	SGR [% day^{-1}]
Week 0	Start	n.a	$19_{♂}$ / $17_{♀}$	624.79 ± 7.45	36.08 ± 0.13	1.31 ± 0.01	4.29 ± 0.08	n.a	n.a
Week 6	CTR	100	$13_{♂}$ / $14_{♀}$	758.36 ± 15.99*	38.74 ± 0.25*	1.29 ± 0.02*	4.02 ± 0.07^{a}*	21.57 ± 2.13^{a}	0.41 ± 0.04^{a}
	N7.5	100	$17_{♂}$ / $10_{♀}$	750.74 ± 15.84*	38.73 ± 0.23*	1.27 ± 0.01*	3.63 ± 0.09^{b}*	19.03 ± 1.09^{a}	0.37 ± 0.02^{a}
	N15	100	$15_{♂}$ / $12_{♀}$	750.24 ± 17.23*	38.60 ± 0.27*	1.28 ± 0.01*	3.66 ± 0.07^{b}*	20.51 ± 0.54^{a}	0.40 ± 0.01^{a}
Week 12	CTR	96.6	$15_{♂}$ / $12_{♀}$	785.21 ± 20.72*	39.98 ± 0.35*	1.22 ± 0.02^{a}*	3.60 ± 0.07^{a}*	26.09 ± 5.32^{a}	0.25 ± 0.07^{a}
	N7.5	100	$19_{♂}$ / $8_{♀}$	804.69 ± 21.48*	40.18 ± 0.30*	1.22 ± 0.01^{a}*	3.32 ± 0.06^{b}*	27.42 ± 3.10^{a}	0.27 ± 0.03^{a}
	N15	94.9	$15_{♂}$ / $12_{♀}$	791.34 ± 22.51*	39.91 ± 0.35*	1.22 ± 0.01^{a}*	3.39 ± 0.07^{ab}*	26.69 ± 7.72^{a}	0.26 ± 0.05^{a}

Table 5: Plasma biochemistry of spotted wolffish fed diets with different level of inclusion of microalgae (*Nannochloropsis oceanica*). Values are means ± SEM. Means in the same column at the same time point with different subscript letter differ significantly ($p < 0.05$).

	Week 6			Week 12		
	CTR	N7.5	N15	CTR	N7.5	N15
Cortisol (mmol/L)	12.62 ± 2.35	8.39 ± 1.68	12.57 ± 2.04	16.78 ± 2.68	13.80 ± 2.92	12.58 ± 2.13
Glucose (mmol/L)	0.65 ± 0.02	0.64 ± 0.02	0.62 ± 0.02	0.59 ± 0.02	0.65 ± 0.03	0.63 ± 0.03
Lactate (mmol/L)	0.71 ± 0.05	0.86 ± 0.07	0.68 ± 0.05	0.41 ± 0.05	0.43 ± 0.03	0.47 ± 0.04
Osmolality (mOsmol/kg^{-1})	353.22 ± 1.47	354.22 ± 1.53	353.43 ± 1.95	369.95 ± 2.16^{a}	358.51 ± 1.73^{ab}	362.92 ± 1.63^{b}
Cl^- (mmol/L)	134.98 ± 1.36	136.47 ± 2.41	135.66 ± 1.62	139.63 ± 0.92	139.28 ± 0.79	137.88 ± 0.83
Na^+ (mmol/L)	182.37 ± 2.78	183.74 ± 2.09	180.02 ± 2.21	190.33 ± 1.15	191.49 ± 1.26	189.06 ± 0.95
K^+ (mmol/L)	4.10 ± 0.08	4.25 ± 0.10	4.12 ± 0.06	3.83 ± 0.07^{a}	4.06 ± 0.06^{b}	3.92 ± 0.05^{ab}
Ca^{2+} (mmol/L)	1.00 ± 0.02^{a}	0.97 ± 0.03^{ab}	0.91 ± 0.01^{b}	1.05 ± 0.01^{a}	1.01 ± 0.01^{b}	0.97 ± 0.01^{b}
pH	7.29 ± 0.02	7.26 ± 0.02	7.21 ± 0.09	7.29 ± 0.01^{a}	7.30 ± 0.00^{ab}	7.32 ± 0.01^{b}

Table 6: Muscle and whole fish proximate composition [%] of spotted wolffish fed diets with different level of inclusion of microalgae (*Nannochloropsis oceanica*). Values are means ± SEM. Means in the same column at the same time point with different subscript letter differ significantly ($p < 0.05$). Means in the same column with subscript * differ significantly from the start of the experiment ($p < 0.05$).

		Crude protein		Crude lipid		Ash		Moisture	
Time	Diet	*Whole fish*	*Muscle*	*Whole fish*	*Muscle*	*Whole fish*	*Muscle*	*Whole fish*	*Muscle*
Week 0	Start	14.33 ± 0.06	16.80 ± 0.10	9.66 ± 0.25	8.36 ± 0.28	1.38 ± 0.02	1.20 ± 0.01	73.94 ± 0.21	73.16 ± 0.22
Week 6	CTR	14.05 ± 0.11*	16.33 ± 0.12*	9.89 ± 0.12	9.29 ± 0.28*	1.34 ± 0.03	1.19 ± 0.03	73.92 ± 0.18	72.85 ± 0.21
	LOW	14.06 ± 0.10*	16.34 ± 0.12*	9.83 ± 0.16	9.43 ± 0.34*	1.35 ± 0.04	1.22 ± 0.02	74.11 ± 0.23	72.79 ± 0.29
	HIGH	14.24 ± 0.10	16.11 ± 0.12*	9.89 ± 0.29	10.07 ± 0.21*	1.36 ± 0.03	1.26 ± 0.03*	73.65 ± 0.35	72.34 ± 0.19*
Week 12	CTR	14.62 ± 0.16^{a}	16.52 ± 0.14	9.73 ± 0.24	9.48 ± 0.40*	1.36 ± 0.03^{ab}	1.32 ± 0.04*	73.61 ± 0.37	72.55 ± 0.32
	LOW	14.12 ± 0.11^{b}	16.28 ± 0.10*	9.47 ± 0.26	9.58 ± 0.24*	1.27 ± 0.04^{a}*	1.24 ± 0.03	74.53 ± 0.25	72.90 ± 0.20
	HIGH	14.43 ± 0.10^{ab}	16.33 ± 0.12*	9.80 ± 0.21	9.62 ± 0.34*	1.42 ± 0.03^{b}	1.34 ± 0.05	73.94 ± 0.29	72.33 ± 0.34*

Table 7: Fast muscle cellularity of spotted wolffish fed diets with different levels of inclusion of microalgae (*Nannochloropsis oceanica*). Values are means ± SEM. Means in the same row with different superscript letters differ significantly ($p < 0.05$). Significance is presented from analysis of data normalized by total length (TL). For this normalization, parameters increasing with increasing TL were divided by ln TL and parameters decreasing with increasing TL were multiplied by ln TL. Means in the same row at the same time point with different subscript letter differs significantly ($p < 0.05$). Means in the same column with subscript * differ significantly from the start of the experiment ($p < 0.05$).

Time	Week 0	Week 12		
	Start	CTR	LOW	HIGH
TCA	2041.0 ± 76.5	2271.1 ± 55.49	2369.5 ± 89.03*	2347.4 ± 94.52*
Fiber number	158703 ± 12826	151173 ± 6261*	154089 ± 9118	163561 ± 7307
Fiber density [fibers mm^{-2}]	84.1 ± 4.12	67.1 ± 3.45*	65.4 ± 3.97*	71.0 ± 4.68*
95^{th} percentile	198.2 ± 5.46	222.1 ± 4.64*	228.5± 6.27*	221.5± 6.65*
D mean of upper 95^{th} percentile	179.7 ± 4.76	246.0 ± 5.02*	257.2 ± 8.19*	247.9 ± 7.18*
90^{th} percentile	121.3 ± 7.93	200.4 ± 4.64*	203.9 ± 5.33*	199.0 ± 6.08*
D mean of upper 90^{th} percentile	204.0 ± 5.58	228.1 ± 4.80*	235.4 ± 6.57*	228.2 ± 6.68*
75^{th} percentile	150.2 ± 4.08	167.5 ± 4.91*	168.3 ± 5.06*	161.2 ± 4.79
D mean of upper 75^{th} percentile	179.9 ± 4.79	200.7 ± 4.57*	204.7 ± 5.50*	197.8 ± 5.81*
D max	277.4 ± 8.45	305.9 ± 6.57	329.7 ± 13.20*	313.5 ± 12.49*
Proportion [%] of white muscle fibers with				
D ≤ 20 µm	0.5 ± 0.18	1.4 ± 0.21*	1.5 ± 0.26*	1.4 ± 0.33*
D > 200 µm	5.7 ± 1.29	11.4 ± 1.78*	12.1 ± 1.72*	10.2 ± 1.42*

838 **Table 8:** Changes in fatty acid content (g 100 g^{-1}) in muscle fillet, liver and whole body of spotted wolffish fed diets with different levels of inclusion of
839 microalgae (*Nannochloropsis oceanica*). Values are means ± SEM. Means in the same column at the same time point with different subscript letter differ
840 significantly ($p < 0.05$). Means in the same column with subscript * differs significantly from the start of the experiment ($p < 0.05$).

Time	Week 0	Week 12		
	Start	CTR	Low	High
Muscle fillet				
C14:0	4.82 ± 0.08	4.64 ± 0.14	4.51 ± 0.03*	4.51 ± 0.07*
C16:0	14.06 ± 0.14	14.10 ± 0.25	13.57 ± 0.09*	13.84 ± 0.11
C18:0	2.39 ± 0.04	2.49 ± 0.06	2.40 ± 0.06	2.48 ± 0.06
C19:0	2.28 ± 0.14	2.15 ± 0.07	2.17 ± 0.02	2.25 ± 0.04
C20:0	2.82 ± 0.00	n.a	n.a	n.a
C16:1	8.64 ± 0.14	8.56 ± 0.19	8.40 ± 0.11	8.62 ± 0.12
C18:1n-9+11	26.03 ± 0.46	27.28 ± 0.45	26.70 ± 0.29	27.01 ± 0.32
C20:1n-9	4.83 ± 0.19	4.20 ± 0.33	4.48 ± 0.05	4.40 ± 0.09
C22:1n-9	4.33 ± 0.08	4.13 ± 0.33	3.97 ± 0.10*	3.88 ± 0.09*
C18:2n-6	7.01 ± 0.14	7.82 ± 0.19*	7.93 ± 0.14*	7.92 ± 0.12*
C20:5n-3 (EPA)	10.09 ± 0.17	9.82 ± 0.23	10.04 ± 0.01	10.06 ± 0.10
C22:5n-3	1.97 ± 0.07	2.86 ± 0.82	1.96 ± 0.05	1.91 ± 0.03
C22:6n-3 (DHA)	13.91 ± 0.13	14.12 ± 0.25	14.19 ± 0.04	14.10 ± 0.11
∑ Saturated	26.38 ± 1.19	23.38 ± 1.56	22.66 ± 1.40	23.07 ± 1.43
∑ Monounsaturated	44.04 ± 0.62	44.16 ± 2.01	43.55 ± 1.94	43.91 ± 1.99
∑ Polyunsaturated	32.98 ± 1.10	34.61 ± 1.24*	34.13 ± 1.33*	33.98 ± 1.30*
∑ EPA+DHA	24.00 ± 0.73	23.94 ± 0.97	24.23 ± 0.93	24.16 ± 0.91
n-6/n-3	1/4	2/7	1/3	1/3
Liver				
C14:0	4.49 ± 0.05	4.10 ± 0.06*	4.01 ± 0.03*	4.13 ± 0.06*
C16:0	14.29 ± 0.09	13.49 ± 0.10^{ab}*	13.16 ± 0.10^{a}*	13.70 ± 0.11^{b}*
C18:0	2.92 ± 0.03	2.93 ± 0.03	2.92 ± 0.04	2.96 ± 0.02

C19:0	1.98 ± 0.07	1.92 ± 0.01[a]	2.03 ± 0.03[b]	2.02 ± 0.03[b]
C16:1	8.58 ± 0.11	8.15 ± 0.12*	8.16 ± 0.10*	8.47 ± 0.06
C18:1n-9+11	30.36 ± 0.33	31.21 ± 0.26	30.65 ± 0.59	30.21 ± 0.45
C20:1n-9	4.66 ± 0.06	4.34 ± 0.04*	4.08 ± 0.11*	4.22 ± 0.01*
C22:1n-9	4.25 ± 0.07	3.68 ± 0.09*	3.46 ± 0.08*	3.59 ± 0.04*
C18:2n-6	6.54 ± 0.08	7.11 ± 0.09[a]*	7.57 ± 0.08[b]*	7.42 ± 0.07[ab]*
C20:5n-3 (EPA)	9.19 ± 0.13	9.17 ± 0.07	9.32 ± 0.01	9.38 ± 0.08
C22:5n-3	1.70 ± 0.05	1.79 ± 0.04	1.86 ± 0.02*	1.86 ± 0.06
C22:6n-3 (DHA)	12.10 ± 0.25	12.40 ± 0.10	12.80 ± 0.17	12.33 ± 0.28
∑ Saturated	23.68 ± 1.70	22.43 ± 1.39[ab]*	22.11 ± 1.35[a]*	22.82 ± 1.41[b]*
∑ Monounsaturated	47.84 ± 1.96	47.38 ± 2.39	46.34 ± 2.36*	46.50 ± 2.31
∑ Polyunsaturated	29.55 ± 0.94	30.47 ± 1.16	31.54 ± 1.19*	31.00 ± 1.15
∑ EPA+DHA	21.30 ± 0.57	21.57 ± 0.73	22.11 ± 0.78	21.71 ± 0.67
n-6/n-3	2/7	1/3	1/3	1/3
Whole fish				
C14:0	1.82 ± 0.04	1.54 ± 0.05*	1.46 ± 0.00*	1.55 ± 0.01*
C16:0	11.30 ± 0.29	9.98 ± 0.27*	9.32 ± 0.03*	9.42 ± 0.09*
C18:0	4.57 ± 0.13	3.94 ± 0.16*	3.90 ± 0.04*	4.00 ± 0.04*
C19:0	n.a	0.90 ± 0.01[a]	0.95 ± 0.02[a]	0.98 ± 0.07[a]
C16:1	7.20 ± 0.26	6.50 ± 0.38	6.08 ± 0.02*	6.38 ± 0.04*
C18:1n-9+11	45.99 ± 0.34	45.10 ± 1.86	45.60 ± 0.59	46.03 ± 1.31
C18:1n-7	4.91 ± 0.21	5.23 ± 0.04[a]	5.56 ± 0.09[b]	5.39 ± 0.04[ab]
C20:1n-9	2.73 ± 0.09	2.66 ± 0.16	2.77 ± 0.01	2.72 ± 0.08
C22:1n-9	1.67 ± 0.02	1.41 ± 0.18	1.41 ± 0.03*	1.44 ± 0.08*
C18:2n-6	3.27 ± 0.12	4.02 ± 0.19*	4.51 ± 0.06*	4.46 ± 0.19*
C20:3n-3	n.a	0.87 ± 0.12[a]	0.87 ± 0.03[a]	1.04 ± 0.00[a]

C20:5n-3 (EPA)	7.34 ± 0.15	7.86 ± 0.34	8.06 ± 0.10*	8.13 ± 0.29*
C22:5n-3	1.40 ± 0.07	1.71 ± 0.08*	1.80 ± 0.05*	1.76 ± 0.09*
C22:6n-3 (DHA)	7.80 ± 0.17	8.84 ± 0.39	8.01 ± 0.28	7.88 ± 0.35
∑ Saturated	17.69 ± 1.21	16.35 ± 1.08	15.63 ± 1.00*	15.95 ± 1.06*
∑ Monounsaturated	62.50 ± 3.87	60.90 ± 4.44	61.41 ± 4.48	61.95 ± 4.53
∑ Polyunsaturated	19.82 ± 0.70	23.31 ± 0.88*	23.24 ± 0.81*	23.27 ± 0.81*
∑ EPA+DHA	15.14 ± 0.14	16.71 ± 0.38	16.07 ± 0.13	16.01 ± 0.21
n-6/n-3	3/8	2/5	3/7	2/5

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Paper III

Proteomic profile of intestine, liver and muscle and evaluation of intestinal morphology of juvenile spotted wolffish (*Anarhichas minor*) fed the microalgae *Scenedesmus obliquus*

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Abstract

The aim of this study was to investigate the proteomic profile of muscle, liver and intestine as well as to evaluate intestinal morphology of juvenile spotted wolffish fed two diets either containing fishmeal as the primary source of protein or microalgae (*Scenedesmus obliquus*) replacing 12% of the fishmeal. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) coupled with mass spectrometry was used to analyze the proteome. Differentially expressed spots were identified using homology search. Muscle, liver and intestinal proteomes were altered as an effect of the dietary treatment. Spots that were upregulated in the algae fed fish were identified as, among others, structural proteins (vimentin, tropomyosin alpha 1-chain, actin, keratin type I, tubulin alpha chain) and proteins involved in regulation of energy homeostasis (in particular creatine kinase). Several proteins involved in glycolysis and gluconeogenesis were also identified among the differentially expressed spots in muscle, liver and intestine. Antioxidant proteins (including glutathione peroxidase, peroxidase and thioredoxin) were identified from downregulated spots in the liver and intestine of algae-fed fish. Glutathione S-transferase was the only protein with antioxidant function identified in an upregulated spot. From the histological evaluation of the intestine we observed no indications of inflammation; fish fed *S. obliquus* did however have thinner distal intestinal muscularis and reduced mean size of acidic mucous cells in the anterior intestine.

Keywords: 2-D PAGE · intestinal health · marine aquaculture · sustainable fish farming · fishmeal replacement · proteomics

1. Introduction

Feed is the highest expense of intensive aquaculture, accounting for 50-70% of the production cost (Rana et al. 2009). In the past, high nutritional value, ready availability and relatively low cost made fishmeal and –oil suitable for use in feed for farmed fish (Olsen and Hasan, 2012, Tacon and Metian, 2015). However, as increased future harvest from wild fish stocks will not be sustainable, the rapidly growing aquaculture industry needs to find alternative feed ingredients (FAO, 2018). Fishmeal alternatives such as plant proteins, micro- and macroalgae, and marine invertebrates have been evaluated in numerous studies, and the recent use of genomic and transcriptomic tools has greatly contributed to a better understanding of the mechanisms and pathways affected by fishmeal replacement (Panserat and Kaushik, 2010; Rodrigues et al. 2012). However, the use of genomic approaches alone are not enough to complete our understanding of the mechanisms affected when replacing fishmeal with novel feed ingredients, many of which are far from the natural diet of the fish. Proteomic analysis represents a powerful tool for obtaining a large amount of information from the complex and dynamic physiological state of a cell, tissue or an organism; the term proteome refers to the entire protein set of a cell, tissue or an organism at a given time under defined conditions. Gel-based techniques such as high-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is the traditional approach to proteomic analysis. In studies of organisms with no or incomplete genome sequence data available, gel-based proteomics plays an important role enabling the use of homology searches using peptide sequences from single proteins (Rogowska-Wrzeinska et al., 2013). Because of this, 2-D PAGE provides a good approach for studying the effects of fishmeal replacement for novel farmed fish species, such as the spotted wolffish.

Spotted wolffish (*Anarhichas minor*) is a bottom-dwelling marine fish with promising potential for cold-water aquaculture production (Foss et al., 2004). High growth-rate at cold temperatures, husbandry-suited behavior, late sexual maturation, ability to tolerate and adapt to a wide range of water quality parameters are some of the traits that make the spotted wolffish favorable for farming (Foss et al., 2001, Foss et al., 2003, Foss et al., 2004). Several countries such as Norway, Canada, Iceland, Sweden and Chile have shown interest in the species, but there is currently only one commercial wolffish producer in the world, Aminor AS, located at Meløy in Norway. Future wolffish production will have to rely on alternative feed ingredients, and it is hence important to evaluate its ability to tolerate ingredients such as microalgae.

This study is part of a larger study and results on muscle growth and chemical composition when partially replacing fishmeal with the microalgae *Scenedesmus obliquus* were reported in Knutsen et al., 2019. No effect of the dietary treatments were observed on fish growth, but tendencies of improved recruitment of muscle fibers as

well as reduced muscle mineral content was observed for the fish fed microalgae (Knutsen et al., 2019). The aim of the present study is to investigate (i) the molecular mechanisms affected by fishmeal replacement with *S. obliquus* using proteomic analysis of muscle, liver and intestine and (ii) fish welfare indices by histological evaluation of anterior and distal intestinal morphology.

2. Materials and methods

The current study was carried out at Nord University research station (Mørkvedbukta, Bodø, Norway). The experiment was approved by the Animal Welfare Committee at FBA, Nord University and was conducted in accordance with the Norwegian Animal Welfare Act (LOV-2009-06-19-97) and the national regulation on the use of animals in research (FOR-2015-06-18-761). Samples analyzed from the present report were collected from the experiment described by Knutsen et al. (2019).

2.1 Experimental diets and feeding trial

Two isonitrogenous, isolipidic and isocaloric diets were formulated; one control diet (CTR) and one diet with the microalgae *Scenedesmus obliquus* replacing 12% of the dietary fishmeal (AL). The composition of the diets are shown in Knutsen et al. (2019), where the control diet is CTR and the algae diet is AL12. The two diets were extruded 2-3 mm pellets produced at Sparos LDa. (Olhão, Portugal). Diets were kept in light-protected and air-tight containers and stored cold (4^{o}C) prior to the experiment and were kept at room temperature for the duration of the experiment. Juvenile spotted wolfish with mean weight 60 g were provided by Aminor AS, Halsa, Norway. Fish were transported to Mørkvedbukta research station, distributed into triplicate groups in circular glass fiber tanks (1 m^3, N = 75 fish per tank) and acclimatized to the laboratory conditions for 7 weeks following transport. During acclimatization the fish were provided a commercial diet (Amber Neptun, Skretting, Stavanger, Norway) and 15 days prior to the initiation of the experiment they were gradually introduced to the experimental diets. Seawater was provided from a flow through water treatment system, supplied from 250 m depth in Saltfjorden, filtered (200 µm) and aerated and with water exchange of 1400 L/h. Salinity (34 ‰), temperature (7.7^{o}C ± 0.005) and oxygen (86.7 ± 0.11 %) was kept stable throughout the trial. Feeding was continuous and in excess between 08:00 and 21:00 during the experiment with feeding rate set to 1.6 % and gradually reduced to 1.4% towards the end of the trial based on appetite and feed waste. The feeding trial lasted 12 weeks, from May 10th to August 3rd 2016.

2.2 Sampling

The sampling procedure is illustrated in Figure 1. Randomly selected individuals were sampled from each replicate tank at the end of the rearing period (n = 4 per tank, 12 per group). All fish were killed by overdose of MS-222 (tricaine methanesulfonate, 0.14 g/L) buffered with equal parts of sodium bicarbonate, followed by a blow to the head. Tissue samples were collected from the liver, muscle fillets and from the anterior intestine for proteomic analysis from two fish per tank (6 per group). All samples were washed in phosphate buffered saline (PBS), immediately frozen in liquid nitrogen and stored at -80^{o}C until further analysis. The intestines of four fish per tank (12 per group) were dissected and small pieces (1 cm length) were cut from the proximate and distal intestine. For the proximate intestine (PI) the piece was cut approximately 0.5 cm posterior to the stomach and for the distal intestine (DI) approximately 0.5 cm anterior to the anal sphincter (Figure 1). Intestinal samples were rinsed in PBS prior to fixation in phosphate buffered formaldehyde (4%, CH_2O, minimum 10 mL / g tissue). The tissue samples were stored at 4^{o}C until further processing.

2.3 Proteomics

2.3.1 Sample preparation and protein extraction

Protein samples from liver, muscle and intestine were individually extracted using a modification of the method described by Wang et al. 2007. In brief, frozen tissue samples were ground to a powder in liquid nitrogen and diluted in PBS containing 0.1% protease inhibitor (GE Healthcare, USA). Samples were sonicated (2x5 s) using an ultrasonic processor (SONICS Vibracell VCX750, USA) followed by centrifugation (10 000 g, 30 min, 4^{o}C). The supernatants were collected and incubated with trichloroacetic acid (TCA, 10 % w/v, Sigma-Aldrich, USA) and dithiotheritol (DTT, 0.1 %, VWR, USA) for 30 min. The samples were then centrifuged (10 000 g, 30 min, 4^{o}C), the obtained pellets were resuspended in a mixture of acetone (VWR, USA) and DTT (0.1 %) for 45 min at -20^{o}C and then centrifuged again (10 000 g, 30 min, 4^{o}C). The obtained pellets were further solubilized in rehydration buffer (9.8 M urea, Sigma-Aldrich, USA; 2% CHAPS (3-[(3-Choladmidopropyl) dimethylammonio]-1-propanesulfonate), VWR, USA; 20 mM DTT, 0.5 % Biolyte (3-10), Bio-Rad, USA; and 0.001 % bromophenol blue, Sigma-Aldrich, USA), vortexed and centrifuged (10 000 g, 30 min, 4^{o}C).

2.3.2 Two-dimensional gel electrophoresis

Protein concentration was estimated using a Qubit® Protein Assay Kit and a Qubit™ fluorometer (Life Technologies, USA) following the manufacturer's protocols. Protein (100 µg, dissolved in 300 µL rehydration buffer) was evenly distributed to an immobilized pH gradient (IPG) strip (17 cm, pH 3-10, Bio-Rad, USA) and left to rehydrate for 15 h. Rehydrated strips were then subjected to iso-electric focusing in a Bio-Rad Protean IEF cell. Focusing was done to 60 000 volt hours using three-step slow ramping with maximum 10 000 V and constant temperature of 20°C. After focusing, the IPG strips were reduced with 0.2 % DTT and alkylated with 0.3 % idoadetamide (VWR, USA) for 15 min each in equilibration buffer (6 M urea, Sigma-Aldrich; 0.375 M tris-HCl (pH 8.8), Bio-Rad; 2 % SDS, Sigma-Aldrich; 20 % glycerol, Sigma-Aldrich). In a Bio-Rad Protean II xi system (USA), equilibrated gel-strips were loaded on 12.5 % polyacrylamide gels. Gels were first run at a constant current of 20 mA/gel for 15 min to ensure even focusing of the protein and then 6 mA/gel overnight (around 16 h). Current was increased to 15 mA/gel the following day to complete the run. Voltage was limited to 250 V at all times during the run. Gels were then stained with Sypro® Ruby Protein gel stain (Thermo-Fisher, USA) diluted with distilled water (1 part Sypro® Ruby, 2 parts water). Gels were imaged using the ChemiDoc™ XRS system (Bio-Rad, USA).

2.3.3 Analysis of gel-images

Gel-images were analyzed using PDQuest™ Advanced 2D analysis software (Bio-Rad, USA) to determine intensity of protein spots. Spot intensities were normalized by the total density of the gel image. Statistical analysis is described in section 2.5. Spots which had significantly higher and significantly lower ($p < 0.05$) spot intensity in the algae-fed group compared to the control-group will be referred to as upregulated (Figure 1-A) and downregulated proteins (Figure 1-B) respectively.

2.3.4 LC-MS/MS

For each of the three tissues, protein from 4-5 fish was pooled and a preparative gel with 300 µg of protein was prepared from the pooled extract following the same protocol as described in section 2.4. The selected differentially expressed spots were manually excised from the gels on a blue light transilluminator (Safe Imager™ 2.0, Blue-Light Transilluminator, Life-Technologies, USA). Spots were then sent to the University Proteomics Platform, University of Tromsø, Norway where LC-MS/MS-analysis was performed. Here, excised spots were trypsinized, reduced in gel, alkylated and subjected to LC-MS/MS-analysis performed with nanoAcquity ultra-performance liquid

chromatography and Q-TOF Ultima global mass spectrometer (Micromass/Waters, MA, USA).

2.3.5 Protein identification using bioinformatics tools

Mascot generic files (mgf) were generated from the LC-MS/MS analysis using the Protein Lynx Global server software (version 2.1, Micromass/Waters, MA, USA). The mgf files were then analyzed using the MASCOT MS/MS Ions search (version 2.4.01) with the SwissProt protein database (06.2018, 557713 sequences; 200130199 residues) as the primary choice and the NCBI non-redundant database (29.04.2018, 152462470 sequences; 55858910152 residues) as a second choice if a hit could not be obtained from SwissProt. The search was limited to the taxonomic class Actinopterygii (ray-finned fishes). Enzyme was set to trypsin and allowing one missed cleavage, with fixed modification carbamidomethyl of cysteine and variable modification oxidation of methionine, peptide charge was set to 2+ and 3+, peptide tolerance to 100 ppm and MS/MS ion tolerance to 0.1 Da. All searches were performed using the decoy search. False discovery rate was kept at 0 % for both peptide matches above identity and for homology threshold. Protein hits with scores above the significant threshold and with at least one unique peptide sequence were identified. If more than one protein was identified, the protein with the highest score was selected.

2.4 Histology

2.4.1 Preparation of histological sections

For processing, embedding and staining of the intestinal samples, the methods described by Roberts (2012) were employed. Paraffin-embedded samples were sectioned at 4 µm thickness. Four non-successive sections (at least 100 µm distance between sections) were made from each sample. Sections were mounted on glass slides and dried overnight. Three of the replicate sections were stained with haematoxylin and eosin (H&E) staining to be used for the morphometric assessment of the intestinal morphology. The fourth section was stained with a combination of Alcian blue and periodic acid Schiff (ABPAS) staining technique, to assess the mucous cells; total mucous cell area per section (TAMC), number and size (area) of neutral mucous cells (N_{NMC}, S_{NMC}) and number and size (area) of acidic mucous cells (N_{AMC}, S_{AMC}).

2.4.2 Morphometric analysis of intestine samples

All sections were examined by light microscopy. For the analysis of villus length (VL), the thickness of tunica muscularis (TM) and the assessment of the mucous cells, whole tissue-sections were scanned at either 4 or 10x magnification using a Leica DM1000 microscope, with a Leica MC170 HD camera and the Leica Application Suite software (v 4.12.0). All measurements were performed using the software ImageJ (https://imagej.nih.gov/ij/, USA). For each of the four replicate sections per fish, a minimum of 10 well-oriented and intact villi were selected. The VL was measured from the base to the tip of the villus. The measurement was done in triplicates for each villus with the final value being the mean of the triplicate measurements. TM was measured in 6 locations, in each of the four replicate sections per sample, with the final value for each section being the mean of these 6 measurements.

TAMC was estimated using color thresholds in ImageJ and calculated from the mean of triplicate measurements of each ABPAS stained section. In short, the estimate was done as follows: color threshold was selected, thresholding method was set to default, threshold color to red and color space to HSB (hue, saturation, brightness). Then brightness was increased until all of the tissue was covered by the threshold color. This area could then be selected and area measured. Brightness was then reduced until only the mucous cells was covered by the thresholding color. The mucous cell area could then be selected and measured. This was used to calculate the percentage of mucous cells per total tissue area, which was used as the final value of TAMC. The estimation of TAMC using color thresholding was done in triplicates with the final value being the mean of the triplicate measurements. The number and size of neutral (pink) and acidic (blue) mucous cells were measured in 10 randomly selected areas of epithelium for each AB/PAS stained section. This was used to calculate the mean number of neutral (N_{NMC}) and acid mucous cells (N_{AMC}) per 1000 μm^2 epithelium and mean size of the neutral (S_{NMC}) and acid mucous cells (S_{AMC}). All values are presented as mean ± SE.

2.5 Statistical analysis

Statistical analysis of spot intensities provided from the analysis of gel images and histology data from the two treatment groups was compared using the software R-3.4.1. All data were tested for normality by the Shapiro-Wilk test and homogeneity of variance by Levene's mean test. When parametric assumptions were met, a t-test was used to compare the two groups. When the data did not display homogeneity of variance a Welch test was used as an alternative, and a Mann Whitney test was used if the data were not normally distributed. In addition, a principal component analysis (PCA) was

253 also performed on intensity of all spots for each tissue using the software R-3.4.1 (R Core
254 Team, 2017).

255

256 ## 3. Results and discussion

257 Each of the tissues investigated are discussed separately. We have used homology
258 search for the identification of protein spots given the paucity of genomic resources of
259 spotted wolffish. The genus *Anarhichas* has only 557 nucleotide sequences in the NCBI
260 Gene bank (509 genomic nucleotide sequences and 48 ESTs). Most of the sequences are
261 from the mitochondrial genome of *Anarhichas sp.* (Lair and Cart, 2016, unpublished,
262 direct submission of sequences in NCBI).

263

264 ### *3.1 Muscle*

265 For spots matched across all muscle 2-DE gels, a total of 202 spots were resolved and
266 of these 13 were found to be significantly altered in abundance ($p < 0.05$) as an effect of
267 the dietary treatment (Figure 1). Of these, 12 out of 13 spots were possible to identify
268 (Table 2), whereof 8 spots were upregulated and 4 spots were downregulated. Spot M10
269 was upregulated in algae-fed fish and identified as vimentin. Vimentin is a type III
270 intermediate filament protein found predominantly in mesenchymal cells, multipotent
271 stromal cells that can differentiate into a variety of cell types (Markl, 1991, Sateli and Li,
272 2011). In higher vertebrates, mature skeletal muscle contains the intermediate filament
273 protein desmin while myoblasts contain vimentin (Tokuyasu et al., 1984). The role of
274 vimentin in fish myogenesis is much less studied. Vimentin has been found in zebrafish
275 (*Danio rerio*) embryos up to 48 h post hatching, after which immunofluorescence
276 detection of vimentin was no longer possible (Costa et al., 2003). Fish myogenesis differs
277 from the myogenesis of mammals and birds as they recruit new muscle fibers for an
278 extended part of their post-embryonic life. The molecular mechanisms behind the post-
279 embryonic muscle hyperplasia is not fully understood so we can only speculate if
280 vimentin plays a similar role in fish myoblasts. Post-embryonic muscle growth in fish is
281 either hypertrophic, where myoblasts are absorbed by an existing fiber, or hyperplastic
282 where myoblasts fuse together on the surface of existing fibers in order to form a
283 myotube that can mature into a new muscle fiber (Johnston et al., 2011). The increased
284 vimentin could be explained by an increased number of myoblasts or it could originate
285 from the mesenchymal lining adjacent to the myotome, from which undifferentiated
286 myoblasts have been suggested to arise (Stoiber and Sänger, 1996). Muscle growth and
287 development for the fish in the present study have been published separately where a
288 tendency of increased muscle fiber recruitment in the algae-fed fish was observed

(Knutsen et al., 2019). If increased spot intensity means upregulated muscle vimentin it could hence support the tendencies observed in our previous report. However, vimentin expression in rainbow trout (*Onchorynchus mykiss*) differs fundamentally from that of higher vertebrates and is restricted to a few non-muscle cell-types only (Herrmann et al. 1996, Markl and Franke, 1988), but it is not known if this is characteristic of all teleost fishes, or if it is unique for rainbow trout.

Macrophages are also known to contain vimentin (Correia et al., 1999); increased expression of vimentin may thus indicate an increase in macrophages in the muscle. In mammals, macrophages are associated with muscle regeneration (Salicer et al., 2013). It is not known if macrophages play a similar role in fish muscle regeneration. However, it could be speculated that macrophages could be involved both in muscle regeneration and in post-embryonic muscle growth of fish; both of which are processes which involve recruitment of new muscle fibers. Although muscle don't play a major role in immune function, it is also possible that an increase in macrophages is related to the immune status of the algae-fed fish. Several studies have shown that dietary provision of microalgae can have a beneficial effect on immune status of fish (Yaakob et al., 2014). Reyes-Becerril et al. (2014) reported both improved immune status, among others through elevated levels of macrophages, and antioxidant capabilities in fish fed the microalgae *Navicula* sp. β-glucans are a group of polysaccharides which are known to enhance immune response in fish, and they are present in several microalga species (Vetvicka et al., 2013, Yakoob et al., 2014). The β-glucan production of *Scenedesmus obliquus* is not known, but the closely related *S. ovalternus* has been found to be a good source of β-glucan (Schulze et al., 2016). In rainbow trout fed dietary β-glucan, there was an increased abundance of tropomyosin alpha-1 chain (Ghaedi et al., 2016); this structural protein was also identified for the upregulated spots M8 and M9 in the present study.

Four of the spots upregulated in the algae-fed group were identified as proteins involved in metabolism and regulation of energy homeostasis. Spot M6 was identified as triosephosphate isomerase, a protein that is involved in both the pathway gluconeogenesis and glycolysis, which is part of carbohydrate biosynthesis and degradation respectively (Wierenga et al., 2010). Spot M3 was identified as beta-enolase, also a protein involved in the glycolysis and gluconeogenesis. Since this observation was made for muscle tissue, it is most likely that the glycolic pathway was affected, as gluconeogenesis mainly happens in liver and kidney of fish and is unlikely to happen in skeletal muscle (Knox et al., 1980). In rodents, beta enolase is involved in embryonic muscle development as well as muscle regeneration and adult fast-twitch muscle fibers are also high in beta enolase (Merkulova et al., 2000). Finally, M4 and M5 were identified as creatine kinase, an enzyme which is involved in maintaining cellular energy homeostasis in tissues with a high and fluctuating energy-demand, such as

skeletal muscle (Wallimann et al., 1992). These results indicate increased muscle energy demand of the algae-fed group which may be explained from increased fast muscle fiber recruitment in the spotted wolfish as reported by Knutsen et al. (2019). Spot M11 was downregulated in the algae-group compared to the control treatment and was identified as keratin type I. Type I keratins are cytokeratins found in vertebrate epithelial tissue. Martin et al. (2003) reported downregulation of other types of cytokeratins, keratin II and tubulin-β, in rainbow trout fed a soybean diet, which was explained by increased requirement for energy for metabolism and hence less energy for the synthesis of structural proteins. The downregulation of a keratin I spot may thus further indicate an increased metabolic energy requirement in the muscle of the algae-fed fish.

3.2 Liver

For protein spots that could be matched across all 2-DE gels, 298 liver spots were resolved. Of the resolved spots, 25 were found to be significantly altered in abundance ($p < 0.05$) as an effect of the dietary treatment. Of these, 20 spots were identified (Table 2). Spot L11 and L16 were both downregulated in algae-fed fish, and identified as triosephosphate isomerase A and glyceraldehyde-3-phosphate dehydrogenase respectively. These two proteins are involved in both glycolysis and in gluconeogenesis (Sirover, 1997; Wierenga et al, 2010); although glycolysis occur in all tissues in fish, the activity of the main glycolytic enzymes has lower relative potential in liver. However, liver and kidney are the main sites for gluconeogenesis (Cowey et al., 1977, Knox et al., 1980). A downregulation of these protein spots in the liver is thus likely an indication of altered liver gluconeogenesis; the results may indicate a protein-sparing effect by reduced use of non-carbohydrate substrates (i.e. protein and lipids) in glucose production. Moderate dietary levels carbohydrates can have both protein-sparing and growth promoting effects in fish as long as they are present in available forms (Hemre et al., 2002). Carbohydrates present in diets of rainbow trout and Nile tilapia (*Oreochromis niloticus*) have been found to repress gluconeogenesis (Cowey et al., 1977, Sánchez-Muros et al., 1996, Shimeno et al., 1993). Carbohydrate in the diets was not directly measured, but by subtracting the measured percent of fat, protein, ash and water from a theoretical 100 %, the algae diets had slightly higher carbohydrate (11.25%) compared to the control group (9.33%). Most teleost fish are adapted to use protein as an energy source and both diets of the present trial were isonitrogenous and with sufficient protein for spotted wolffish (Cowey, 1995, Foss et al., 2004). Cathepsin D, a lysosomal protease, was identified for another downregulated spot (L15); as this might indicate changes in protein degradation, it also further supports the theory of a protein-sparing effect. The expression of Cathepsin D was increased in the liver-proteome of starved rainbow trout (*Oncorhynchus mykiss*) (Martin et al., 2001). The

downregulation of Cathepsin D in the present trial suggest that the fish were fed to satiation. Upregulation of spot L8 identified as 60S ribosomal protein, a component of the ribosome responsible for protein synthesis, further suggests modified protein synthesis complimentary to the protein-sparing effect in the liver of the algae-fed fish.

Spot L1 was upregulated in algae-fed fish, and identified as creatine kinase, which was unexpected. In all vertebrates that has been investigated, except from humans, creatine kinase has either been non-existing in liver or present in minute amounts, which could have been derived from blood vessels or blood cells present in the sample (Wallimann and Hemmer, 1994; Wyss and Kaddurah-Daouk, 2000). The presence of creatine kinase in human liver could be normal or an indication of an unknown disease (Wallimann and Hemmer, 1994). Given that L1 is correctly identified, this observation most likely originate from the presence of blood in the sample. Creatine kinase is particularly abundant in blood macrophages (Wallimann and Hemmer, 1994), which may be related to the theorized role of macrophages in muscle fiber recruitment as discussed for muscle. Alternatively, it is also possible that upregulation of creatine kinase indicates stimulation of immune responses in the algae-fed fish through elevated levels of macrophages.

Interestingly, several of the differentially expressed protein spots in liver were identified as proteins involved in mechanisms related to the protection of cells against oxidative damage caused by reactive oxygen species (ROS) through antioxidant and detoxification activity. ROS includes hydroxyl radicals (OH), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO). The glutathione (GSH) dependent system is a major part of the endogenous ROS defense system. In short, superoxide dismutase (SOD) is the first enzyme which is activated as a response to exposure to ROS; SOD catalyzes the radical into H_2O_2. Following this reaction, H_2O_2 will be transformed into water and oxygen by catalase (CAT) or glutathione peroxidase (GPx). GPx uses H_2O_2 to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Finally, glutathione reductase (GRx) regenerates GSH from GSSG. Spot L9 was downregulated in the algae-fed fish and identified as GPx. Spot L7 was also downregulated and identified as peroxiredoxin. Peroxiredoxins are a family of enzymes with antioxidant function, which also protects cells from oxidative damage by detoxifying peroxides. They catalyze the thiol-specific reduction of hydrogen peroxide into water and alcohols (Valero et al., 2015). Given the observations of proteins involved in the GSH dependent system, it seems that the algae-fed fish show changes in enzymes involved in removing H_2O_2 from the cells. Heat-shock proteins (HSP), are a family of proteins that protect cells during exposure to stress and inflammation, including protection against ROS. Specifically, they are involved in the regulation of protein homeostasis (Jacquier-Sarlin et al. 1994). Spots L18 and L19 were both found to be downregulated and identified as heat shock cognate 71 kDa (HSC71). Tetratricopeptide repeat proteins (TRPs) are structural motif proteins

405 that mediate protein-protein interactions of which several interact with the major
406 members of the HSP family, such as HSP70; they are necessary for correct regulation of
407 protein folding and transport (Ballinger et al. 1999). Spot L6 was downregulated in the
408 algae-fed group and identified as tetratricopeptide repeat protein. This could also be
409 connected to the downregulation of the endogenous antioxidants in the algae-fed fish.

410 Microalgae are exposed to high oxygen and free radical stress due to their phototropic
411 lifestyle. To protect themselves from damage they have a well-developed antioxidant
412 system, which is of interest to utilize both in functional foods and in pharmaceutical
413 compounds (Pulz and Gross, 2004). Antioxidant compounds found in microalgae are
414 considered very promising due to their bioavailability and also due to the fact that the
415 antioxidant action is believed to be a synergistic function of many different antioxidants
416 (Yaakob et al. 2014). *S. obliquus* and related *Scenedesmus* species have been shown to
417 be a good source of potent antioxidants, such as the carotenoids astaxanthin, β-
418 carotene and lutein (Chan et al., 2013, Macías-Sánches et al., 2010, Qin et al., 2008).
419 Carotenoids are well known for their strong antioxidant properties; they protect cells
420 against ROS involving both a physical component as well as a chemical reaction between
421 the carotenoid and the ROS (Krinsky, 1989). Therefore, it can be argued that *S. obliquus*
422 contributed with dietary antioxidants. Hence there is less need for the fish to synthesize
423 their own antioxidant enzymes, specifically the ones involved in removing peroxides.
424 From our previous report from the same trial, Knutsen at al. (2019) reported increased
425 skin yellowness in the algae fed fish showing that the carotenoids in the diet indeed
426 were metabolized by the fish. Pan et al. (2010) found that dietary supplemented
427 carotenoids, especially astaxanthin, increased the antioxidant capacity and protection
428 of liver in *Hyphessobrycon callistus* during hypoxia stress; the authors explained the
429 improved antioxidant capacity by lower activity of antioxidant enzymes, including GPx.

430 The downregulation of endogenous antioxidants could also be explained by
431 imbalanced amino acid content of the diet. The content of methionine in the algae diet
432 was only half (0.824 g 100 g^{-1}) of that of the control-diet (1.62 g 100 g^{-1}) (Knutsen et al.,
433 2019). Methionine is an essential amino acid and has antioxidant properties on its own,
434 protecting especially against lipid oxidation (Patra et al., 2001). Methionine is also taken
435 up in the liver for direct synthesis of another antioxidant, GSH (Reed and Orrenius,
436 1977). However, this does not explain the upregulation of glutathione S-transferase
437 (GST) spot, which is another enzyme that uses GSH. Spot L10 was upregulated in the
438 algae-fed fish and was identified as GST. These are a family of enzymes which catalyzes
439 the conjugation of lipophilic xenobiotics to reduced GSH for the purpose of inactivating
440 toxic xenobiotic agents (Salinas and Wong, 1999).

441

3.3 Intestine

For protein spots that could be matched across all 2-DE gels, 442 spots were resolved for intestine. Of the resolved protein spots, 35 spots for intestine were found to be significantly altered in abundance ($p < 0.05$) as an effect of the dietary treatment. Of these, 25 spots were identified by mapping to the protein database (Table 2). Two of the intestinal spots, G10 and G20, were found to be downregulated in the algae-fed fish and were identified as peroxiredoxin and thioredoxin domain-containing protein 17, both of which are involved in antioxidant functions and detoxification. As these are proteins with peroxidase activity, these observation further support the finding of downregulation of antioxidant proteins involved in peroxide removal as described for liver (section 3.2). The downregulated spot G11 was identified as another heat shock protein 90-beta (HSP90-beta). This protein is involved in stabilization of proteins when the cells are exposed to stress and could again be connected to the previously discussed role of HSP in defense against ROS (section 3.2).

Spot G13 and G20 were both downregulated in the intestine of algae fed fish and were identified as fatty-acid binding protein and trypsin respectively. The latter is an enzyme with endopeptidase activity which could indicate lower digestibility of the microalgae for the fish. The microalgae in the present study were present as whole algal cells; reduced availability of the nutrients and energy in the diet can be caused by inefficient disruption of the algal cell wall during feed processing or in the digestive tract. Spot G4 was identified as fructose-biphosphate adolase B and was upregulated in the intestine; fructose-biphosphate adolase, or just aldolase, is an enzyme involved in the glycolytic pathway. An increase in aldolase-B has also been observed in rainbow trout (*Oncorhynchus mykiss*) fed a diet high in soybean, which the authors explained from metabolic changes due to the increased energy demand of the fish (Martin et al., 2003). Intestine spot G2 was upregulated in algae-fed fish and identified as citrate synthase; this protein is involved in the tricarboxylic acid cycle, which is a part of the carbohydrate metabolism. This corresponds to the theory of a protein sparing effect of a slightly more carbohydrate rich diet as was suggested from the results described for liver (section 3.2).

Spot G17 was upregulated in the algae-fed fish and identified as creatine kinase. Although mostly found in cells with high energy demand, creatine kinase is also present in intestinal epithelial cells where it supplies energy for brush-border contraction (Wallimann and Hemmer, 1994). Spots G5, G15, G21 and G9 were also upregulated in algae-fed fish and identified as the structural proteins tropomyosin alpha-1 chain, actin, keratin type I cytoskeletal 13 and tubulin alpha chain, respectively. Actins are proteins which function in muscle contraction and are involved in various types of cell motility; they are the main proteins making up the microvilli of the brush border. This is quite interesting as other feed replacement studies for juvenile marine fish have found

changes in expression of corresponding genes. In a transcriptomic analysis of intestinal gene expression of juvenile Atlantic cod (*Gadus morhua*) fed diets with camelina oil replacing fish oil, Morais et al. (2012) found an upregulation of tropomyosin alpha-1 chain, actin alpha cardiac muscle 1 and creatine kinase. Murray et al. 2010 replaced fishmeal with soybean-meal for juvenile Atlantic halibut (*Hippoglossus hippoglossus*) and found the exact opposite of both the present study and the study of Morais et al. (2012). Here, tropomyosin and other structure related genes as well as creatine kinase were downregulated over time. Keratin type I cytoskeletal 13 and tubulin alpha chain were also downregulated in soybean-fed trout in the study by Martin et al. (2003). Spot G18 was found to be downregulated in algae fed fish and was identified as adenylate kinase 2. Adenylate kinase 2 is an enzyme that catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. It plays an important role in cellular energy homeostasis and in adenine nucleotide metabolism. Interestingly, it was found to be upregulated in the study by Murray et al. (2010), again the opposite of the findings of the present study.

In both intestine and muscle, we have observed an upregulation of the spots identified as several structural proteins as well as proteins involved in regulation of energy homeostasis. Although differing in the observed expression patterns, both Martin et al. (2003), Morais et al. (2012), Murray et al. (2010) and our study all reported altered expression of the same proteins. The intestine serves an important function in not only the digestion and absorption of nutrients, but it also plays a vital role in immune regulation and protection against pathogens. Changes in structural proteins in the intestine could potentially affect contractile activity, nutrient absorption and the overall health of the fish. The upregulation of structural proteins and proteins involved in regulation of energy homeostasis might be explained by increased energy demand for protein synthesis and cell-growth. Although not significant, there was a trend of slightly longer villi length (VL) from the histological evaluation (Table 1), which may support this theory.

From the histological evaluation of intestinal morphology, we also observed reduced thickness of the tunica muscularis ($p = 0.047$) in the distal intestine, potentially negatively affecting the contractile activity in the distal intestine. The mucous layer is a vital component of the digestive tract: it protects the mucosal epithelium from chemical and physical damage, it lubricates the mucosal surface and functions as a barrier against infection from pathogenic organisms (Ringø et al., 2003). Mucins are secreted by goblet cells. The histological evaluation also showed reduced mean size of the acid mucin containing goblet cells ($p = 0.001$) in the algae fed fish, though the number of goblet cells were not significantly different. Increased number and size of goblet cells indicate increased protection and lubrication response of the mucosa, as it has been reported in several studies reporting negative effects of feed ingredients in fish (Baeverfjord and

519 Krogdahl, 1996, Olsen et al., 2007, van den Ingh et al., 1991). Hence, the decrease in size
520 of goblet cells probably does not indicate any inflammatory response of the diets in the
521 intestine of the fish. However, it could indicate reduced mucus production and hence a
522 compromised mucous barrier.

523

524 ## 4. Conclusion

525 In summary, muscle, liver and intestinal proteome was altered as an effect of the
526 dietary incorporation with the microalgae *S. obliquus*. The proteins that were identified
527 as differentially expressed included structural proteins and proteins involved in
528 regulation of energy homeostasis might be explained by increased energy demand for
529 protein synthesis and cell-growth in muscle and intestine. The dietary treatment altered
530 liver gluconeogenesis which might indicate a protein-sparing effect by reduced use of
531 non-carbohydrate substrates in glucose production. Several proteins involved in
532 antioxidation (including GPx, peroxidase and thioredoxin) were downregulated in the
533 algae-fed fish; we suggest that this could be explained by less need for endogenous
534 antioxidant enzymes due to dietary supplementation of antioxidants from *S. obliquus*.
535 From the morphological evaluation we observed no indications of inflammation in the
536 intestine, but the decreased size of goblet cells may indicate a compromised mucous
537 barrier in the algae fed fish.

538

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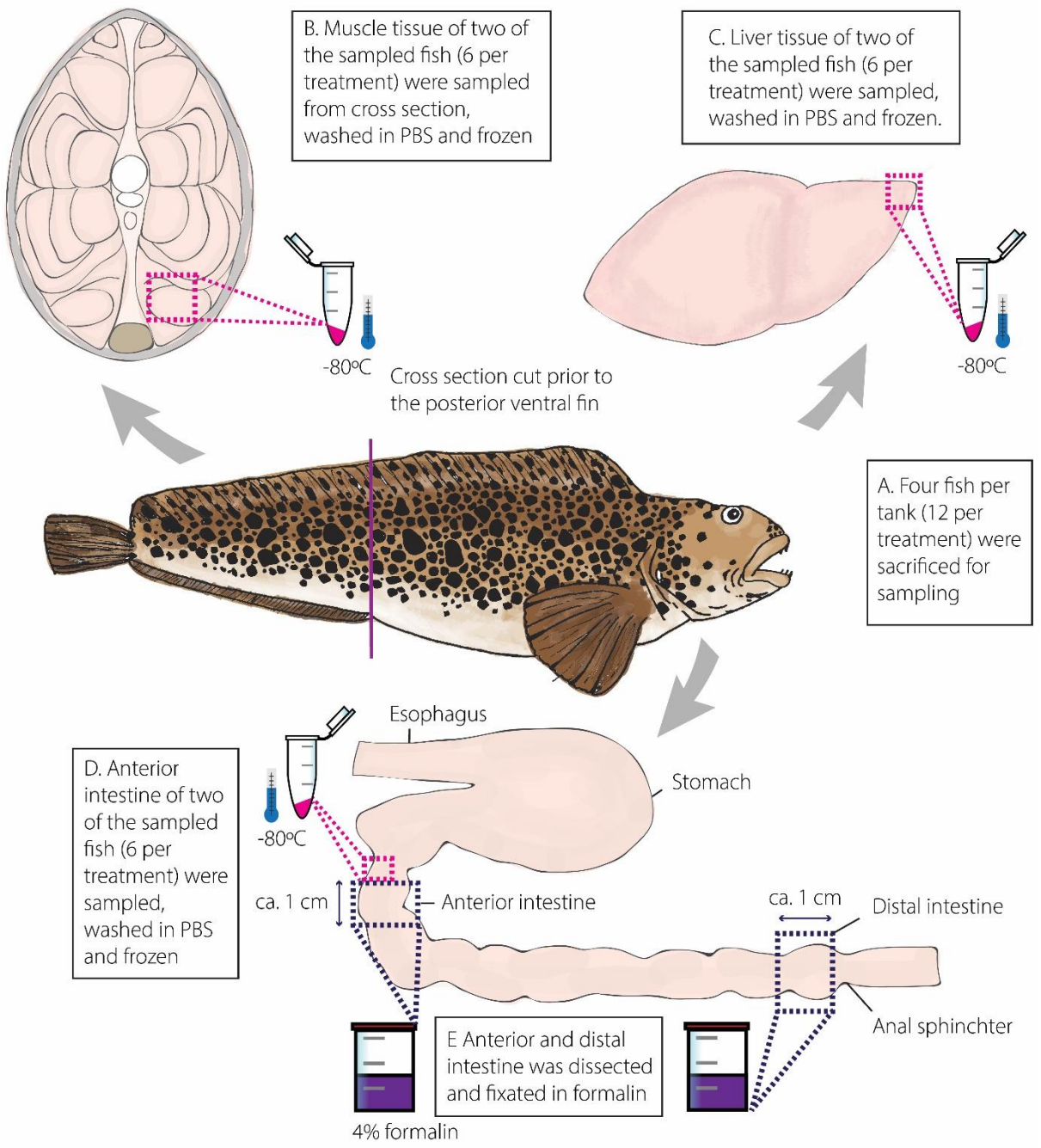


705
706 **Figure 1:** Workflow for sampling spotted wolffish for proteomic and histological analysis.

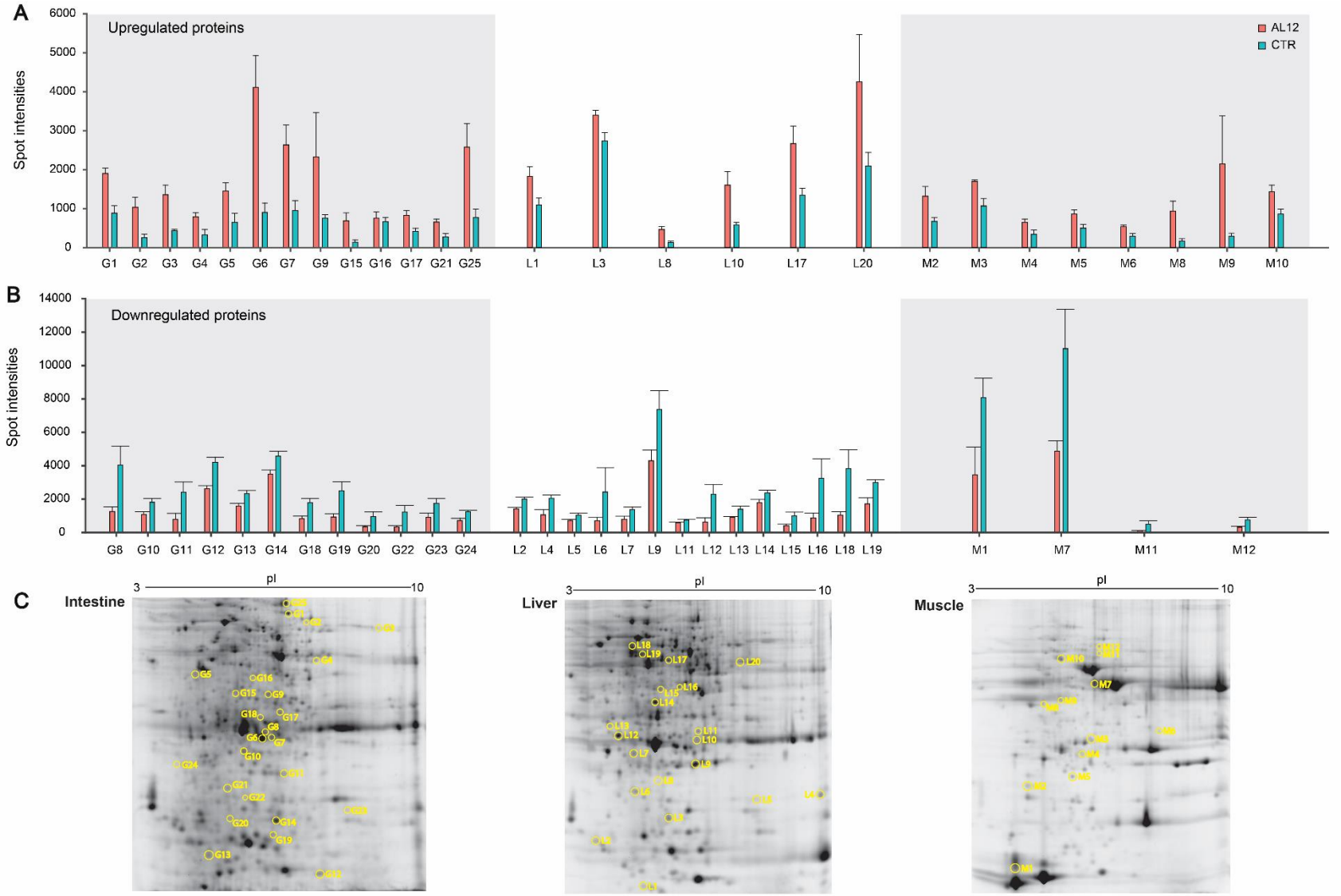
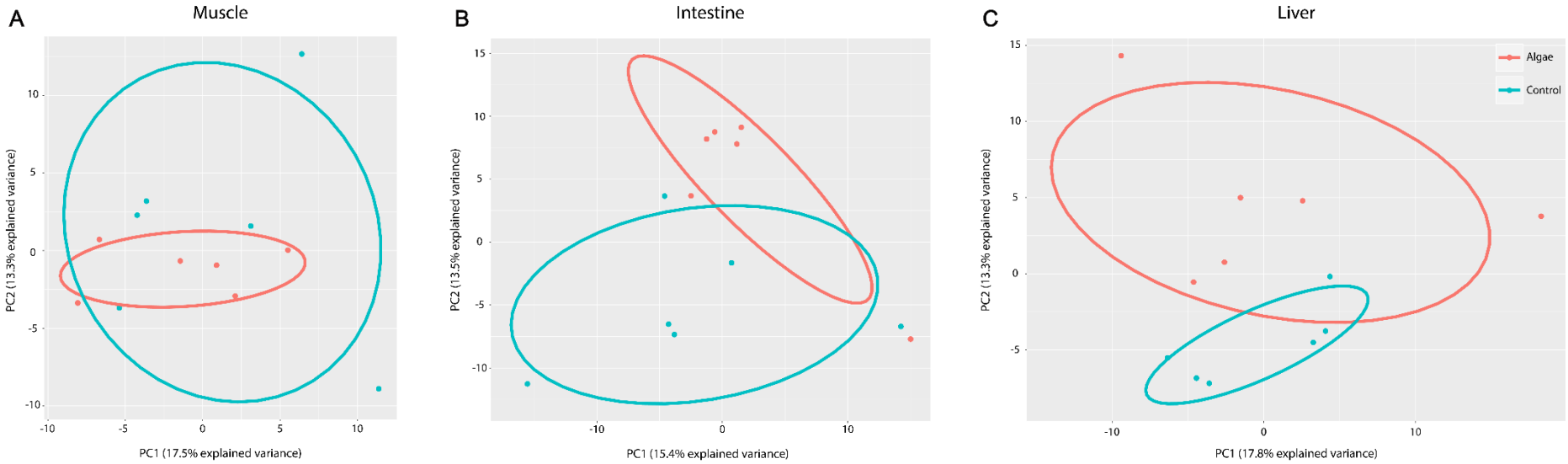


Figure 2: Spot intensities of identified differentially expressed proteins. A: Protein spots upregulated in the AL12 treatment. B: Protein spots downregulated in AL12 treatment. C: Spot-identities on a representative gel of the three different tissues; intestine (G), liver (L) and Muscle (M).



710
711 **Figure 3:** Principle component analysis of the three different tissues. One dot represents one fish. A: Muscle. B: Intestine. C: Liver.

712 **Table 1:** Morphometric measurements of histological sections of intestine.

	Proximal intestine			Distal intestine		
	Fishmeal diet (FM)	Algae diet (AL)	p-value	Fishmeal diet (FM)	Algae diet (AL)	p-value
TM (µm)	124.20 ± 10.74	136.55 ± 6.85	0.396	**116.96 ± 8.75**	**96.38 ± 6.36**	**0.047**
VL (µm)	2503.60 ± 180.77	2945.95 ± 170.70	0.109	1769.62 ± 70.06	1716.73 ± 68.97	0.598
TAMC (%)	3.40 ± 0.39	2.27 ± 0.38	0.067	4.30 ± 0.57	3.59 ± 0.37	0.309
S_{AMC} (μm^2)	**171.70 ± 5.91**	**131.16 ± 7.24**	**0.001**	190.77 ± 9.43	189.10 ± 7.42	0.893
N_{AMC} (per 1000 μm^2 epithelium)	0.52 ± 0.04	0.50 ± 0.02	0.754	0.58 ± 0.02	0.61± 0.05	0.863
S_{NMC} (μm^2)	120.76 ± 7.77	102.45 ± 4.92	0.154	169.66 ± 12.97	167.71 ± 15.98	0.928
N_{NMC} (per 1000 μm^2 epithelium)	0.02 ± 0.01	0.02 ± 0.01	0.727	0.06 ± 0.01	0.04 ± 0.01	0.115

Note: TM: Tunica muscularis thickness, VL: villus length, N_{NMC}: number of neutral mucous cells / 1000 μm^2 epithelium, N_{NMC}: number of acid mucous cells / 1000 μm^2 epithelium, S_{AMC}: size of acid mucous cells, S_{NMC}: size of neutral mucous cells, TAMC: total mucous cells area / Total tissue area

713

Table 2: Differentially expressed proteins in liver (L), muscle (M) and intestine (G) of juvenile spotted wolffish fed diets with and without the microalgae *Scenedesmus obliquus* identified using MASCOT. Shown in the table is spot ID, name of the identified protein, accession number, apparent and calculated isoelectric point (pI) and molecular weight (MW), significant threshold, protein score and unique sequence protein score (Up), peptide sequence, GO terms and the data base of the search. For peptide sequences, unique sequences are highlighted in red. For the calculated pI, values outside of the pI range (4.5-8.5) are noted as <4.5 and >8.5

Spot ID	Protein name (*Species*)	Accession number	Apparent pI/MW	Calculated pI/MW	Significant threshold/ p-value	Protein score/Up	SC (%)	Peptide sequence	GO terms	Data base
L1	Creatine kinase, testis isozyme (*Oncorhynchus mykiss*)	P24722	6.2/43262	5.1/12100	23/p < 0.03	36/36	3	KGGDDLDPNYVISSRV	*ATP binding(GO:0005524), Catalytic activity (GO:0003824)	Swiss Prot
L2	Hemoglobin subunit alpha-2 (*Anarhichas minor*)	P83271	8.89/15789	<4.5/16900	21/p < 0.05	123/123	16	KSADAIGADALGRM KNVMSGVALAVSKI	Oxygen transport (GO:0015671)	Swiss Prot
L3	Hemoglobin subunit beta-2 (*Anarhichas minor*)	P83273	6.2/ 16392	5.5/19700	21/ p < 0.05	76/76	16	KMDYEVVGPAALSRC KFMAVVVSALGRQ	Oxygen transport (GO:0015671)	Swiss Prot
L4	Hemoglobin subunit alpha-2 (*Anarhichas minor*)	P83271	8.89/15789	>8.5/24100	31/ p < 0.005	186/186	39	KAAVIAMWGKI KSADAIGADALGRM KNVMSGVALAVSKI KDFTPEAHVSMDKF KFFCGLSLALAEKY	Oxygen transport (GO:0015671)	Swiss Prot
L5	Hemoglobin subunit alpha-1 (*Anarhichas minor*)	P83270	9.33/15753	7/22200	25/p < 0.02	150/150	22	KAAEIGSDALSRM RMLVVYPQTKT KSVMGGVADAVMKI	Oxygen transport (GO:0015671)	Swiss Prot
L6	Tetratricopeptide repeat	A8E7I5; Q5U3F6	4.91/20407	5/22600	21/ p < 0.05	42/42	5	RAAALGSEFARQ	Cilium assembly (GO:0060271)	Swiss Prot

	protein 36 (*Danio rerio*)									
L7	Peroxiredoxin (*Oncorhynchus mykiss*)	Q91191	6.95/22248	5/26700	22/ p < 0.049	37/37	5	RQITINDLPVGRC	Cell redox homeostasis (GO:0045454)	Swiss Prot
L8	60S ribosomal protein L11 (*Ictalurus punctatus*)	Q90YV7	10.07/20635	5.4/24400	24/ p < 0.025	49/49	7	KVLEQLTGQTPVFSKA	Protein localization to nucleus (GO:0034504), translation (GO:0006412)	Swiss Prot
L9	PREDICTED: glutathione peroxidase 1-like (*Lates calcarifer*)	XP_018542016	6.84/21569	5.9/25300	53/ p < 0.025	412/153	20	KLLTGETFNFSSLQGKV KFLIGPDGVPFKR KFLIGPDGVPFKRY RKFLTSDIEGDIKK RKFLTSDIEGDIKK KFLTSDIEGDIKK	Response to oxidative stress (GO:0006979)	NCBI prot
L10	Glutathione S-transferase, partial (*Syacium gunteri*)	AIO11191	8.52/23017	6/28500	53/ p < 0.025	66/66	3	RFGLCEERY	*Glutathione transferase activity (GO:0004364)	NCBI prot
L11	Triosephosphate isomerase A (*Danio rerio*)	Q1MTI4; Q7ZWB0; Q90XF9	4.90/27179	6/28900	20/ p < 0.05	27/27	6	RVVLAYEPVWAIGTGKT	Gluconeogenesis (GO:0006094), glycolytic process (GO:0006096)	Swiss Prot
L12	Insulin-like growth factor 2 mRNA-binding	Q08CK7	8.88/65831	4.6/29200	23/ p < 0.03	28/28	1	KVTAEDLVKT	mRNA transport (GO:0051028), regulation of	Swiss Prot

	protein 1 (*Danio rerio*)								translation (GO:0006417)	
L13	PREDICTED: tumor protein D54 isoform X1 (*Lates calcarifer*)	XP_018538375	5.48/24636	4.5/30900	45/ p < 0.02	117/117	8	KVEEEINTLRQ KLGLSPLNELRQ		NCBI Prot
L14	40S ribosomal protein S3 (*Ictalurus punctatus*)	Q90YS2	9.81/27044	5.3/33900	22/ p < 0.03	141/141	18	KAELNEFLTRE RTEIIILATRT RELTAVVQKR RGLCAIAQAESLRY KLLGGLAVRR	Regulation of translation (GO:0006417), DNA repair (GO:0006281), cell cycle (GO:0007049)	Swiss Prot
L15	Cathepsin D (*Chionodraco hamatus*)	O93428	5.77/43387	5.3/35800	21/ p < 0.05	198/101	13	KFDGILGMAYPRI RISVDGVAPVFDNIMS QKK RISVDGVAPVFDNIMS QKK RNPDTEPGGELLLGGT DPKY RNPDTEPGGELLLGGT DPKY RQAYWQIRV	Proteolysis (GO:0006508)	Swiss Prot
L16	Glyceraldehyde-3-phosphate dehydrogenase (*Danio rerio*)	Q5XJ10; Q1RM54	8.20/35989	5.7/56400	25 / p < 0.02	63/63	4	RGASQNIIPASTGAAK A	Glucose metabolic process (GO:0006006), glycolytic process (GO:0006096)	Swiss Prot

L17	Methanethiol oxidase (*Danio rerio*)	Q6PHD9	5.83/51521	5.4/41600	32/ p < 0.004	78/78	3	RLILPSLISSRI RQYDITDRK	Protein transport (GO:0015031)	Swiss Prot
L18	Heat shock cognate 71 kDa protein (*Danio rerio*)	Q90473	5.18/71158	5/44600	23/ p < 0.03	81/56	5	KVEIIANDQGNRT KSINPDEAVAYGAAVQ AAILSGDKS	Cellular response to unfolded protein (GO:0034620), chaperone cofactor dependant protein folding (GO:0051085)	Swiss Prot
L19	Heat shock cognate 71 kDa protein (*Oryzias latipes*)	Q9W6Y1	5.80/76577	5.1/42600	30/ p < 0.005	250/250	10	KVEIIANDQGNRT KDAGTISGLNVLRI RIINEPTAAAIAYGLD KK RIINEPTAAAIAYGLD KK RIINEPTAAAIAYGLD KKV KSINPDEAVAYGAAVQ AAILSGDKS KNGLESYAFNMKS	Cellular response to unfolded protein (GO:0034620), chaperone cofactor dependant protein folding (GO:0051085)	Swiss Prot
L20	Serotransferrin (*Paralichthys olivaceus*)	O93429	6.06/76545	6.8/40400	24/ p < 0.02	97/97	2	KEADAMAVDGGQVYTA GKC KEADAMAVDGGQVYTA GKC	Iron ion homeostasis (GO:0055072), ion transport (GO:0006811)	Swiss Prot
M1	Parvalbumin beta 2 (*Oncorhynchus mykiss*)	P86432;	4.27/ 11392	<4.5/15200	21/ p < 0.05	146/80	19	KSGFLEEDELKL KLFLQNFSASARA	*Calcium ion binding (GO:0005509)	Swiss Prot

M2	Parvalbumin alpha (*Cyprinus carpio*)	P09227	4.43/11501	4.8/22800	25/ p < 0.02	50/50	9	KSGFIEEDELKL	*Calcium ion binding (GO:0005509)	Swiss Prot
M3	Beta-enolase (*Salmo salar*)	B5DGQ7; B5DGQ6	6.61/47656	6.2/29100	21/ p < 0.05	201/201	13	RAAVPSGASTGVHEALELRD RAAVPSGASTGVHEALELRD KDATNVGDEGGFAPNILENNEALELLKT KVNQIGSVTESIKACKL	Glycolytic process (GO:0006096)	Swiss Prot
M4	Creatine kinase, testis isozyme (*Oncorhynchus mykiss*)	P24722	6.2/43262	6/27400	21/ p < 0.05	85/85	3	KGGDDLDPNYVISSRV	*ATP binding(GO:0005524), Catalytic activity(GO:0003824)	Swiss Prot
M5	Creatine kinase, testis isozyme (*Oncorhynchus mykiss*)	P24722	6.2/43262	5.9/24300	28/ p < 0.01	48/48	3	KGGDDLDPNYVISSRV	*ATP binding(GO:0005524), Catalytic activity(GO:0003824)	Swiss Prot
M6	Triosephosphate isomerase B (*Danio rerio*)	Q90XG0; Q7T315	6.45/27096	8.1/29900	28/ p < 0.01	154/154	16	KGAFTGEISPAMIKD KVVLAYEPVWAIGTGKT KTNVSEAVANSVRI	Gluconeogenesis (GO:0006094), glycolytic process (GO:0006096)	Swiss Prot
M7	Creatine kinase, testis isozyme (*Oncorhynchus mykiss*)	P24722	6.2/43262	6.1/39700	20/ p < 0.05	129/129	3	KTFLVWVNEEDHLRV KTFLVWVNEEDHLRV KTFLVWVNEEDHLRV	*ATP binding(GO:0005524),	Swiss Prot

									Catalytic activity(GO:0003824)	
M8	Tropomyosin alpha-1 chain (*Liza aurata*)	P84335	4.69/32767	5/35500	33/ p < 0.003	256/256	34	KLDKENALDRA KGTEDELDKYSEALKD KKATDAEGDVASLNRR RRIQLVEEELDRA RRIQLVEEELDRA KDEEKMELQEIQLKE KMELQEIQLKE KEDKYEEEIKV KTIDDLEDELYAQKL KAISEELDHALNDMTS I	*Actin filament binding (GO:0051015), protein heterodimerization (GO:0046982) and homodimerization (GO:0042803) activity	Swiss Prot
M9	Tropomyosin alpha-1 chain (*Danio rerio*)	P13104	4.7/32760	5.6/35600	28/ p < 0.01	349/349	21	KLDKENALDRA KDAQEKLELAEKK RIQLVEEELDRA KMELQEIQLKE KCSELEEELKT KTIDDLEDELYAQKL KTIDDLEDELYAQKL	*Actin filament binding (GO:0051015), protein heterodimerization (GO:0046982) and homodimerization (GO:0042803) activity	Swiss Prot
M10	Vimentin (*Cyprinus carpio*)	Q92155	5.13/52516	5.6/44700	25/ p < 0.02	116/116	6	REYQDLLNVKM KMALDIEIATYRK KLLEGEESRI KLLEGEESRI	Structural molecule activity (GO:0005198)	Swiss Prot

M11	Keratin, type I cytoskeletal 13 (*Oncorhynchus mykiss*)	Q8JFQ6	5.17/51938	6.4/48500	28/ p < 0.01	55/55	1	KLAADDFRT	Structural molecule activity (GO:0005198)	Swiss Prot
M12	Creatine kinase, testis isozyme (*Oncorhynchus mykiss*)	P24722	6.2/43262	6.4/48500	28/ p < 0.01	59/59	3	KGGDDLDPNYVISSRV	*ATP binding(GO:0005524), Catalytic activity (GO:0003824)	Swiss Prot
G1	WD repeat-containing protein 1 (*Larimichthys crocea*)	KKF15520	6.56/70514	6.6/51100	52/ p < 0.03	158/101	4	KYAPSGFYIASGDASGKI KDIAWTEDSKRI	maturation of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)(GO:0000466)	NCBI Prot
G2	Citrate synthase, mitochondrial (*Katsuwonus pelamis*)	Q6S9V7;	8.47/52345	7/48400	21/ p < 0.05	52/52	5	KIVPNVLLEQGKA KSMSTDGLMTLVGAKS	Carbohydrate metabolic process (GO:0005975), tricarboxylic acid cycle (GO:0006099)	Swiss Prot
G3	PREDICTED: ATP synthase subunit alpha, mitochondrial-like	XP_013858090	9.18/59880	8/47200	57/ p < 0.01	237/237	6	KMGTAEVSSILEEKI RVLSIGDGIARV RVVDALGNAIDGKG	*NA*	NCBI prot

	(*Austrofundulus limnaeus*)									
G4	Fructose-bisphosphate aldolase B (*Sparus aurata*)	P53447	8.43/ 40190	7.3/39100	20/ p < 0.05	83/83	3	KGILAADESTGTMGKR	Glycolytic process (GO:0006096)	Swiss Prot
G5	Tropomyosin alpha-1 chain (*Liza aurata*)	P84335	4.69/32767	4.3/3600	30/ p < 0.005	85/85	9	KMELQEIQLKE KLVIIEGDLERT KLVIIEGDLERT KEDKYEEEIKV	*Actin filament binding (GO:0051015), protein heterodimerization (GO:0046982) and	Swiss Prot
G6	Proteasome subunit alpha type-2 (*Carassius auratus*)	O73672	5.99/25918	6/27100	19/ p < 0.05	46/46	8	KLVQIEYALAAVAAGA PSVGIKA	Ubiquitin dependent protein catabolic process (GO:0006511)	Swiss Prot
G7	Peroxiredoxin (*Oncorhynchus mykiss*)	Q91191	6.95/22248	6.3/2700	20/ p < 0.05	42/42	5	RQITINDLPVGRC	Cell redox homeostasis (GO:0045454)	Swiss Prot
G8	Mitochondrial proton/calcium exchanger protein (*Danio rerio*)	Q1LY46; B8JIA3	5.97/87192	6.1/28200	25/ p < 0.02	28/28	1	MASILLTRSRT	Mitochondrial calcium ion homeostasis (GO:0051560)	Swiss Prot
G9	Tubulin alpha chain (*Oncorhynchus mykiss*)	P30436	4.93/50683	6.1/33100	21/ p < 0.05	27/27	3	RAIFVDLEPTVIDEVR T	Microtubule based process (GO:0007017)	Swiss Prot

G10	Peroxiredoxin (*Oncorhynchus mykiss*)	Q91191	6.95/22248	5.5/25400	23/ p < 0.03	75/75	10	RQITINDLPVGRC RLVQAFQFTDKH	Cell redox homeostasis (GO:0045454)	Swiss Prot
G11	Heat shock protein HSP 90-beta (*Danio rerio*)	O57521; Q6P0Y9; Q90475	4.90/83704	6.5/23100	21/ p < 0.05	48/48	1	KSIYYITGESKD	Protein folding (GO:0006457) Regulation of cellular protein localization (GO:1903827)	Swiss Prot
G12	Histone H4 (*Oncorhynchus mykiss*)	P62797; P02304; P02305	11.36/11360	7.3/14000	21/ p < 0.05	27/27	11	RDNIQGITKPAIRR	Nucleosome assembly (GO:0006334), DNA templated transcription initiation (GO:0006352)	Swiss Prot
G13	Fatty acid-binding protein, liver-type (*Epinephelus coioides*)	Q8JJ04	6.62/13941	4.6/16100	21/ p < 0.05	26/26	10	KSISEIEENGDDFKV	*Fatty acid binding (GO:0005504)	Swiss Prot
G14	Unnamed protein product, partial (*Tetraodon nigroviridis*)	CAG06134	4.85/23207	6.3/18800	50/ p < 0.05	64/54	5	KSDYMVGSYGPRD	*NA*	NCBI prot
G15	Actin, cytoplasmic 1 (*Oreochromis mossambicus*)	P68143; P53484	5.30/42082	5.3/33100	31/ p < 0.05	130/130	11	KAGFAGDDAPRA RGYSFTTTAERE KEITALAPSTMKI KQEYDESGPSIVHRK	*ATP binding (GO:0005524)	Swiss Prot

G16	PREDICTED: N-acetyl-D-glucosamine kinase (*Lates calcarifer*)	XP_018540415	6.10/37375	5.8/36300	57/ p < 0.01	125/125	7	KCIETINDMVQRA RGGVVLISGTGSNCKL	*NA*	NVBI Prot
G17	Creatine kinase, testis isozyme (*Oncorhynchus mykiss*)	P24722	6.20/43262	6.4/30100	21/ p < 0.05	90/90	4	RFCTGLTKI KGQSIDDLMPAQK	*ATP binding(GO:0005524), Catalytic activity (GO:0003824)	Swiss Prot
G18	Adenylate kinase 2, mitochondrial (*Danio rerio*)	Q1L8L9; Q7T3D7	5.53/26884	5.9/29500	23/ p < 0.03	72/72	4	RAILLGPPGAGKG RAILLGPPGAGKG	ATP (GO:0046034), AMP (GO:0046033) metabolic process	Swiss Prot
G19	40S ribosomal protein S12 (*Oreochromis niloticus*)	O13019	6.30/14726	6.6/17200	22/ p < 0.04	39/39	6	KDVIEEYFKS	Translation (GO:0006412)	Swiss Prot
G20	Thioredoxin domain-containing protein 17 (*Epinephelus coioides*)	T1SH39	5.43/14285	5.2/18600	24/ p < 0.025	70/70	13	KLSGVPTLLRY KLVEEECFKA	Oxidation reduction process (GO:0055114)	Swiss Prot
G21	Keratin, type I cytoskeletal 18 (*Oncorhynchus mykiss*)	O57607	5.39/48805	5.2/22000	28/ p < 0.01	71/71	2	RQSVEADIVGLRK	Structural molecule activity (GO:0005198)	Swiss Prot
G22	PREDICTED: trypsin-1-like	XP_012694795	6.15/26987	5.6/20900	53/ p < 0.02	100/100	5	RVTEGNEQFIKSSRV	Serine type endopeptidase	NCBI prot

	(*Clupea harengus*)								activity (GO:0004252)	
G23	Histone H4 (*Oncorhynchus mykiss*)	P62797; P02304; P02305	11.36/11360	7.8/18600	21/ p < 0.05	55/55	21	RDNIQGITKPAIRR RISGLIYEETRG	Nucleosome assembly (GO:0006334), DNA templated transcription initiation (GO:0006352)	Swiss Prot
G24	Nascent polypeptide-associated complex subunit alpha (*Danio rerio*)	Q8JIU7; Q0P3U9	4.51/23386	4/23900	21/ p < 0.05	36/36	6	KIEDLSQQAQLAAAEK F	Myofibril assembly (GO:0030239), protein transport (GO:0015031)	Swiss Prot
G25	Serotransferrin (*Paralichthys olivaceus*)	O93429	6.06/76545	6.7/23900	21/ p < 0.05	173/173	2	KEADAMAVDGGQVYTA GKC KEADAMAVDGGQVYTA GKC	Iron ion homeostasis (GO:0055072), ion transport (GO:0006811)	Swiss Prot

719 NA – Not available

720 Asterisk (*) denotes molecular function of the protein, due to unavailability of GO terms for biological process

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